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The Surface Protein Superfamily of *Trypanosoma cruzi* Stimulates a Polarized Th1 Response That Becomes Anergic

Amanda E. Millar, Monika Wlekliński-Lee, and Stuart J. Kahn

*Trypanosoma cruzi* is an obligate intracellular parasite that chronically infects mammals. Extracellular mammalian stage trypomastigotes simultaneously express and release multiple members of the parasite’s surface protein superfamily; these extracellular proteins should stimulate MHC class II-restricted CD4 T cells. The surface protein superfamily, however, encodes variant epitopes that may inhibit this CD4 response. In this report the surface protein-specific CD4 response was investigated. CD4 cells isolated from acutely and chronically infected mice did not proliferate when stimulated with surface proteins. Adoptive transfer of surface protein-specific CD4 clones or immunization with a peptide encoding a surface protein T cell epitope protected mice during *T. cruzi* infection. These data strongly suggested that surface proteins were expressed and presented to CD4 cells during infection.

Limiting dilution analysis identified an expanded population of surface protein-specific CD4 cells during the acute and chronic infection. These surface protein-specific CD4 cells did not produce IL-2 or IL-4, but did produce IFN-γ. Enzyme-linked immunosorbent assays confirmed that many of the surface protein-specific CD4 cells produce IFN-γ. Together these results suggest that during *T. cruzi* infection a potentially protective CD4 response becomes anergic. It is possible that this anergy is induced by variant T cell epitopes encoded by the surface protein superfamily. *The Journal of Immunology*, 1999, 162: 6092–6099.

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Chagas disease is caused by *Trypanosoma cruzi*, an obligate intracellular protozoan parasite. During the acute infection trypomastigotes are detected in the blood as they disseminate throughout the host and invade host cells, where they transform into amastigotes, the replicative form. As the chronic phase ensues, parasites become difficult to detect, yet they persist in the tissues, and individuals remain infected for life. *T. cruzi*-infected mice deficient in MHC class I- and class II-restricted T cells suffer higher parasitemia and decreased survival (1–4). T cells responses, however, during *T. cruzi* infection appear to be depressed, as they fail to produce IL-2, and their proliferation to Con A, anti-CD3 Ab, and parasite lysate is decreased (5, 6). The mechanisms of T cell depression have not been clearly defined, although deficiencies in Ag presentation and IL-2 production and inhibitory effects of IL-10, TGF-β, and nitric oxide may contribute (5–9). In addition, a massive polyclonal activation of T cells occurs during the infection and may contribute to the depressed T cell responses (10). Perhaps because the T cells are depressed, T cell responses to specific *T. cruzi* proteins during an infection have not been determined.

During *T. cruzi* infection of mice, a robust Th1 response does not occur (11, 12). IFN-γ-secreting CD4 T cells, however, are observed, but their expansion appears to be inhibited by the production of nitric oxide (6). The administration of IL-2 or IFN-γ to mice improves their immune response against the parasite, suggesting that a more robust Th1 response may eradicate *T. cruzi* (9, 13).

Amino acid changes within a T cell epitope can generate altered or variant epitopes that inhibit T cell responses. These variant epitopes have been shown to induce T cell anergy, to function as T cell antagonists or partial agonists, and to favor the development of Th2 responses (14–17). Evidence that infectious organisms express variant T cell epitopes that improve their survival come from studies on malaria that demonstrate the selection of *Plasmodium* strains that express variant epitopes that inhibit T cell responses (18). Other studies suggest that during HIV and hepatitis B viral infections, variant epitopes that inhibit the cytotoxic T cell response are generated (19, 20). The *T. cruzi* SA85-1 proteins encode variant epitopes that may inhibit the T cell responses (21).

SA85-1 surface proteins are a subfamily of the *T. cruzi* sialidase surface protein superfamily. There are >100 genes in the SA85-1 family, and these SA85-1 proteins appear to be 70–80% homologous to each other at the amino acid level throughout the length of the proteins. Each trypomastigote simultaneously expresses these polymorphic proteins and releases them into the extracellular milieu (21). Therefore, the SA85-1 proteins should be presented by MHC class II molecules and should stimulate Th responses. The MHC class II-restricted T cell response to the polymorphic surface proteins has not been investigated. In this report, studies were performed to initiate analysis of the CD4 T cell response to the surface proteins.

