ABSTRACT—Trypanosoma cruzi–infected mice display increased susceptibility to shock induced by injection of lipopolysaccharide (LPS), anti-CD3, or resulting from interleukin (IL)-10-defective response to the parasite itself, but the basis of such susceptibility remains unknown. Herein, we tested the susceptibility of mice inoculated with virulent and avirulent T. cruzi to staphylococcal enterotoxins (SE), potent inducers of inflammatory cytokine secretion. Mice infected with T. cruzi CL-strain or inoculated with the avirulent clone CL-14, a clone that does not induce disease or polyclonal lymphocyte activation, succumb suddenly to low doses of staphylococcal enterotoxin B (SEB, but not to staphylococcal enterotoxin A (SEA). High plasma levels of TNF, IFN-γ, and liver transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were found in these mice, indicating lethal toxic shock. Sensitization to shock required inoculation of live avirulent trypanosomes and a time interval before challenge with SEB. We found no prior skewing of T cell receptor (TCR) Vβ repertoire in CL-14-inoculated mice that could be responsible for sensitization. Splenocytes from CL-14-inoculated mice proliferated more under anti-Vβ8 than anti-TCRβ stimulation when compared with normal mice, but were suppressed to SEB stimulation. Both SEB and anti-Vβ8 antibodies stimulated splenocytes from T. cruzi–inoculated mice to secrete higher levels of inflammatory cytokines than normal controls. Taken together, our results show that T. cruzi inoculation can sensitize mice to lethal SEB-induced shock even in the absence of tissue damage, polyclonal lymphocyte activation, or previously increased levels of inflammatory cytokines, and they suggest that altered reactivity of Vβ8 lymphocytes may be involved in the phenomenon.

KEYWORDS—Repertoire, clone CL-14, CL strain, staphylococcal enterotoxin B, sepsis, T lymphocyte, TNF-α, IFN-γ

INTRODUCTION

Staphylococcal enterotoxin B (SEB) is a bacterial superantigen that interacts with major histocompatibility complex (MHC) class II-bearing cells and activates mainly Vβ8 T cells in mice to produce several inflammatory mediators such as TNF and IFN-γ, leading to systemic shock (1). It can be lethal in low doses when synergizing with lipopolysaccharide (LPS) (2) given to glucocorticoid-depleted (3), irradiated-reconstituted (4), or mice administered with t-Gal (5). Virus infection can also sensitize mice to lethal SEB-induced shock, as observed in Vβ8.1 T cell receptor (TCR) transgenic mice infected with Lymphocytic Choriomeningitis Virus (LCMV), but not in wild-type mice (6). However, infection with *Listeria monocytogenes* (7) or inoculation of *Candida albicans* (8) did not sensitize mice to SEB-induced shock, indicating that susceptibility is not related to a general inflammatory state.

*T. cruzi* infection sensitizes mice to shock induced by anti-CD3 administration, a phenomenon dependent on CD8 cells and on secretion of TNF and IFN-γ (9). Also, IL-10 knockout mice undergo lethal toxic shock partially mediated by CD4 cells and TNF levels during acute *T. cruzi* infection, indicating that the parasite itself can induce lethal proinflammatory cytokine production in the absence of appropriate immune regulation (10, 11). In fact, high levels of TNF and IFN-γ were observed during acute *T. cruzi* infection (13), and infected mice die upon injection of low TNF doses (14), which could explain their increased susceptibility to shock. As *T. cruzi* glycosphosphatidylinositol-mucins induce secretion of high levels of IL-12 and TNF (15), such molecules could hypothetically sensitize *T. cruzi*-infected mice to any further inflammatory stimulation. In agreement with these data, *T. cruzi*-infected mice succumb to injection of low doses of LPS (16). Polyclonal T cell activation (17), previously elevated basal levels of inflammatory cytokines, and acute liver damage (11) are candidates to explain the sensitization to toxic shock induced by *T. cruzi* infection.

