Identification and Characterization of the Trypanosoma cruzi B-cell Superantigen Tc24

Sarah M. Gunter, Kathryn M. Jones, Bin Zhan, Heather T. Essigmann, Kristy O. Murray, Melissa N. Garcia, Rodion Gorchakov, Maria Elena Bottazzi, Peter J. Hotez, and Eric L. Brown

Center for Infectious Diseases, University of Texas School of Public Health, Houston, Texas; Baylor College of Medicine, National School of Tropical Medicine, Section of Tropical Medicine, Houston, Texas

Abstract. Trypanosoma cruzi causes life-long disease after infection and leads to cardiac disease in 30% of infected individuals. After infection, the parasites are readily detectable in the blood during the first few days before disseminating to infect numerous cell types. Preliminary data suggested that the Tc24 protein that localizes to the T. cruzi membrane during all life stages possesses B-cell superantigenic properties. These antigens facilitate immune escape by interfering with antibody-mediated responses, particularly the avoidance of catalytic antibodies. These antibodies are an innate host defense mechanism present in the naïve repertoire, and catalytic antibody–antigen binding results in hydrolysis of the target. We tested the B-cell superantigenic properties of Tc24 by comparing the degree of Tc24 hydrolysis by IgM purified from either Tc24 unexposed or exposed mice and humans. Respective samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, silver stained, and the degree of hydrolysis was measured. Data presented in this report suggest that the T. cruzi Tc24 is a B-cell superantigen based on the observations that 1) Tc24 was hydrolyzed by IgM present in serum of unexposed mice and humans and 2) exposure to Tc24 eliminated catalytic activity as early as 4 days after T. cruzi infection.

INTRODUCTION

Chagas disease is a parasitic infection caused by the hemoflagellate protozoan Trypanosoma cruzi. There are an estimated 8–9 million prevalent human cases worldwide and an estimated 300,000 to 1 million infections in the United States. This vector-borne parasite is transmitted to humans and a wide variety of other mammalian hosts by the triatomine bug found throughout South and Central America, Mexico, and portions of the southern United States. Infection occurs when the parasite is released in the feces of a feeding triatomine and introduced into the host at mucosal surfaces or via skin abrasions. Less commonly, T. cruzi is transmitted congenitally, through blood transfusion, transplantation, or from consumption of contaminated food or beverages. Once inside a mammalian host, T. cruzi disseminates systemically and parasitic nests are most commonly found in the tissues of the heart and digestive tract.

Acute Chagas disease can be asymptomatic, but is often characterized by the appearance of a chagoma, an inflammatory nodule at the site of inoculation, followed by early clinical manifestations that can range from asymptomatic to general fever and facial edema in healthy persons. In immuno-suppressed persons, symptoms can range from myocarditis, hepatomegaly, splenomegaly, and meningoencephalitis that occasionally can result in death. After the acute phase, the majority of people enter an indeterminate stage that is largely asymptomatic, but approximately 30% will develop cardiomyopathy or more rarely megacolon or megaesophagus. The current etiological treatments for Chagas disease, nifurtimox or benznidazole, are not Food and Drug Administration-approved medications and are associated with several severe adverse effects. Moreover, neither nifurtimox nor benznidazole reverse existing pathology. Because of the limited efficacy of available medications, a vaccine or novel therapeutic approach would be cost effective and benefit the prevention and treatment of Chagas disease. A better understanding of host/pathogen interactions including immune evasion strategies used by T. cruzi would facilitate these efforts.

Antibodies that develop as a consequence of antigen exposure and affinity maturation bind microbial antigens non-covalently and are central to immune defense against microbial pathogens. Of the immunoglobulins, IgM and homologous molecules were the first selected evolutionarily and the first to develop ontogenetically after exposure to antigen. IgM production by germ line–encoded immunoglobulin genes combines conserved evolutionary memory with effective effector functions in the absence of somatic hypermutation and develop in germ-free and antigen-free mice and represent an innate, first line of defense. Some of these IgM molecules are also catalytic antibodies that are capable of hydrolyzing target antigens because of the presence of specific amino acid sequences (e.g., the catalytic triad comprises Ser27a, His93, and Asp1) encoded by variable region germ line antibody genes. Because of their innate production and lack of somatic hypermutation, IgMs possess the most efficient antigen-specific catalytic activity (IgM > IgA > IgG).