This study focused on the CD4 T cell response to a specific *T. cruzi* superfAMILY protein, the SA85-1.1 protein, during the acute and chronic infection. The investigations failed to detect a proliferative response to several SA85-1 proteins, including the SA85-1.1 protein. These negative responses suggested that either these members of the superfAMILY were not expressed, or that the responses to them were impaired. SA85-1.1-specific adoptive transfer and immunization experiments demonstrated protection, strongly suggesting that during *T. cruzi* infection the SA85-1.1 protein was expressed. Further studies identified SA85-1.1-specific CD4 cells that secrete IFN-γ, but do not proliferate; these properties are characteristic of anergic T cells (22).
Materials and Methods

Mice
C57BL/6 mice were used in all experiments (Bantin & Kingman, Fremont, CA). Experiments involving T. cruzi-infected mice were initiated with 8- to 10-wk-old mice.

Parasites
T. cruzi CL strain subclone 3 trypomastigotes (23) were obtained from culture supernatants of infected 3T3 cells grown in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated calf serum (BioWhittaker) and 50,000 U of penicillin/streptomycin (BioWhittaker). Parasites were removed from naive mice and mashed between sterile frosted glass slides, and the cells were suspended in 5 ml of DMEM supplemented with penicillin and streptomycin. Splenocytes were then incubated for 10 min at room temperature in 5 ml of 1.66% NH4 Cl to lyse RBCs. Cell suspensions were washed three times in DMEM by centrifugation at 200 × g for 10 min, irradiated (3250 rad), washed, and suspended in complete RPMI medium.

Antigens
The SA85-1.1-III protein (a protein that encodes the carboxy region of the SA85-1.1 protein), SA85-1.2 protein, SA85-1.8 protein, SA85-1.9 protein, SA85-1.10 protein, and GST were expressed in Escherichia coli and affinity purified as previously described (21). KLH, 3 which was not expressed in. coli, was purchased from Pierce (Rockford, IL). Peptide c, which encodes the SA85-1.1 epitope 1, was synthesized on a Gilson Multiplex Synthesizer (Gilson, Middleton, WI) (21) and was coupled to OVA.

Parasitemia determination
Parasitemia was monitored every second day by venaecenesis of the tail. Two microliters of blood was diluted in 18 ml of 0.89% ammonium chloride in PBS, and the trypomastigotes were counted on a hemocytometer (24). Parasitemias are expressed as the mean of five mouse samples ± SEM.

T cell clones
All clones were obtained from the lymph nodes of immunized C57BL/6 mice. The isolation of the SA85-1.1-specific Th1 T cell clones, 1C11 and 0.3C4, and the KLH-specific Th1 clone, CD6, have been previously described (21, 25). The CD6 clone was a gift from Immunex (Seattle, WA) and was provided by Dr. M. Sweetser (University of Washington, Seattle, WA). The clones were maintained by stimulation every 2 wk with SA85-1.1-III or KLH and supplemental IL-2 (Chiron Therapeutics, Emeryville, CA). T cells were grown in complete medium (RPMI 1640 (BioWhittaker) supplemented with 5% heat-inactivated FCS (HyClone, Logan, UT), 2 mM t-glutamine, 1 mM sodium pyruvate, 50,000 U of penicillin/streptomycin, 10 mM HEPES, and 50 μM 2-ME).

Adoptive transfer
T cell clones were rested for 2 wk before adoptive transfer. Groups of five mice received either 107 T cells in 200 μl of medium or 200 μl of medium only by i.v. infusion. Twenty-four hours after T cell infusion each mouse was injected i.p. with 5 × 104 trypomastigotes.

Immunization
Groups of five mice were immunized subcutaneously with proteins in CFA and then 2 and 4 wk later with the same protein in IFA. Peptide c-OVA, OVA, and SA85-1.1-III protein were diluted in PBS and then emulsified with an equal volume of adjuvant to allow immunizations of each mouse with 40 μg of protein in 200 μl. Mice were immunized at four sites of the back each time. Each mouse was injected i.p. with 5 × 104 trypomastigotes 2 wk after the last immunization.