Susceptibility to septic shock may vary greatly among individuals, and prior contact with microorganisms could be a factor underlying such variability (18). Herein, we investigated whether prior contact with *T. cruzi* could sensitize mice to shock. To rule out general health as a determinant of susceptibility to shock, we used the avirulent clone CL-14, which does not induce tissue lesions (19, 20) or immune abnormalities (21, 22) in mice.

MATERIALS AND METHODS

**Mice and parasites**

Adult BALB/c female mice were used in all experiments. The study was approved by our institutional Animal Care and Use Committee. CL-14 trypomasti-
gotes were obtained by in vitro metacyclogenesis and were purified by diethylami-
noethyl-chromatography as described elsewhere (19). Immunization with CL-14
was performed by injecting 2 × 10^6 to 10^7 live trypomastigotes i.p. in 0.2 mL of
phosphate-buffered saline (PBS). CL strain was maintained by serial blood passages
through BALB/c mice, and infection was performed by inoculation of blood
containing 10^7 trypomastigotes. Fixed parasites were obtained by adding 1 mL of
1% formaldehyde-PBS to CL-14 trypomastigote pellets (3 × 10^6) for 1 min and then
washing them in PBS. Freeze-thawed (F/T) parasites were obtained by freezing
pellets of CL-14 trypomastigotes (~180°C) and thawing it (100°C) 10 times.

**Antibodies and superantigens**

Vβ1β (KJ-25), Vβ4 (KT4), Vβ6 (44.22.1), Vβ7 (TR310), Vβ8.1 + 8.2 (KJ-16),
Vβ10 (B21.5), Vβ11 (RR3-15), and Vβ14 (14-2) were produced as cell culture
supernatants. Other antibodies were purified from cell culture supernatants or asci,
nes fluid (anti-CD4, GK1.5; CD8a, 53-6.7; TCRβ, H57-597; Vβ2, B20.6; Vβ5,
MR9.4; Vβ8.1 + 8.2, KJ-16; Vβ8.1 + 8.2 + 8.3, F23.1; and CD3, 145-2C11) and
were conjugated to fluorescein isothiocyanate (FITC; Sigma, St Louis, MO). Anti-
CD4-PE (H12/9.19) and anti-CD8a-PE (53-6.7; Pharmingen, San Diego, CA) bio-
tinylated anti-hamster cocktail (PharMingen), anti-rat antibody (Amersham,
Buckinghamshire, UK), and R-phycocerythrin (PE)-conjugated streptavidin (Caltag,
South San Francisco, CA) were used at concentrations recommended by the manu-
facturer. SEB and staphylococcal enterotoxin A (SEA; Sigma), which in BALB/c
mice react mainly with Vβ8.1 + 8.2, were produced as cell culture
mice react mainly with Vβ7/Vβ8 and Vβ10 respectively, were diluted in ster-
ile PBS.

**Flow cytometry**

Splenocyte suspensions were hemolyzed in red blood lysis buffer, while mono-
nuclear blood cell suspensions were obtained by Ficoll-Hypaque separator. Rat and
murine Ig (1 µg of each/10^6 cells) were added to block binding to Fc receptors.
Cell samples were stained directly with FITC- and PE-conjugated antibodies diluted
in cold 5% fetal calf serum (FCS)-PBS for 1 h. Indirect immunofluorescence was
performed by sequential incubation of primary unlabeled antibodies, cognate
secondary biotinylated antibodies (anti-rat or hamster), and streptavidin-PE accom-
dpanied by either anti-CD4-FITC or CD8a-FA (Amersham, Buckinghamshire, UK).
Splenocytes (3 × 10^6) were stained for 15 min with 4-aminofluorescein
(Invitrogen, Carlsbad, CA) and data acquired in an EPICS Elite (Coulter Electronics,
Seattle, WA). Live mononuclear cells were gated using light scatter parameters, and
10,000–20,000 (blood) or 50,000–100,000 (spleen) cells were acquired inside this gate.

