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-Primining

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

J Immunol 2007; 179:6889-6900; doi: 10.4049/jimmunol.179.10.6889

References This article cites 58 articles, 23 of which you can access for free at: http://www.jimmunol.org/content/179/10/6889.full#ref-list-1

Why The JI? Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
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.
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. CDA4−/− and CD8−/− mice were provided courtesy of T. W. Mak (Ampere 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 Dipetalogaster maxima reduvid bugs and BALB/c mice as described previously (22, 23). Blood-form trypanastigotes (BFT) and IMT were obtained from hepatimized mouse blood and reduvid excreta, respectively. For some experiments we prepared “TS immune-recovered” BFT parasites (TSIR BFT) by first immunizing wild-type mice with the Tulahuen strain T. cruzi and then transferring the immune sera to naive mice. 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 protein was purified in a similar manner and used as a negative control protein as described previously (21).

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 oligodeoxynucleotid (ODN) 1826 (TC CATGACGTTCCTGACGTT, with CpG underlined; courtesy of A. Krieg, Copley Pharmaceutical Group) mixed with purified negative control bacteriophage 10 protein, trans-sialidase, or T. cruzi lysate. In some experiments, the negative control ODN devoid of Cpg motifs (TCCAG GCTTTCCTCAGGT; also provided by A. Krieg, Copley 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 or 20–50 μg of recombinant TS protein. Mice were immunized with 60 mg/kg of ketamine and 5 mg/kg of 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 preinfection, 3 days after systemic infection, and 11–14 days after oral challenge (see figure legends for details). TS-specific serum IgG and fecal 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 × 105 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-IIIM automated harvester, dried, placed in sample bags with 4.0 ml of Ultima Gold 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 control A20 cells, A20 cells transfected with plasmid providing early CMV promoter-driven expression of the TS154 protein (29), A20 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 × \[1 - \left(\frac{\text{percentage of naive CFSE}^{low}}{\text{percentage of sample CFSE}^{low}}\right)\].

**In vitro CTL Assays**

CTL assays were performed using a modified version of a previously reported method (35). Effector cell populations were prepared by culturing 3 × 10^6 spleen cells with 1 × 10^6 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-STEM without Con A (Collaborative Biomedical Products) was added and cultures were incubated for 4–6 h at 37°C. Twenty-five-microliter aliquots of these culture supernatants were mixed with 150 μl of Wallac OptiPhase SuperMix scintillation fluid and counted in a Wallac TriLux MicroBeta 1450 liquid scintillation counter. The percentage of lysis was calculated using the following formula: \[(\text{sample dpm} - \text{spontaneous release dpm})/(\text{maximum release dpm} - \text{spontaneous release dpm})\] × 100.

**Parasite challenges and assessment of protective immunity**

Vaccinated mice were challenged 4–6 wk after their final immunization. BFT were obtained from highly parasitemic, heparinized BALB/c blood and diluted to 50,000 BFT/ml with PBS. Mice were challenged s.c. at the base of the tail with 5,000 BFT and survival was followed for >3 mo. IMT 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 with 5,000 IMT orally. Mice were sacrificed 11–13 days after IMT challenge for quantitative studies of parasite replication within the initial site of mucosal invasion, previously shown to be through the proximal gastric epithelia following the oral delivery of parasites (36). T. cruzi-specific parasitic DNA was quantified in gastric DNA and quantitative culture assessment of recoverable viable parasites from draining gastric lymph nodes were done as described previously (36).

**CpG effects on CD80 and MHC-I surface expression**

Spleen cells from naive BALB/c mice were treated overnight with 100 μM concentrations of control ODN 1982 or CpG motif-containing ODN 1826 and then stained for flow cytometry using anti-CD8 microbeads (Miltenyi Biotec). Spot development was performed as previously described (21). Spots were enumerated using the scanning service and ImmunoSpot scanning software from Cellular Technology.

**In vivo CTL assays**

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 × \[1 - \left(\frac{\text{percentage of naive CFSE}^{low}}{\text{percentage of sample CFSE}^{low}}\right)\].