Isolation of CD4 T cells
Mice were infected with 105 trypomastigotes i.p. (a sublethal dose) and were sacrificed at the indicated times. Spleens or periaortic and inguinal lymph nodes were removed from mice, mashed between sterile frosted glass slides, and the cells were suspended in 5 ml of DMEM supplemented with penicillin and streptomycin. Splenocytes were then incubated for 10 min at room temperature in an equal volume of 1.66% NH4 Cl to lyse RBCs. Cell suspensions were washed three times in DMEM by centrifugation at 200 × g for 10 min. Live cells were then quantitated with a hemocytometer by trypan blue exclusion and suspended at 5 × 106 cells/ml in supernatants of the following mAbs: RA3-3A1 (anti-B220), 53-6.72 (anti-CD8), and M5/114.15.2 (anti-I-A) for 15 min on ice (21). Cells were washed in DMEM, suspended in 10 ml PBS containing 1% BSA, 5 mM EDTA, and 0.01% azide. Cells were then suspended at 107 cells/90 μl and mixed with 10 μl of microbeads conjugated to anti-rat Abs (Miltenyi Bio-tech, Auburn, CA), incubated at 4°C for 30 min, and passed over a MACS depletion column (Miltenyi Biotech) according to the manufacturer’s protocol. The purity of the CD4 T cells isolated varied from 70–90%.

Isolation of APCs from spleens
Spleens were removed from naive mice and mashed between sterile frosted glass slides, and the cells were suspended in 5 ml of DMEM supplemented with penicillin and streptomycin. Splenocytes were then incubated for 10 min at room temperature in 5 ml of 1.66% NH4 Cl to lyse RBCs. Cell suspensions were washed three times in DMEM by centrifugation at 200 × g for 10 min, irradiated (3250 rad), washed, and suspended in complete RPMI medium.

Proliferation and cytokine assays
CD4 cells (2 × 105) and APCs (5 × 105) in 200 μl of complete medium with the indicated proteins were added to wells of flat-bottom 96-well plates and incubated at 37°C. For proliferation assays, after 72 h 1 μCi of [3H]thymidine was added to each well, and 16 h later the cells were analyzed for [3H]thymidine incorporation (21). For cytokine assays, at 24 or 72 h supernatants were harvested and analyzed by cytokine ELISAs. In some experiments the anti-MHC class II I-A1 mAb M5/114.15.2 or the anti-MHC class II I-E mAb 14.4.5S were present during the entire incubation at 30 μg/ml.

Limiting dilution assays
Graded numbers of CD4 cells (47–6000 cells/well, 36 wells/concentration) were cultured from 37°C for 10–14 days in 200 μl of complete medium with 5 × 105 APCs and 20 U/ml rIL-2 (Chiron Therapeutics), and with and without 20 μg of Ag (SA85-1.1-III or KLH) in flat-bottom 96-well plates. Each plate contained 24 control wells that contained APCs only. Then cells were washed three times and were cultured for an additional 24 h in complete medium with Con A (5 μg/ml). Supernatants were harvested, and each well was scored as positive or negative based on the presence or the absence of IL-2 using a highly sensitive CTL cell assay (26). Responder cell frequencies were estimated from the Poisson distribution relationship between the number of cells cultured and the logarithm of the fraction of negative wells (26).

High sensitivity CTLL assays
IL-2-dependent CTL cells were washed four times to remove any IL-2 and were cultured (50 cells/well) with an equal volume of supernatant taken from each limiting dilution assay well, in a final volume of 200 μl, for 3 days at 37°C. Ten microliters per well of rat Con A-activated spleen cell supernatant was added, and cells were incubated for an additional 4 days at 37°C. [3H]Thymidine (1 μCi/well) was added, and 20 h later the cells were analyzed for [3H]thymidine incorporation (27). Wells were scored as positive if the value was >3 SD above the mean of background cultures. Wells containing no CD4 cells were used to calculate the background (26).