**Proliferation assays**

Splenocytes (3 × 10^5) were placed in 10% FCS-RPMI (supplemented with 2 mM L-
glutamine and 5 × 10^−4 M 2-mercaptoethanol) and were stimulated with plate-
bound antibodies (purified anti-Vβ8.1 + 8.2 + 8.3 or -TCRβ) or SEB for 72 h at
37°C in 96-well plates. Cultures were pulsed with 1 µCi H-methyl thymidine
(DuPont, Boston, MA) for the last 8 h, washed, harvested on glass fiber filters, and
counted in a beta counter. Values obtained for unstimulated cultures were
discounted from those obtained for antibody-stimulated cultures (Δcpm). The prolif-
eration index represents the ratio between Δcpm obtained for CL-14-inoculated
mice and Δcpm for nontreated mice.

**Stimulation in vitro for cytokine production**

Plates (24-well) were incubated with 0.5 mL of 2% normal mouse serum-RPMI
plus 0.5 mL of culture supernatants from hybridomas (anti-Vβ8.1 + 8.2 and Vβ7),
or were incubated with 1 mL of 0.5% normal mouse serum-RPMI supplemented
with 2 mL L-glutamine and 5 × 10^−4 M 2-mercaptoethanol plus 1 µg of purified
anti-Vβ8.1 + 8.2 + 8.3, anti-Vβ4, anti-Vβ10, 10 µg of purified anti-CD3, TCRβ, or
SEB overnight at 4°C. Splenocytes (5 × 10^6) in 0.1 mL of RPMI were added to each
well and were incubated at 37°C for 24 h. Two hundred microliters of cell-free
supernatants was assayed for cytokine detection.

**Enzyme-linked immunoabsorbant assays (ELISA) for
cytokine quantitation**

Cytokines production was assayed by the sandwich ELISA technique using capture
capture antibodies according to the manufacturer’s instructions
(Pharmingen). Recombinant cytokines diluted in 10% FCS-PBS (2000–31.25
pg/mL of TNF-α or 15,000–234 pg/mL of IFN-γ) were used as standards. Assays were
performed in duplicates or triplicates. The assay was developed with o-phenylene-
diamine-dihydrochloride (Sigma), stopped with HCL 3 N, and read in a microplate
reader (3500-UV; Bio-Rad, Hercules, CA). In some assays, we incubated plates
with streptavidin-alkaline phosphatase (Amersham, Buckinghamshire, UK) and
developed the assay with p-NPP (Zymed, San Francisco, CA).

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**Liver transaminases assay**

Plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase
(ALT) were assayed by a colorimetric method using a kit from Analisa Diagnostica
(Belo Horizonte, MG, Brazil).

**RESULTS**

T. cruzi inoculation sensitizes mice to SEB-induced hyperproduction of inflammatory cytokines and
toxic shock

Different from normal controls, mice infected with the CL
strain of T. cruzi succumbed to low doses of SEB administered
i.p. at various moments postinfection (Fig. 1). Conversely, administration of SEA did not kill T. cruzi-infected mice.
Susceptibility to SEB was associated with higher serum levels of
TNF and IFN-γ in infected than in normal mice 90 min after
administration (Fig. 1). CL strain-infected mice injected with SEB presented severe signs of acute shock early
after injection, such as tachycardia, hyperventilation, and vaso-
dilation. Surprisingly, mice inoculated with CL-14, which do
not present disease or polyclonal lymphocyte activation,
also showed increased susceptibility to SEB-induced shock and
overrelease of the inflammatory cytokines TNF and IFN-γ
(Fig. 2). In this case, however, shock followed a slower kinet-
ic, mice had lower levels of inflammatory cytokines than
that observed in challenged CL-infected mice, and a proportion
of these mice could survive SEB-induced shock depending on the
time interval after parasite inoculation. CL-14-inoculated mice
that received SEA (50–100 µg) had no mortality.