**FIGURE 1.** TS immunity protects against acute T. cruzi infection and persists during chronic infection without evidence for immune escape in subsequent generations of surviving parasites. A, Mice chronically infected with T. cruzi maintain potent TS-specific immunity. Control mice, mice hyperimmunized with TS154 DNA (last immunization 1 mo before harvest), and mice repeatedly challenged and chronically infected with T. cruzi (last parasite challenge 3 mo before harvest) were sacrificed and their spleen cells used for IFN-γ ELISPOT assays. Control A20J cells, A20J cells pulsed with a previously identified H-2K d-restricted TS peptide (IYNVGQVSI), A20J cells expressing the consensus TS enzymatic domain, and T. cruzi infected A20J cells (NC A20J, TS CD8 peptide A20J, TS transfected A20J, and T. cruzi infected A20J, respectively) were used to stimulate responses. Results are representative of multiple experiments with pooled samples. B, Mice chronically infected with T. cruzi mediate effective TS-specific in vivo cytolytic activity. Control mice (NC), mice hyperimmunized with TS154 DNA (1 mo after their last immunization), and mice repeatedly challenged and chronically T. cruzi-infected (3 mo after their last challenge) were injected with an equivalent mixture of two BALB/c spleen cell preparations differentially labeled with CFSE and pulsed with either a control peptide or the TS-specific CD8 peptide (IYN VGQVSI). The next day, spleen cells were harvested and studied by flow cytometry for the relative persistence of control and TS-specific peptide pulsed APC. Results are representative of multiple experiments studying mice individually (2–3 mice per group in each experiment). C. T. cruzi parasites previously exposed to TS-specific immunity remain susceptible to protective TS-specific immune responses. Parasites were recovered from TS-immunized mice that survived virulent T. cruzi challenge and amplified by passage through SCID mice (TSIR BFT). The BFT recovered were used to challenge new mice previously vaccinated with control DNA or TS DNA. NC, Control; WT, wild type.
either PE-labeled anti-MHC class I (H-2D\(^d\)) or anti-CD80 (BD Pharmingen). 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 \(\mu\)M ODN (control no.1982 or CpG no.1826) with and without 100 \(\mu\)g/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 \(\times\) 10^5) with 1 \(\times\) 10^5 A20J cells pulsed or not pulsed with 2.5 \(\mu\)M 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 virulent Tulahüen 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 Tulahüen 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 parasites/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\(^d\)-restricted TS epitope (IYNVGQVSI) known to stimulate CD8\(^+\) T cell responses, and unpulsed negative control A20J cells. The data shown in Fig. 1A clearly demonstrate that during chronic T. cruzi infection potent TS-specific, IFN-\(\gamma\)-producing T cells (including CD8\(^+\) T cells) are present. In fact, the numbers of TS-specific, CD8\(^+\) IFN-\(\gamma\)-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 T. cruzi 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\(^+\) T cell responses in lymphocytes harvested from mice chronically infected with T. cruzi by using in vitro assays. Next, we investigated whether these TS-specific CD8\(^+\) 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 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 T. cruzi. These results confirm that TS-specific T cell immunity remains effective in vivo during chronic T. cruzi infection.

We further explored the possibility that T. cruzi 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 T. cruzi challenge. After passage through SCID mice to increase numbers of BFT, these parasites that survived TS-specific immunity were used to challenge new TS-immunized BALB/c mice. In Fig. 1C, we demonstrate that both parasites recovered from TS-immune mice and parasites not previously exposed to vaccine-induced TS immunity were similarly lethal in naive control mice but equivalently protected against TS-immunized mice. Therefore, we were unable to find evidence for a TS-specific immunologic escape mechanism relevant for the persistence of T. cruzi infection. We cannot rule out the possibility that during the expansion of BFT in SCID mice the loss of immune pressure may have allowed for parasite phenotype reversion. However, at the very least there clearly was no stable immune escape induced by TS-specific immune pressure. These results support the further development of TS-specific vaccines for Chagas disease despite the large family of related genes present within the parasite’s genome.