ELISPOT
Ninety-six-well plates (MultiScreen-HA plates, Millipore, Millpore, Bedford, MA) were coated with 5 μg/ml of anti-mouse IFN-γ mAb (R4-6A2, PharMingen, San Diego, CA) in 1% BSA/PBS and incubated overnight at 4°C. Plates were washed four times with PBS and blocked for 2 h at 37°C with complete RPMI medium, and graded numbers of CD4 cells (three wells per dose) were cultured overnight at 37°C in 200 μl of complete medium with 105 APCs, with and without 20 μg/ml of SA85-1.1-III. Plates were then washed four times with PBS/Tween-20 (0.05%, v/v) and twice with PBS. Biotinylated anti-IFN-γ mAb (XM1G1.2, PharMingen; 5 μg/ml) was added, and the plates were incubated overnight at 4°C. Plates were washed four times with PBS/Tween-20 and twice with PBS, incubated with streptavidin-peroxidase (Genzyme, Cambridge, MA) for 1 h at room temperature, washed four times with PBS/Tween-20 and twice with PBS, and the substrate (50 mM Tris (pH 7.5), dianinobenzidine (1 mg/ml), and H2O2 (0.014%)) was added for 10 min at room temperature. The plates were washed extensively with H2O after removal of the silicone backing. Plates were allowed to dry at room temperature, and spots were quantitated using a dissecting microscope (28). Triplet wells with too many spots to count are not reported.

Cytokine ELISAs
IL-2, IL-4, and IFN-γ capture Abs were diluted in 0.1 M carbonate buffer, pH 9.5, as described by the manufacturer (Genzyme), to coat 96-well flat-bottom plates. Plates were incubated at 4°C overnight, washed twice with
PBS/Tween-20 (0.05%, v/v), and blocked with 4% BSA/PBS for 1 h at 37°C. Plates were washed twice with PBS/Tween, standards and samples were added and incubated for 3 h at 37°C and washed four times, and the detection Abs, diluted in 1% BSA/PBS, were added and incubated at 37°C for 1 h. The plates were then washed six times before adding streptavidin-peroxidase. After the final 1-h incubation and six washes, 100 μl/well of ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and the color was allowed to develop at room temperature. Plates were read immediately at 405 nm using an ELISA plate reader (EL311, Bio-Tek Instruments, Winooski, VT). The results are expressed as the mean of triplicate wells minus the background levels. The minimal value of the standard curves were: IL-2, 0.2 pg/ml; IL-4, 15 pg/ml; and IFN-γ, 15 pg/ml.

Statistics
The p values for parasitemia were determined using single-factor ANOVA (Microsoft Excel, Microsoft Corp., Redmond, WA). The p values for survival were calculated by determining the log rank statistic using Kaplan-Meier survival analysis (SPSS, Chicago, IL).

Results
CD4 cells isolated from T. cruzi-infected mice do not proliferate in response to SA85-1.1 protein
During an infection, T. cruzi surface proteins are released in the extracellular milieu and thus should stimulate a CD4 T cell response. The surface proteins, however, are encoded by a superfamily of variant epitopes that may subvert this CD4 T cell response (21). To investigate the response of CD4 T cells to the surface proteins during T. cruzi infection, enriched CD4 cells from lymph nodes or spleens of acutely and chronically infected mice were examined in in vitro proliferation assays (Fig. 1). CD4 T cells isolated from lymph nodes on day 14 of infection did not proliferate in response to the SA85-1.1-III protein (a protein that encodes the carboxyl region of the SA85-1.1 protein) or several other SA85-1 proteins (Fig. 1a) (21). In contrast, CD4 T cells isolated from lymph nodes following immunization with SA85-1.1 protein did proliferate in response to the SA85-1.1-III protein, indicating that the SA85-1.1 protein is immunogenic and that the enriched CD4 T cells can proliferate in response to SA85-1.1-III protein in the in vitro assay (Fig. 1b) (21). In addition, CD4 T cells isolated from the spleens of acutely and chronically infected mice did not proliferate in response to this same protein (Fig. 1c); CD4 T cells did proliferate in response to Con A, again indicating that these cells were alive and able to divide in this assay (Fig. 1c). Three possible explanations for the absence of proliferation by the SA85-1.1-specific CD4 T cells were that 1) these SA85-1 proteins were not expressed by the parasites during the infection, and therefore T cell responses to them were not stimulated; 2) the SA85-1 proteins were expressed, but the level of their expression or the state of APC activation was insufficient for naïve T cell stimulation; or 3) these SA85-1 proteins were expressed, but the T cell proliferative responses were inhibited. Direct analysis of SA85-1.1 protein expression during T. cruzi infection with anti-SA85-1 mAbs was not possible, as Abs with sufficient sensitivity were not available (data not shown). Instead, the ability of adoptively transferred SA85-1.1-specific CD4 T cell clones to specifically respond and provide protection against T. cruzi were used to determine whether the SA85-1.1 protein was expressed.