The naïve antibody repertoire present in humans is derived from a large pool of B cells expressing diverse B-cell receptors (BCRs) generated by approximately 500 different germ line genes encoding the V_{L}/V_{H} diversity, and joining segments that also hold the potential of generating thousands of antibodies of various classes (e.g., IgM, IgG, and IgA); each with a unique antigen-binding specificities. The combinatorial IgM repertoire derived from germ line V, D, and J segments can encode approximately $4 \times 10^{9}$ $V_{L}$/$V_{H}$ domain pairs and does not include expansion due to junctional diversification. This naïve or natural antibody pool in both humans and animals has been shown to possess catalytic activities that range from promiscuous, that is, sequence-independent recognition of peptides to the hydrolysis of specific target antigens resulting from specific, noncovalent antigen recognition mediated by a serine protease mechanism.

Nucleophilic sites encoded by germ line V genes (without the need for antigen stimulation through the BCR) and selected over millions of years are universally expressed by antibodies resulting in promiscuous catalytic antibody activity, suggesting...
that this innate (promiscuous) activity provides a homeostatic function.\textsuperscript{15} For example, high and low levels of promiscuous catalytic activity has been linked to decreased and increased risk of death in patients with sepsis, respectively.\textsuperscript{16,17} In contrast to antibodies with promiscuous activity, antibodies with noncovalent antigen-binding specificities to respective antigens are endowed with the ability of hydrolyzing specific targets. That is, although different antibodies may have the potential for enzymatic activity, lysis of specific targets is the result of initial antigen specific, noncovalent binding followed by target hydrolysis.\textsuperscript{10} Because of the ability of catalytic antibodies to hydrolyze and inactivate their targets, their potential role in neutralizing microbial antigens is significant; however, their role in protection against microbial pathogens remains largely unexplored.

A subset of antigens have been defined as B-cell superantigens (BC-SAgS) because 1) antibodies present in the naive repertoire encoded by germ line sequences have specificity for these antigens, for example, staphylococcal protein A and the extracellular fibrinogen-binding protein, and human immunodeficiency virus (HIV) gp120\textsuperscript{10,18–21} and 2) exposure to BC-SAgS results in the depletion of the B-cell subsets secreting antibodies with BC-SAg hydrolyzing potential, leaving a “hole” in the catalytic repertoire.\textsuperscript{10,11} We hypothesize that BC-SAgS evolved in part to counter catalytic antibody-mediated hydrolysis of target antigens.

As described above, the HIV gp120 glycoprotein represents one example of a BC-SAg that can be hydrolyzed by IgM purified from uninfected people\textsuperscript{22} and it’s hydrolysis is lost after BC-SAg exposure as a consequence of clonal B-cell depletion.\textsuperscript{22} As described for HIV/acquired immune deficiency syndrome, \textit{T. cruzi} may also produce a potential BC-SAg. For example, Tc24 is a member of the excretory/secretory antigen family\textsuperscript{23} that localizes to the membrane during all life stages and is primarily expressed at the flagellar pocket.\textsuperscript{24,25} B cells exposed to Tc24 have been shown to clonally expand both in vivo and in vitro\textsuperscript{26} and the immunoglobulin response elicited by Tc24 is nonspecific and composed primarily of IgM, similar to what occurs after B-cell stimulation by mitogens. In addition, injection of Tc24 into athymic mice suggested that the observed B-cell activation occurred independent of T cell help.\textsuperscript{24} Data presented in this report suggest that Tc24 exhibits BC-SAg properties based on the observation that exposure to Tc24 via either antigen injection or infection with \textit{T. cruzi} significantly reduced IgM-mediated cleavage of Tc24.

MATERIALS AND METHODS

\textbf{Recombinant proteins.} Recombinant Tc24 was expressed in \textit{Escherichia coli} BL21(DE3) by cloning an \textit{E. coli} codon-optimized DNA coding for full-length Yucatan strain \textit{T. cruzi} 24 kDa flagellar calcium-binding protein (Tc-24E-Yucatan, amino acids 1–211) in frame into pET41a vector between NdeI and EcoRI sites. Na-ASP-2 (\textit{Ancylostoma-secreted protein}) was cloned from human hookworm \textit{Necator americanus} and expressed as a recombinant protein in \textit{Pichia pastoris} X-33 as described previously.\textsuperscript{27} The recombinant \textit{Staphyloccus aureus} S component of the Panton–Valentine bicomponent pore-forming leukotoxin (LukS-PV) and the enterotoaggregative \textit{E. coli} strain O42 dispersin protein have been described in previous work.\textsuperscript{28–31} Recombinant \textit{S. aureus} toxic shock syn-

drome toxin (TSST) was purchased from IBT Bioservices (Gaithersburg, MD).