**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 prophylactic 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 I-related protective immunity in numerous mouse models of human intracellular infectious diseases, including T. cruzi (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-\(\gamma\) responses (Fig. 2C). More importantly, TS/CpG vaccination induced complete protection against normally lethal T. cruzi BFT challenges (Fig. 2D; \(n = 5\) per group; \(p < 0.01\), 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 T. cruzi 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 TS-specific, H2-K^d-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 (\(\mu\)MT) 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 (\(n = 5/\text{group}; p < 0.01\), 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. Fig. 7 demonstrates that the failure to induce protective immunity in TS-immunized B cell knockout mice was unique to mice vaccinated with TS protein mixed with CpG adjuvant and was not seen in B cell knockout mice immunized with TS/DNA vaccine (7A). This failure of B cell knockout mice to be protected was associated with the absence of the TS-specific CD8+ T cell responses detectable 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 cells. Indeed, Fig. 8 demonstrates that purified B cells pulsed with TS protein and CpG ODN adjuvant can induce cross-priming of TS-specific naive CD8+ T cells. Naive B cells were purified by anti-CD19 primary immunomagnetic selection and pulsed overnight in vitro with or without 100 μg of TS protein and 100 μg of CpG ODN 1826 or a non-CpG ODN 1982 control (NC). The next day these B cells were washed and studied by flow cytometry for MHC class I (A) and CD80 (B) surface expression or used to stimulate naive highly purified CD8+ T cells for 7 days in the presence of 10 ng/ml IL-2. Expanded CD8+ T cells were restimulated overnight in IFN-γ ELISPOT assays pulsed with the IYNVGQVSI peptide epitope (C). CpG plus TS protein treatment of CD19+ B cells induced significantly increased numbers of naive CD8+ T cells to produce IFN-γ in response to TS protein restimulation 7 days later compared with non-CpG ODN 1982 alone, CpG ODN 1826 alone, or TS protein plus non-CpG ODN 1982 (p < 0.01, by paired t tests). Results shown are representative of multiple experiments with multiple replicates of pooled samples.

Discussion

Sequencing of the T. cruzi genome revealed several important and unexpected findings (24). Over 50% of the entire genome is composed of repeated sequences, including large families of related genes. The T. cruzi gene family with the largest number of homologues is the trans-sialidase or TS superfamily. The TS family includes 1,430 separate genes, only 12 of which are thought to
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 challenges (29, 31, 49, 51) and can be major targets for CD8+ T cell responses during natural infection in both mice and humans (50). In addition, we further show in the present work that BALB/c mice chronically infected with T. cruzi maintained potent TS-specific immunity detectable both in vitro and in vivo (Fig. 1, A and B, respectively). In addition, T. cruzi parasites that survive in vivo exposure to TS-induced vaccine immunity remained susceptible to TS-specific immunity (Fig. 1C). Therefore, we failed to detect evidence for TS-specific immune evasion during either acute or chronic T. cruzi infection.

However, it is still possible that during chronic infection minor subsets of T. cruzi intracellular amastigotes express TS isoforms with altered T cell epitopes that cannot be recognized by or optimally trigger effector T cell responses. This would allow a lower level of infection to persist while TS-specific immunity could contribute to suppression of the majority of parasite replication during the long periods of asymptomatic infection associated with the indeterminate phase of T. cruzi infection. Techniques capable of identifying differential TS gene expression in individual infected host cells, which are not yet available, will be necessary to address this latter possibility. Even if this were shown to be the case, the induction of TS-specific immunity could still be an important goal for vaccine development because the majority of parasites during acute and chronic infection appear to be targeted by these immune responses, and reductions in parasite burden without complete clearance of parasite infection have been associated with protection against chagasid disease progression (6, 8). However, if some partial immune escape were shown to be present during chronic infection, it would be even more important than currently thought to develop multicomponent vaccines that combine consensus TS epitopes with other T. cruzi epitopes shown to be expressed by residual subpopulations of intracellular amastigotes not susceptible to consensus TS epitopes. In this regard, a number of potential vaccine candidates expressed in different stages of the parasite have been recently described (52).