Adoptive transfer of SA85-1.1-specific T cell clones protects against T. cruzi
An SA85-1.1-specific Th1 clone, 1C11, that responds to epitope 1 within the SA85-1.1-III protein or a KLH-specific Th1 clone, CD6, was adoptively transferred into C57BL/6 mice. One day later the mice were infected with T. cruzi, and parasitemia and survival were determined (Fig. 2, a and b) (21). In the 1C11 recipient mice, parasitemia was lower (p < 0.002 on day 16; Fig. 2a), and survival was prolonged (log rank statistic, p < 0.002; Fig. 2b). In addition, mice were either adoptively transferred with the SA85-1-1 epitope 1-specific Th1 clone, 0.3C4, or mock adoptively transferred with medium (Fig. 2, c and d). In the 0.3C4 recipient mice parasitemia was lower (p < 0.05 on day 15; Fig. 2c), and survival was prolonged (log rank statistic, p < 0.002; Fig. 2d). These experiments strongly suggested that the SA85-1.1 protein was being expressed by T. cruzi and was being presented to CD4 T cells during the infection at levels sufficient to stimulate the adoptively transferred SA85-1.1-specific clones. These adoptive transfer experiments also suggested that immunization with a peptide encoding epitope 1 could provide protection against T. cruzi.

Immunization with SA85-1.1 epitope 1 also protects against T. cruzi
Mice were immunized with either a peptide that encodes epitope 1 (peptide c) coupled to OVA, the SA85-1.1-III protein, or OVA (21). Two weeks after completion of the immunizations, mice were infected with T. cruzi, and parasitemia and survival were monitored. Parasitemia was lower in mice immunized with peptide c (p < 0.008 on day 17) or SA85-1.1-III (p < 0.007 on day 17) than in mice immunized with OVA (Fig. 3a). Furthermore, survival was prolonged in the mice immunized with peptide c or SA85-1.1-III protein compared with that in the mice immunized...
with OVA (p < 0.008; Fig. 3b). Again, these data strongly argued that epitope 1 and the SA85-1.1 protein were expressed and presented to epitope 1-specific CD4 cells during the infection. Therefore, the failure of the naive, endogenous SA85-1.1-specific CD4 T cells to proliferate in the in vitro assays was not due to an absence of epitope presentation, but may be due to 1) low levels of SA85-1.1-protein expression, 2) inadequate APC activation, or 3) inhibition of the SA85-1.1-specific CD4 cells. Since the naive SA85-1.1-specific CD4 cells failed to proliferate, evidence of a specific response to SA85-1.1 protein during acute and chronic T. cruzi infection was investigated using sensitive limiting dilution analysis that did not depend on detection of proliferation. This assay is not sufficiently sensitive to detect Ag-specific MHC class II-restricted CD4 T cells, which are estimated to be present at a frequency of 1 in 10^6 (29).

**Limiting dilution analysis detects an SA85-1.1-specific CD4 T cell response**

Graded numbers of CD4 T cells isolated from spleens of T. cruzi-infected mice were cultured for 10 days with irradiated APCs from normal mice, specific Ag, and IL-2. In previously studied systems only CD4 cells stimulated by TCR-specific Ag remained viable after 10 days of culture (26). The cells were then washed and stimulated with Con A, and 24 h later the supernatants of each well were assayed for IL-2 (26, 27, 30). The detection of IL-2 indicates the presence of viable CD4 cells or a positive well. During the acute and chronic infection expanded populations of SA85-1.1-specific CD4 cells were detected (Fig. 4, a and b). The SA85-1.1 responder frequency on day 14 of infection was ~1 cell in 2900 (Fig. 4a), and that on day 81 was ~1 cell in 1200 (Fig. 4b). The KLH responder frequency was too small to be detected by this limiting dilution assay (Fig. 4, a and b). Additional studies have shown that the SA85-1.1-specific responder frequency is not detectable until after the fourth day of infection (our manuscript in preparation). These data indicate that during T. cruzi infection, the SA85-1.1 protein was expressed and that it specifically stimulated naive CD4 cells. These results did not indicate that these CD4 cells produce IL-2 during the natural infection or that they were able to produce IL-2 when stimulated with SA85-1.1 protein. The inability of CD4 cells to proliferate in response to SA85-1.1 protein in vitro assays suggests that these CD4 cells had become anergic.