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**FIG. 1. Effects of superantigen administration in T. cruzi-infected mice.** Mice were infected with 10^7 bloodstream forms of CL strain 2 or 9
weeks prior to superantigen administration. Top panel, Mortality in CL-in-
fected mice after SEB or SEA inoculation. Intervals after parasite inoculation,
dose of superantigen injected, and number of individuals per group are indi-
cated in the figure. Bottom panel, Serum levels of TNF and IFN-γ in CL-in-
fected mice (2 weeks postinoculation) 90 min after inoculation with 100 µg of
SEB. Each bar shows cytokine levels in pooled plasma samples from three
mice and is representative of three to four independent experiments
performed.
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Severe hepatic damage is caused by SEB administration to CL-14-inoculated mice

The liver is an important target for injury in toxic shock (23). To evaluate whether mice undergo relevant hepatic injury after inoculation with CL-14 (3 weeks) or challenge with SEB, we measured the levels of AST and ALT in the plasma of normal and CL-14-inoculated mice 8 h after PBS or SEB (100-µg) injection. The plasma levels of AST and ALT were similar among normal and CL-14-inoculated mice that received PBS injection before sacrifice (Fig. 2), confirming previous histopathological observations that CL-14 does not cause detectable hepatic lesion by itself (20). Nevertheless, CL-14-inoculated mice had higher levels of AST and ALT than normal mice 8 h after challenge with SEB (Fig. 2).

Time interval and viable parasites are required to sensitize mice to SEB-induced shock

To test whether T. cruzi molecules could synergize with SEB to increase inflammation, we treated mice with live, freeze-thawed, or formaldehyde-fixed CL-14 trypomastigotes 4 weeks or 24 h prior to inoculation with 200 µg of SEB. Only mice treated with live CL-14 trypomastigotes 4 weeks prior to challenge succumbed to SEB (Table 1). None of these treatments performed 24 h prior to challenge with SEB were able to sensitize mice to shock.

CL-14-inoculated mice do not display increased frequencies of SEB-reactive cells

We investigated whether the frequency of SEB-reactive T cells was correlated with increased susceptibility to shock, such as happens in other models (6, 24). To this end, we studied TCR Vβ-usage in splenocytes from CL-14-inoculated mice at a moment when they were susceptible to SEB-induced shock (4–5 weeks postinoculation). CL-14-inoculated mice expressed a stable repertoire of TCR Vβ segments among CD4 (Fig. 3a) and CD8 (Fig. 3b) splenocytes, contrasting with the preferential expansion of Vβ subsets previously found in the mice infected with the parental CL (25). A reduction in Vβ8.1 + 8.2 usage was detected among CD4 peripheral blood lymphocyte (PBL; Fig. 3c). Similar results were obtained with Vβ8.1 + 8.2 + 8.3 antibodies (data not shown). The density of expression of Vβ8.1 + 8.2 among CD8 PBL was significantly decreased in CL-14-inoculated compared with normal mice (Fig. 3d, insert), an alteration that could reflect a particular state of activation of these cells (26).

No significant changes in the density of TCR expression were detected among other Vβ subsets from CD8 compartment or among CD4 cells.

Vβ8 splenocytes from CL-14-inoculated mice proliferate more than normal cells in response to TCR stimulation with anti-Vβ8, but not with SEB

To assess the activation status of Vβ8 cells, the proliferative response of splenocytes from normal controls and CL-14-inoculated mice to stimulation with anti-Vβ8.1 + 8.2 + 8.3, anti-TCRβ and SEB was studied at various time points post-CL-14 inoculation. Splenocytes from CL-14-inoculated mice were more responsive to anti-Vβ8.1 + 8.2 + 8.3 than normal controls from 4 to 8 weeks postinoculation (Fig. 4), but had normal or less increased proliferation in response to anti-TCRβ
stimulation, indicating that cells from CL-14-inoculated mice were preferentially responsive to anti-Vβ8 stimulation. Intriguingly, cells from CL-14-inoculated mice had consistently suppressed responses to SEB-induced proliferation at all intervals postinoculation (Fig. 4).