In addition to demonstrating the efficacy of TS-specific immunity against T. cruzi acute and chronic infection, as well as against mucosal invasion and systemic replication, our current work provides important preliminary data for the development of a feasible strategy for the vaccination of humans. Previous TS-specific immune responses shown to protect mice against T. cruzi have been induced with naked DNA vaccines encoding TS sequences. Although DNA vaccines have been highly immunogenic in murine models, the immunogenicity of DNA vaccination in humans has so far been disappointing, and the safety of DNA vaccines has not been proven in large human trials. Protein subunit vaccines advantageous with single-stranded short ODN containing unmethylated CpG motifs have been shown to be safe and immunogenic in humans (53, 54). Furthermore as we have shown, protein/CpG vaccines can be delivered mucosally and induce both potent mucosal and systemic immunity. Naked DNA vaccines are degraded in mucosal secretions and/or are not well absorbed by mucosal surfaces. Therefore, our current results provide the “proof of principle” that a vaccination strategy feasible for use in humans, the intranasal delivery of TS protein mixed with a CpG motif-containing an 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 naive 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 vaccine designs 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 surrogate markers for use in future human vaccine trials, and functional diversity of helper T lymphocytes. Nature 383: 787–793.

4. O’Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 8: 275–283.

5. Szczylik, T. F., S. R. Schell, G. Nau, and F. W. Fitch. 1989. Regulation of T cell activation: differences among T-cell subsets. Immunol. Rev. 111: 79–110.

6. Fitch, F. W., M. D. McKisic, D. W. Lancki, and T. F. Gajewski. 1993. Differential regulation of murine T lymphocyte subsets. Annu. Rev. Immunol. 11: 29–48.

7. Coffman, R. L., K. Karkila, P. Scott, and R. Chatelain. 1991. Role of cytokines in the differentiation of CD4+ T-cell subsets in vivo. Immunol. Rev. 123: 189–207.

8. Chen, Y., V. J. Kuchroo, J. Imbe, D. A. Hafler, and H. L. Weiner. 1994. Regulation of CD8+ T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265: 1237–1240.

9. Fukaura, H., S. C. Kent, M. J. Pietruszewicz, S. J. Khouby, H. L. Weiner, and D. A. Hafler. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transcriptional growth factor-b-secretion T8 cells by oral administration of myelin in multiple sclerosis patients. J. Clin. Invest. 98: 70–77.

10. Hoff, D. F., A. R. Schnapp, C. S. Eickhoff, and S. T. Roodman. 2000. Involvement of CD4+ Th1 cells in systemic immunity protective against primary and secondary challenges with Trypanosoma cruzi. Infect. Immun. 68: 197–204.

11. Schnapp, A. R., C. S. Eickhoff, D. Sizemore, R. Curtis III, and D. F. Hoff. 2002. Czuzapin induces both mucosal and systemic protection against Trypanosoma cruzi in mice. Infect. Immun. 70: 5065–5074.

12. Hoff, D. F., and C. S. Eickhoff. 2002. Type 1 immunity provides optimal protection against both mucosal and systemic Trypanosoma cruzi challenges. Infect. Immun. 70: 6715–6725.

13. Hoff, D. F., and C. S. Eickhoff. 2005. Type 1 immunity provides both optimal mucosal and systemic protection against a mucosally invasive, intracellular pathogen. Infect. Immun. 73: 4934–4940.

14. Atwood, J. A. III, D. B. Weatherly, T. A. Minning, B. Bundy, C. Cavola, F. R. Opperoedos, R. Orlando, and R. L. Tarleton. 2005. The Trypanosoma cruzi proteome. Science 309: 409–415.

15. Schenkman, S., M. Jiang, G. W. Hart, and V. Nussenzweig. 1991. A novel cell surface trans-sialidase of Trypanosoma cruzi generates a stage-specific epitope required for invasion of mammalian cells. Cell 65: 1117–1125.

16. Bueno, M., and M. E. Pereira. 1995. Trypanosoma cruzi trans-sialidase: enhancement of virulence in a murine model of Chagas’ disease. J. Exp. Med. 181: 1693–1703.