**CD4 T cells isolated from infected mice and stimulated with SA85-1.1 protein produce IFN-γ**

Since anergic T cells, when stimulated with Ag, fail to proliferate or secrete IL-2, but may produce other cytokines, such as IFN-γ, CD4 T cells isolated from the spleens of acutely and chronically infected mice were cultured, and the supernatants were assayed for IFN-γ. (Fig. 4c, d). As expected, a large number of Ag-specific CD4 T cells produced IFN-γ in response to stimulation with SA85-1.1 protein. These data show that, in contrast to proliferation, IFN-γ production by anergic CD4 T cells was not blocked by the anergic state. This observation suggests that the anergic state did not impair cytokine production, but rather impaired other aspects of T cell function.
infected mice were stimulated with SA85-1.1-III protein and analyzed for the secretion of IL-2, IL-4, and IFN-γ by ELISA (22). The ELISAs did not detect any IL-2 or IL-4 secretion, but did detect IFN-γ secretion from acutely (Fig. 5a) and chronically (Fig. 5b) infected mice. The secretion of IFN-γ was inhibited by the anti-MHC class II I-A<sup>d</sup> mAb M5/114.15.2, and not by the control anti-MHC class II I-E mAb 14.4.4s (Fig. 5c), demonstrating that the secretion of IFN-γ was caused by the MHC presentation of SA85-1.1 epitopes to the CD4 T cells. These results indicate that during T. cruzi infection the SA85-1.1 protein stimulates the development of IFN-γ-producing CD4 cells. These cells appear to be long-lived as they are detected during the acute and chronic infection, yet their failure to proliferate to SA85-1.1 protein suggests that they have become anergic (22).

IFN-γ ELISPOT analyses suggest that many SA85-1.1-specific CD4 cells have differentiated into IFN-γ-producing cells

To further investigate the SA85-1.1-specific response during T. cruzi infection IFN-γ ELISPOT assays were performed on CD4 T cells isolated from acutely and chronically infected mice (Fig. 6). This assay demonstrated that the frequency of SA85-1.1-responsive CD4 cells that produce IFN-γ is ∼1 in 3000 CD4 T cells during the acute and chronic infection (Fig. 6). These data suggest that during T. cruzi infection many of the SA85-1.1-specific CD4 cells differentiate into IFN-γ-producing cells that become anergic.

Discussion

This study of the SA85-1-specific CD4 response during T. cruzi infection was initiated because 1) the T. cruzi surface proteins are encoded by a superfamily that encodes variant T cell epitopes; and 2) variant epitopes or altered peptide ligands have been shown to function as partial agonists and to impair normal T cell responses (15, 17, 21, 31). Initially, CD4 cells from acutely and chronically infected mice were assayed for proliferation following stimulation with several SA85-1 proteins (Fig. 1). No proliferative response was detected, suggesting that 1) these SA85-1 proteins were not expressed or were expressed at levels insufficient for naive T cell activation; 2) APC activation was inadequate; or 3) the SA85-1.1-specific T cells were inhibited. Adoptive transfer of SA85-1.1-specific T cell clones and immunization with a peptide encoding a SA85-1.1-specific CD4 T cell epitope protected mice from T. cruzi infection, strongly arguing that the SA85-1.1 protein was expressed (Figs. 2 and 3). Additional analyses of the endogenous
CD4 T cells revealed that during the infection the SA85-1.1-specific cells had expanded and differentiated into IFN-γ-producing cells (Figs. 4–6). Together these data suggested that during T. cruzi infection a potentially protective SA85-1.1-specific response becomes inhibited. T cell anergy can be defined as a failure of T cells to proliferate to Ag when they are provided with optimal in vitro conditions (22). Anergic CD4 T cells fail to produce IL-2, but the production of effector cytokines, such as IFN-γ, can occur (22). In this report CD4 cells isolated from T. cruzi-infected mice and presented with SA85-1.1 protein by normal APCs fail to proliferate or produce IL-2, but produce IFN-γ. Thus, the SA85-1.1-specific CD4 cells appear to be anergic; the mechanism of anergy induction remains unclear.