\textbf{Parasites and mice.} \textit{Trypanosoma cruzi} CL strain expressing tdTomato (CL-tdTomato)\textsuperscript{32} was provided by Rick Tarleton (University of Georgia, GA) and trypomastigotes were maintained by continuous in vitro passage in C2C12 cells. \textit{Trypanosoma cruzi} H1 strain previously isolated from a human case in Yucatan, Mexico\textsuperscript{33} was provided by Eric Dumonteil (Universidad Autónoma de Yucatán) and maintained by serial passage in Balb/c mice. Female Balb/c mice, 6–8 weeks old, were obtained from Taconic Biosciences (Hudson, NY).

\textbf{Immunizations and infections.} Naive mice were injected twice, 2 weeks apart with 25 μg recombinant Tc24 protein adsorbed to 200 μg Alhydrogel\textsuperscript{36} (Brenntag Biosector, Frederikssund, Denmark) (referred to as alum for the remainder of the report), or alum only. Blood was collected 2 weeks after the second injection by cardiac puncture under anesthesia (150 mg/kg ketamine/15 mg xylazine). Pooled sera from noninfected and noninfected Balb/c, C57BL/6, CD-1, and non-Swiss albino mice were also obtained from Innovative Research (Novi, MI).

For acute infection studies, naive mice were infected with 500 blood-form trypomastigotes of \textit{T. cruzi} H1 and killed at 4, 7, 21, 28, or 49 days after infection. For chronic infection studies, mice were infected with 1,000 tissue culture trypomastigotes of \textit{T. cruzi} CL-tdTomato by intraperitoneal injection and killed at 166 and 238 days after infection. At the designated time points, mice were exsanguinated by cardiac puncture under anesthesia as described above. All mouse studies were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

\textbf{Human samples.} Written informed consent was obtained before human serum samples were collected and the study was approved by the Baylor College of Medicine Institutional Review Board. Blood samples were collected from individuals identified as potentially Chagas positive by the Gulf Coast Regional Blood Bank (Houston, TX), as previously described.\textsuperscript{34} Human samples were divided based on serology into either Chagas-positive or Chagas-negative controls. Chagas positive individuals were tested for Chagas on six different diagnostic tests: ortho \textit{T. cruzi} enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics, Rochester, NY), radioimmunoprecipitation assay, Stat-Pak (Chembio Diagnostic Systems, Medford, NY), DPP (ChemBio Diagnostic Systems), Chagatest (Wiener Laboratories, Santa Fe, Argentina), and immunofluorescence antibody assays. Chagas-negative individuals were defined as testing negative for Chagas using all serological tests described above. Serum was isolated from blood samples of 13 Chagas-positive individuals and five Chagas-negative controls. Chagas-positive individuals were further divided by the presumed region where the infection was acquired. Infections acquired in the United States were defined by no significant history of travel to endemic regions or having received blood or tissue transplants.\textsuperscript{35} To rule out congenital transmission, these individuals and their mothers also had to be born in the United States. Participants not matching the definitions for locally acquired infections were considered to have been infected in an endemic area as described previously.\textsuperscript{35}

\textbf{IgM and IgG purification.} IgM and IgG antibodies were purified from mouse or human serum samples using CaptureSelect™ Affinity Matrix from Life Technologies.
nitrocellulose paper and probed with rabbit anti-mouse antibodies. Mouse IgM or IgG preparations were blotted onto specific peroxidase conjugated (Sigma-Aldrich Corporation) at 37°C with shaking. Reactions were stopped at various time points by adding 4× nonreducing SDS-PAGE loading buffer (Bio-Rad, Hercules, CA). Hydrolysis of target antigens was conducted using antibody concentrations ranging between 10.4 μg/mL (diluted in PBS). Hydrolysis rates were determined and expressed as percentage Tc24 hydrolysis using the following equation: 

\[
\frac{[s-Ab]/[s-BC]}{100}
\]

In this equation [s-Ab] and [s-BC] represent the intensity of the intact protein band for the antibody-containing reaction and the control reaction containing only buffer, respectively. Band intensities were determined using ImageJ software (Bethesda, MD).