**Splenocytes from T. cruzi-sensitized mice produced higher amounts of TNF and IFN-γ in response to SEB and anti-Vβ8 stimulation than normal cells in vitro**

To investigate whether specific TCR stimulation of the main SEB-reactive subset in BALB/c mice (Vβ8) could trigger the production the cytokines associated with shock, we stimulated splenocytes from normal or CL-14-immune mice (4–5 weeks postinoculation) in vitro for 6 or 24 h with various stimuli and assayed culture supernatants for cytokine detection.

Increased production of IFN-γ by cells from CL-14-inoculated mice was verified as early as 6 h after anti-TCRβ or SEB stimulation in vitro, whereas splenocytes from normal mice produced low levels of this cytokine when stimulated with SEB (Fig. 5a). These levels of IFN-γ remained elevated in the supernatants after 24 h of anti-TCRβ or SEB stimulation (Fig. 5a, insert). No TNF production could be detected 6 h after stimulation of cells from normal or CL-14-inoculated mice with SEB in vitro. After 24 h, higher levels of TNF were produced by stimulation of splenocytes from CL-14-inoculated mice with anti-TCRβ, SEB, or anti-Vβ8.1 + 8.2 than by stimulation of cells from normal animals (Fig. 5b). Similar experiments were performed with splenocytes from CL-infected mice (14–16 days postinoculation), and we found a proportionally higher production of IFN-γ when splenocytes were stimulated for 24 h with anti-Vβ8.1 + 8.2 or SEB than with anti-CD3 (Fig. 5c). Although monoclonal antibodies and superantigens are different in their competence to stimulate cytokine production, these results indicate an overall hyperreactivity of T. cruzi-sensitized mice to TNF and IFN-γ production after stimulation of SEB-reactive cells.

**DISCUSSION**

In this study, we have shown that mice inoculated with the avirulent T. cruzi clone CL-14 that do not display tissue damage or polyclonal lymphocyte activation succumb to SEB-induced shock. Different from T. cruzi-infected mice (11), the plasma levels of TNF, IFN-γ, and liver transaminases are normal in CL-14-inoculated mice and cannot explain their increased susceptibility to shock, such as happens in severely injured mice (27). Nevertheless, upon SEB challenge, these plasma levels increased sharply, indicating development of a systemic shock.

The molecular and cellular mechanisms by which T. cruzi sensitizes mice to SEB-induced lethal shock are still unknown. As T. cruzi also sensitizes mice to shock induced by LPS (13, 16) and anti-CD3 (9), one could argue that the inflammatory properties of T. cruzi molecules (28) synergize with the T cell stimulation to produce high levels of proinflammatory cyto-
In addition, the expression of costimulatory molecules by antigen-presenting cells after immunization with CL-14 could enhance the production of cytokines by T cells in response to SEB presentation.

Although targeting the same TCR, the proliferative response of lymphocytes to stimulation with SEB and anti-Vβ8 produced different results: whereas SEB-stimulated cells from CL-14-inoculated mice had a somewhat reduced proliferation, cells stimulated with anti-Vβ8 proliferated well above controls since the 4th week postinoculation. It remains to be established whether this difference is related to different transducing pathways in response to these stimuli, activation-induced death of CL-14-primed cells by SEB, or any other phenomena. Another possibility that we cannot exclude is that despite the overrelease of inflammatory cytokines by SEB-stimulated splenocytes in vitro, the major reactive population is located somewhere else. A good candidate to test this hypothesis is the predominant Vβ8 NK1.1 liver population. This subset closely interacts with Kupffer cells to enhance production of IFN-γ (30), and activation of Kupffer cells could represent a link between SEB- and LPS-induced shock in T. cruzi-sensitized mice.