Mouse T cells have been shown to become anergic following 1) CTLA-4 receptor engagement with B7 molecules, 2) TCR stimulation in the absence of costimulation, 3) T cell exposure to IL-10, 4) T cell stimulation in the presence of excess Ag, and 5) TCR stimulation with altered peptide ligands or variant epitopes (22, 29, 32). Data are not available to determine whether these mechanisms contribute to the SA85-1.1-specific CD4 T cell response during T. cruzi infection. Aspects of T cell costimulation have been investigated. One study revealed decreased CD28 expression by peripheral T cells isolated from T. cruzi chronically infected patients, suggesting that inadequate costimulation may contribute to an anergic T cell response (33); however, other studies have demonstrated increased B7-2 and CD40 expression on T. cruzi-infected mouse macrophages, suggesting that costimulation is adequate during T. cruzi infection (34, 35). In addition, during T. cruzi infection IL-10 is abundantly produced and has been shown to inhibit the immune response, but the effect on the T cell response has not been examined (7, 36).

In the CL strain of T. cruzi, two families of the sialidase surface protein superfamily, the SA85-1 and FL160 families, have been characterized (37–40). The SA85-1 proteins, encoded by >100 genes, are expressed by trypomastigotes and amastigotes, whereas the FL160 proteins, encoded by >750 genes, are expressed by trypomastigotes only (37, 38, 40). These two large families appear to represent a small fraction of the surface protein superfamily (38, 40, 41). In vitro experiments have suggested that surface protein variation may inhibit the T cell response as many T. cruzi surface protein variant epitopes are processed and presented to T cells, and some of these variant epitopes function as partial agonists (21).
These in vitro studies, however, did not demonstrate that *Trypanosoma cruzi* variant epitopes induce anergy (21). In this report a robust CD4 T cell response to the SA85-1 proteins has not been detected. Rather, the response of SA85-1.1-specific CD4 cells is consistent with an anergic response, and this response may be stimulated by variant epitopes encoded by the SA85-1 proteins and the related surface protein superfamily members.

Precedence for infectious agents using variant T cell epitopes to evade T cell responses comes from studies on malaria. Two strains of *Plasmodium* were identified that encode variant forms of a MHC class I-restricted T cell epitope (18). In vitro these variant epitopes inhibit T cells responding to the epitope of the other strain (18). In vivo these two strains are found to cohabit at an increased frequency only in those individuals whose HLA type permitted presentation of the variant epitopes (18). These data indicate that antagonistic variant epitopes facilitate parasite survival in vivo (18). Further evidence that infectious agents use variant T cell epitopes to evade T cells comes from studies on HIV and hepatitis B virus (19, 20). During an infection these viruses mutate and appear to generate MHC class I-restricted variant epitopes that inhibit protective T cell responses (19, 20). *Trypanosoma cruzi*, rather than mutate and generate variant epitopes during an infection, appears to express a superfamily of surface proteins that encodes many variant epitopes capable of inhibiting the CD4 T cell response (21). It is also possible that the *Trypanosoma cruzi* surface proteins encode variant epitopes capable of inhibiting the MHC class I-restricted CD8 T cell response.

During *Trypanosoma cruzi* infection of mice, T cells secreting IFN-γ have been detected, but a robust Th1 response has not been observed (12, 42). One study isolated splenocytes from infected mice and stimulated them in ex vivo assays with epimastigote lysates. Some IFN-γ production was noted only during the acute phase before peak parasitemia occurred (11). Another study examined CD4 cells isolated from acutely infected mice by stimulating them in vitro assays with Con A or trypomastigote lysate; these cells secreted IFN-γ, but no IL-2 or IL-4. The proliferation of these cells was inhibited by nitric oxide, suggesting that their development into a robust Th1 response was inhibited (6). It is possible that the SA85-1.1-specific CD4 response is also depressed by nitric oxide. In the previous study, however, the CD4 T cell proliferation improved with APCs from uninfected mice (6), whereas in the current study the SA85-1.1-specific T cells failed to proliferate in assays using APCs from uninfected mice (Fig. 1). In addition, inhibition of nitric oxide production in vivo during *Trypanosoma cruzi* infection and in vitro in subsequent T cell proliferation assays did not restore SA85-1.1-specific proliferation (our manuscript in preparation).