To determine kinetics of the hydrolysis reaction, hydrolysis was measured at increasing concentrations of Tc24 and data fit to the Michaelis–Menten equation. The ability of protease inhibitors to inhibit respective hydrolysis reactions was assessed by adding either a serine protease inhibitor, 4-(2-Aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (1 mM); or a metalloprotease inhibitor, ethylenediaminetetraacetic acid (EDTA) (2 mM).

Binding assays. Binding of IgG antibody to immobilized Tc24 was measured in triplicate using an ELISA as previously described. In brief, bound mouse IgG was detected using a goat anti-mouse γ-chain specific AP-conjugated antibody. The reaction was developed by adding 100 μL of 1 mg/mL Sigma 104 Phosphatase substrate (Sigma-Aldrich Corporation) dissolved in 1 M diethanolamine, 0.5 mM MgCl₂, pH 9.8 for 1 hour. The optical density was measured at 405 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

RESULTS

IgM-mediated hydrolysis of Tc24. A hallmark of BC-SAGs is their susceptibility to hydrolysis by catalytic antibodies present in the naive repertoire. Because IgM antibodies are derived from germ line immunoglobulin sequences with the potential of mediating hydrolysis of respective BC-SAg targets and previously demonstrated to possess the highest levels of enzymatic activity, IgM was purified from pooled mouse sera and from the sera of five healthy human negative-control donors. Pooled, polyclonal IgM preparations were subsequently incubated in the presence of Tc24 and hydrolysis determined by subjecting samples to SDS-PAGE and measuring Tc24 depletion (Figure 1A and B).

![Figure 1](image-url)

**Figure 1.** Hydrolysis of Tc24 by IgM purified from unexposed mice and humans. IgM was pooled from the sera of naive Balb/c mice and from the sera of five individuals that tested negative for Chagas and Tc24 hydrolysis was assessed over a period of 48 hours. (A) Dose-dependent hydrolysis of Tc24 by mouse or human IgM (0, 25, 50, and 100 μg/mL). (B) Representative silver-stained gel demonstrating hydrolysis of Tc24 (2.6 μM) by either human or mouse IgM (100 μg/mL). Parent band depletion was measured using ImageJ.
IgM purity, specificity, and nature of the enzymatic reaction. The purity of each IgM preparation used was confirmed by both silver staining and immunoblot analyses demonstrating that respective preparations were electrophoretically homogenous (Figure 2A and B). Purity was assessed for all IgM preparations used in subsequent hydrolysis experiments (data not shown).

We observed saturation kinetics consistent with the Michaelis–Menten equation (Table 1). The apparent \(5.11 \times 10^3\) (M\(^{-1}\) min\(^{-1}\)) \(k_{cat}/K_M\) is similar in enzyme efficiency to previously reported catalytic antibodies with reactivity against different substrates.\(^{39,37,38}\) Presence of the serine protease inhibitors, AEBSF and leupeptin (also a cysteine and threonine protease inhibitor), reduced IgM-mediated hydrolysis of Tc24 compared with no inhibition observed for the metalloprotease inhibitor EDTA. The diminished inhibitory effect of leupeptin relative to that of AEBSF was not surprising as leupeptin has been shown to be a less effective serine protease inhibitor, and in one study failed completely to inhibit serum IgA-mediated hydrolysis.\(^{39,40}\) These experiments demonstrated that catalytic antibody–mediated hydrolysis of Tc24 occurred via a serine protease mechanism as previously described for other catalytic antibody-mediated hydrolysis reactions (Figure 2C).\(^{19}\)

The specificity of the IgM-mediated hydrolysis reaction for Tc24 was demonstrated by incubating non-BC-SAg recombinant proteins generated from various pathogens including *N. americanus* (Na-ASP-2), a major protein released by infective hookworm larvae, *S. aureus* (LukS-PV and TSST), and *E. coli* (dispersin). Only Tc24 was hydrolyzed by IgM purified from alum-only immunized animals (Figure 3).