Herein, we have shown that T. cruzi-inoculated BALB/c mice succumb to toxic shock induced by low doses of SEB, but not to SEA. As Vβ8 represents the most expressive fraction of BALB/c TCR Vβ repertoire, these results do not allow one to conclude promptly that Vβ8 T cells are preferentially activated over other Vβ subsets in T. cruzi-sensitized mice (3). On the other hand, the stimulation of low numbers of superantigen-reactive cells is usually sufficient to induce high production of IFN-γ by lymphocytes, particularly SEA in BALB/c mice (31), and also to induce lethal shock under various circumstances (24, 29, 32). Also, the proliferative response of cells from CL-14-inoculated mice to anti-Vβ8 stimulation suggests that T. cruzi somehow alter the response of these cells. Another evidence of altered reactivity is that both anti-Vβ8 and SEB elicited the production of higher levels of TNF and IFN-γ by cells from T. cruzi-sensitized mice than by normal controls, whereas other anti-Vβ stimulated cytokine production less efficiently. However, as different antibodies and superantigens have distinct mechanisms of action and stimulatory capacities (3), additional studies are required to determine whether preferential activation of a subset of cells is responsible for the susceptibility to SEB-induced shock found in T. cruzi-sensitized mice. An attractive possibility is that the increased proliferative response of cells from T. cruzi-infected mice to stimulation with anti-human Vβ5.3 (an antibody reactive to Vβ8.2 cells in mice) (33), the expansion of Vβ5 cells after incubation

![Image](Image)

FIG. 5. Production of cytokines by splenocytes from T. cruzi-inoculated mice after TCR stimulation. Splenocytes from normal and CL-14-inoculated mice (4 weeks postinoculation) were stimulated in vitro for 6 (a) or 24 h (b) with anti-Vβ8.1 + 8.2 + 8.3 (graph a only), -Vβ8.1 + 8.2, -Vβ7, -TCRδ, and SEB, and culture supernatants were assayed in duplicate for cytokine detection. Insert in a shows IFN-γ production after 24 h of SEB and anti-TCRδ stimulation. In c, splenocytes from CL-infected mice (2 weeks postinfection) were stimulated in vitro for 24 h and supernatants were assayed for IFN-γ detection. Stimulation of spleen cells from CL-14-inoculated or CL-infecte...
of blood lymphocytes from Chagas’ disease patients with T. cruzi antigens (34), and the overrepresentation of Vβ cells in the hearts of infected mice (35) reflect preferential activation of Vβ subsets bearing specificities similar to Vβ cells in our model. Irrespective of whether preferential activation of a Vβ subset is involved in sensitization to shock, our results warn to the silent hyperreactivity to T cell stimulation that can be induced even by vaccinal strains of T. cruzi.

ACKNOWLEDGMENTS

This work was partially supported by Programa de Apoio a Núcleos de Excelência Conselho Nacional de Pesquisas, Fundação de Amparo a Pesquisa de Estado do Rio de Janeiro, and the World Health Organization (grant no. 960853). C.N.P. was supported by Conselho Nacional de Pesquisas (Ph.D.) and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (postdoctoral) fellowships. The authors thank Drs. George A. dos Reis and Ricardo Ribeiro dos Santos for helpful discussions. Dr. Marcolino Araujo for careful review of the manuscript, Dr. Morgan T. L. Castelo-Branco for help with flow cytometry preparations, and Deise A. Costa, discussions, Dr. Marcelo Bozza for careful review of the manuscript, Dr. Morgana Silva JS, Morrissey PJ, Grabstein KH, Mohler KM, Anderson D, Reed SG: Instituto de Biologia of the University of Rio de Janeiro, and the World Health Organization (grant no. 960853). C.N.P. the hearts of infected mice (35) reflect preferential activation of Vβ cells in our model. Irrespective of whether preferential activation of a Vβ subset is involved in sensitization to shock, our results warn to the silent hyperreactivity to T cell stimulation that can be induced even by vaccinal strains of T. cruzi.

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