17. Pereira-Chioccola, V. L., F. Costa, M. Ribeirao, I. S. Soares, F. Arena, S. Schenkman, and M. M. Rodrigues. 1999. Comparison of antibody and protective immune responses against Trypanosoma cruzi infection elicited by immunization with a parasite antigen delivered as naked DNA or recombinant protein. Parasite Immunol. 21: 103–110.

18. Fujimura, A. E., S. S. Kinoshita, V. L. Pereira-Chioccola, and M. M. Rodrigues. 2001. DNA sequences encoding CD4+ and CD8+ T-cell epitopes are important for efficient protective immunity induced by DNA vaccination with a Trypanosoma cruzi gene. Infect. Immun. 69: 5477–5486.

19. Machado, A. V., J. E. Cardoso, C. Claser, M. M. Rodrigues, R. T. Gazzinelli, and D. A. Hafler. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transcriptional growth factor-b-secreting T8 cells by oral administration of myelin in multiple sclerosis patients. J. Clin. Invest. 98: 70–77.

20. Chen, Y., V. J. Kuchroo, J. Imbe, D. A. Hafler, and H. L. Weiner. 1994. Regulation of CD8+ T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265: 1237–1240.

21. Hoff, D. F., and C. S. Eickhoff. 2002. Type 1 immunity provides optimal protection against both mucosal and systemic Trypanosoma cruzi challenges. Infect. Immun. 70: 6715–6725.

22. Hoff, D. F., and C. S. Eickhoff. 2005. Type 1 immunity provides both optimal mucosal and systemic protection against a mucosally invasive, intracellular pathogen. Infect. Immun. 73: 4934–4940.

23. Atwood, J. A. III, D. B. Weatherly, T. A. Minning, B. Bundy, C. Cavola, F. R. Opperoedos, R. Orlando, and R. L. Tarleton. 2005. The Trypanosoma cruzi proteome. Science 309: 473–476.

24. Schenkman, S., M. Jiang, G. W. Hart, and V. Nussenzweig. 1991. A novel cell surface trans-sialidase of Trypanosoma cruzi generates a stage-specific epitope required for invasion of mammalian cells. Cell 65: 1117–1125.

25. Bueno, M., and M. E. Pereira. 1995. Trypanosoma cruzi trans-sialidase: enhancement of virulence in a murine model of Chagas’ disease. J. Exp. Med. 181: 1693–1703.

26. Pereira-Chioccola, V. L., F. Costa, M. Ribeirao, I. S. Soares, F. Arena, S. Schenkman, and M. M. Rodrigues. 1999. Comparison of antibody and protective immune responses against Trypanosoma cruzi infection elicited by immunization with a parasite antigen delivered as naked DNA or recombinant protein. Parasite Immunol. 21: 103–110.