**TABLE 1**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Apparent (K_M)</th>
<th>(k_{cat}/K_M) (min(^{-1})) or apparent (V_{max}) (μM substrate/μM IgM/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgM</td>
<td>4.3 ± 3.9</td>
<td>0.022 ± 0.006</td>
</tr>
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\(^{50}\) μg/mL IgM purified from alum-only vaccinated mouse serum was incubated with varying concentrations of Tc24 (2.6, 5.2, 10.4, and 20.8 μM) for 19 hours at 37°C with shaking and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 15% Tris-Gly gels. Percent cleavage calculated using ImageJ. Apparent \(k_{cat}\) and \(K_M\) values were calculated by fitting data to the Michaelis–Menten equation.
Tc24 injection reduces IgM-mediated hydrolysis of Tc24. Hydrolysis of respective antigens by antibodies present in the naive repertoire is not sufficient to define a BC-SA g. A second criterion requisite of BC-SAgs is their ability to reduce catalytic activity specific to that BC-SA g after exposure. This is accomplished as a consequence of the signals resulting from the antibody–BC-SA g interaction, that is, B-cell clones capable of binding and hydrolyzing respective BC-SAgs are deleted from the repertoire.19,22 This criterion was assessed by measuring hydrolysis of Tc24 after incubation with IgM preparations purified from mice injected with Tc24 in the presence of alum, or from mice injected with 200 μg alum only (negative control) (Figure 4A). The IgM concentration tested was 50 μg/mL for IgMs purified from Tc24 injected or alum-only injected mice. This concentrations is 40 times less than physiologic IgM concentrations. Representative silver-stained gels demonstrate hydrolysis of the parent Tc24 band by IgM purified from alum-only injected mice (Figure 4C) compared with hydrolysis carried out by IgM purified from mice injected with 25 μg Tc24 + 200 μg alum (Figure 4B). These data suggest that the IgM pool purified from mice exposed to Tc24 was devoid of a subset of IgM molecules present in the other IgM preparations that mediated Tc24 hydrolysis.

Trypanosoma cruzi infection reduces IgM-mediated hydrolysis of Tc24. To examine the impact of T. cruzi infection on the hydrolysis of Tc24, IgM was purified from the serum of either T. cruzi-infected mice or from humans diagnosed with Chagas disease on the basis of serology. As observed after immunization with Tc24, T. cruzi infection also resulted in a significant reduction in Tc24 hydrolysis by IgM preparations purified from the respective groups (Figure 5A and B).

We next assessed the temporal effects of Tc24 exposure on catalytic activity after infection with T. cruzi H1 strain. IgM was purified from individual mice that were killed at 4, 7, 21, or 28 days postinfection and tested in Tc24 hydrolysis assays. These data demonstrated a significant and time-dependent reduction in hydrolytic activity detectable as early as day 4 postinfection (Figure 6). This temporal reduction in catalytic activity was associated with a parallel increase in the IgM concentration after infection suggesting that the reduction in catalytic activity is independent of the ability to generate an IgM response (Figure 6).

DISCUSSION

Data presented in this report demonstrated that Tc24 is a BC-SA g based on the observations that: 1) Tc24 was
hydrolyzed by IgM purified from mice and humans not previously exposed to Tc24 by either injection or T. cruzi infection and 2) after exposure to Tc24, the subset of IgM antibodies mediating Tc24 hydrolysis is absent similar to what has been described for other BC-SAgs.19,37 Responses to BC-SAgs (i.e., reduction in catalytic activity) are opposite to responses generated after exposure to conventional antigens, which are recognized by complementarity determining regions leading to class-switching and affinity maturation.41 Antibodies with catalytic potential are an innate immune defense mechanism not associated with adaptive immunity that likely evolved as a means of hydrolyzing antigenic targets. BC-SAgs represent a microbial response to this host defense mechanism that is important to pathogens such as T. cruzi that spend a period of time in the blood stream.

We observed significant hydrolysis of Tc24 at concentrations of IgM approximately 40 times less than the 2 mg/mL present in serum. These data suggested that the presence of catalytic IgM in the preimmune serum may provide some degree of protection against acute forms of disease if the catalytic activity were to remain present long enough to prevent T. cruzi replication and dissemination. On the basis of the observation that catalytic activity was significantly reduced by day-4 postinfection, it is likely that protective catalytic responses would be marginal at best, which may explain why catalytic responses are not protective in the context of acute T. cruzi infections. That IgM plays an important role during the early stages of infection and provides some level of innate protection is supported by the observation that rats deficient in IgM had an increased susceptibility to acute T. cruzi infection.42 What level of protection would be achieved if catalytic activity were not abolished remains unknown at this time. Therefore, developing vaccine formulations that stimulate rather than reduce catalytic activity against Tc24 may potentially result in a robust immune response with the potential of controlling the initial infection.

Another variable potentially affecting susceptibility to infection is heterogeneity of hydrolysis rates between individuals and species. This was tested by purifying IgM from different mouse strains and demonstrated that these strains possessed different hydrolysis rates (Supplemental Figure 2). At this juncture, it would be premature to speculate as to the significance of these differences in hydrolysis because there are conflicting results in the literature regarding what mouse strains are more or less resistant to T. cruzi infection.43–46 In addition, IgM-mediated Tc24 hydrolysis rates between Balb/c mice and humans were different. However, these differences may be a reflection of how the heterogeneity of the immune response affects acute and chronic disease progression.

The specificity of the Tc24 hydrolysis reaction was demonstrated by the ability of IgMs purified from non-Tc24-exposed mice to hydrolyze Tc24 but not other non-BC-SAgs, including Na-ASP-2 from N. americanus, TSST and LuKS-PV from...
S. aureus, or dispersin from E. coli suggesting that the activity reported was Tc24 specific. Hydrolysis of Tc24 occurred via a serine-protease mechanism similar to previously described catalytic antibody-mediated hydrolysis reactions. Although exposure to BC-SAgs reduced catalytic activity, conventional IgG-mediated recognition of alternative epitopes on Tc24 was readily observed (Supplemental Figure 1A). However, no detectable hydrolysis of Tc24 by purified IgG from alum-injected mice was observed as previously demonstrated for other BC-SAgs (Supplemental Figure 1B).

We hypothesize that the B cells that produce Tc24 hydrolyzing IgM antibodies are either deleted or anergized after exposure to Tc24 (either as a result of immunization or infection) and that suppression of catalytic responses is sustained throughout T. cruzi infections since Tc24 is secreted and expressed by all T. cruzi developmental stages. This impact to the IgM response appears to be limited to the B cells secreting catalytic IgM antibodies since the IgM concentration following T. cruzi infection increased, suggesting that IgMs with specificities to other targets may not play a significant role in controlling the infection and that any benefit conferred by the catalytic response would be during the early stages of infection (Figure 6). A Tc24-specific IgG binding (but not catalytic as described in the context of other BC-SAgs) response was also observed and was likely mounted in response to epitopes on Tc24 that are not superantigenic. “SILENCING” of the anti-Tc24 catalytic response early in the infection process may represent a key immune escape mechanism that allows the parasite to survive in the bloodstream after infection and may leave the host more susceptible to subsequent infections since there is now a “hole” in the catalytic IgM repertoire. As the infection progresses and T. cruzi parasites intracellular niches, the presence of antibodies in general may not play as an important a role in containment of the organism compared with early in the infection process. Although, a robust IgG response develops (Supplemental Figure 1), we do not at this time know how this response impacted the early stages of infection.

This report is the first to describe a BC-SAg produced by a protozoan. In comparison with bacteria and viruses, protozoans and helminths are highly complex organisms and it is likely that Tc24 is the first of many BC-SAgs produced not only by T. cruzi but also other parasites with blood stages (e.g., Schistosomes and Plasmodium sp.). Data presented in this report suggest that the measurement and manipulation of catalytic antibody responses could be used to more effectively manage Chagas disease for which current diagnostic, treatment and vaccine options remain limited.

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Authors’ addresses: Sarah M. Gunter, Heather T. Essigmann, and Eric L. Brown, Department of Epidemiology, Human Genetics and Environmental Sciences, University of Texas School of Public Health, Houston, TX, E-mails: sarah.k.murphy@uth.tmc.edu, heather.t.essigmann@uth.tmc.edu, and eric.l.brown@uth.tmc.edu. Kathryn M. Jones, Bin Zhan, Kristy O. Murray, Melissa N. Garcia, Rodion Gorchakov, Maria Elena Bottazzi, and Peter J. Hotez, Department of Pediatrics, Section of Tropical Medicine, Baylor College of Medicine, Houston, TX, E-mails: kathrynjn@bcm.edu, kmurray@bcm.edu, bzhans@bcm.edu, mnolan@bcm.edu, rodion@bcm.edu, bottazzi@bcm.edu, and hotez@bcm.edu.

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REFERENCES

THE T. CRUZI TC24 PROTEIN IS A B-CELL SUPERANTIGEN