27. Fujimura, A. E., S. S. Kinoshita, V. L. Pereira-Chioccola, and M. M. Rodrigues. 2001. DNA sequences encoding CD4+ and CD8+ T-cell epitopes are important for efficient protective immunity induced by DNA vaccination with a Trypanosoma cruzi gene. Infect. Immun. 69: 5477–5486.
of the cellular immune response generated by immunization with a DNA vaccine containing a *Trypanosoma cruzi* gene. Infect. Immun. 67: 3855–3863.
35. Schnapp, A. R., C. S. Eickhoff, J. Scharfstein, and D. F. Hoff. 2002. Induction of B- and T-cell responses to cruzipain in the murine model of *Trypanosoma cruzi* infection. Microbes Infect. 4: 805–813.
36. Hoff, D. F., P. L. Farrar, K. Kratz-Owens, and D. Shaffer. 1996. Gastric invasion by *Trypanosoma cruzi* and induction of protective mucosal immune responses. Infect. Immun. 64: 3800–3810.
37. Krieg, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20: 709–760.
38. Corral, R. S., and P. B. Petray. 2000. CpG DNA as a Th1-promoting adjuvant in immunization against *Trypanosoma cruzi*. Vaccine 19: 234–242.
39. Frank, F. M., P. B. Petray, S. I. Cazorla, M. C. Munoz, R. S. Corral, and E. L. Malchiiodi. 2003. Use of a purified *Trypanosoma cruzi* antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypanmastigotes. Vaccine 22: 77–86.
40. Araujo, A. F., B. C. de Alencar, J. R. Vasconcelos, M. I. Hiyane, C. R. Marinho, M. L. Penido, S. B. Boccardi, D. F. Hoff, R. T. Gazzinelli, and M. M. Rodrigues. 2005. C8Δ9-T-cell-dependent control of *Trypanosoma cruzi* infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect. Immun. 73: 6017–6025.
41. Tarleton, R. L. 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. J. Immunol. 144: 717–724.
42. Tarleton, R. L., B. H. Koller, A. Latour, and M. Postan. 1992. Susceptibility of β2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. Nature 356: 338–340.
43. Tarleton, R. L., J. Sun, L. Zhang, and M. Postan. 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitemia in experimental Chagas’ disease. Infect. Immun. 62: 1820–1829.
44. Tarleton, R. L., M. J. Grusby, M. Postan, and L. H. Glimcher. 1996. *Trypanosoma cruzi* infection in MHC-deficient mice: further evidence for the role of both class I- and II-restricted T cells in immune resistance and disease. Int. Immunol. 8: 13–22.
45. Cresswell, P., A. L. Ackerman, A. Giodini, D. R. Peaper, and P. A. Wearsch. 2005. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol. Rev. 207: 145–157.
46. Rock, K. L., and L. Shen. 2005. Cross-presentation: underlying mechanisms and role in immune surveillance. Immunol. Rev. 207: 166–183.
47. Bevan, M. J. 2006. Cross-presentation. Nat. Immunol. 7: 363–365.
48. Heit, A., K. M. Huster, F. Schmitz, M. Schiemann, D. H. Busch, and H. Wagner. 2004. CpG-DNA aided cross-presentation by cross-presentation B cells. J. Immunol. 172: 1501–1507.
49. Millar, A. E., M. Wklinski-Lee, and S. J. Kahn. 1999. The surface protein superfamily of *Trypanosoma cruzi* stimulates a polarized Th1 response that becomes anergic. J. Immunol. 162: 6092–6099.
50. Martin, D. L., D. B. Weatherley, S. A. Lancellia, M. A. Cabinian, M. T. Crim, S. Sullivan, M. Heiges, S. H. Craven, C. S. Rosenberg, M. H. Collins, et al. 2006. CD8+ T-Cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. PLoS Pathog. 2: e77.
51. Wizel, B., N. Garg, and R. L. Tarleton. 1998. Vaccination with trypanastagote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* infection. Infect. Immun. 66: 5073–5081.
52. Garg, N., and V. Bhatia. 2005. Current status and future prospects for a vaccine against American trypanosomiasis. Expert. Rev. Vaccines 4: 867–880.
53. Cooper, C. L., H. L. Davis, J. B. Angel, M. L. Morris, S. M. Eller, I. Seguin, A. M. Krieg, and D. W. Cameron. 2005. CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. AIDS 19: 1473–1479.
54. Krieg, A. M. 2006. Therapeutic potential of Toll-like receptor 9 activation. Nat. Rev. Drug Discov. 5: 471–484.
55. Kumar, S., and R. L. Tarleton. 2001. Antigen-specific Th1 but not Th2 cells provide protection from lethal *Trypanosoma cruzi* infection in mice. J. Immunol. 166: 4596–4603.
56. Kleindienst, P., and T. Brocker. 2005. Concerted antigen presentation by dendritic cells and B cells is necessary for optimal CD4 T-cell immunity in vivo. Immunology 115: 556–564.
57. Crawford, A., M. Macleod, T. Schumacher, L. Corlett, and D. Gray. 2006. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. J. Immunol. 176: 3498–3506.
58.McClellan, K. B., S. Gangappa, S. H. Speck, and H. W. Virgin. 2006. Antibody-independent control of γ-herpessivirus latency via B cell induction of anti-viral T cell responses. PLoS Pathog. 2: e58.