Many factors can influence the development of CD4 Th cells into Th1 or Th2 subsets (43). These factors include the cytokine environment, the type of APC, the costimulatory signals, the amount of Ag, and the affinity of the epitope-TCR interaction (43). During *Trypanosoma cruzi* infection, all these factors may influence the SA85-1.1-specific CD4 T cell response. In particular, the Ag dose and the affinity of the epitope-TCR interaction may be influenced by the variant epitopes encoded by the *Trypanosoma cruzi* surface proteins. The effect of Ag dose on Th development is controversial; some studies have suggested that a low Ag dose favors Th1 responses and a high Ag dose favors Th2 responses; other studies have suggested the opposite (43). It is possible that *Trypanosoma cruzi*, by expressing many variant surface proteins rather than a single surface protein, decreases the Ag dose of each surface protein epitope (21). If the surface protein variation does decrease the Ag dose, then the results presented here (Figs. 5 and 6) suggest that a low Ag dose favors the development of Th cells that produce IFN-γ.

The adoptive transfer studies indicate that an increased responder frequency of SA85-1.1-specific IFN-γ producing CD4 cells provides protection against *Trypanosoma cruzi* (Fig. 2). This protection may be due to the early response of large numbers of transferred T cells that subsequently become inhibited in the same way as the endogenous SA85-1.1-specific T cells became inhibited. Alternatively, it is possible that during *Trypanosoma cruzi* infection the differentiated transferred Th1 clones cannot be inhibited. Determining whether the T cell inhibition is specific for naïve T cells might be ascertained by analyzing the response of both adoptively transferred SA85-1.1-specific Th1 clones and SA85-1.1-specific naïve T cell clones during *Trypanosoma cruzi* infection (44). It will also be of interest to determine whether the inhibited T cell response is specific for highly polymorphic surface proteins. If the inhibition of the SA85-1.1-specific T cells is caused by the epitope variation, then T cells specific for nonpolymorphic proteins will not be inhibited. These studies will provide insights into the mechanisms used by *Trypanosoma cruzi* to inhibit the T cell response.

Previous studies have not identified *Trypanosoma cruzi* immunodominant T cell Ags, and perhaps because immunodominant Ags have not been identified, Ag-specific responses during *Trypanosoma cruzi* infection have not been examined in detail. Immunodominant T cell Ags may not exist because of both the T cell inhibition and the massive polyclonal T cell activation that occur during *Trypanosoma cruzi* infection (45). The results presented here permit further analyses of the SA85-1-specific T cell response, and the mechanisms affecting this Ag-specific response.

During *Trypanosoma cruzi* infection, endogenously produced or exogenously administered IFN-γ are protective, suggesting that the production of more IFN-γ during *Trypanosoma cruzi* infection would improve the protective response (13, 46, 47). Since IFN-γ stimulates the development of Th1 responses, one can speculate that increased amounts of IFN-γ during *Trypanosoma cruzi* infection would stimulate the development of a more robust Th1 response against the parasite (43). The anergic status of the SA85-1.1-specific CD4 cells may limit the expansion of these Ag-specific IFN-γ secreting and compromise the development of a more robust Th1 response. Why the host-parasite interactions evolved to stimulate such a SA85-1.1-specific response remains unclear. One can speculate that the development of this inhibited SA85-1.1-specific IFN-γ response attains a compromise that benefits the parasite by limiting its growth, while permitting its survival, and benefits the host by providing protection, while limiting autoimmune or inflammatory damage.

The results presented here are consistent with previous studies that the T cell response during *Trypanosoma cruzi* infection is depressed (45). The results demonstrate for the first time during *Trypanosoma cruzi* infection 1) the depression of a specific MHC class II-restricted response, and 2) the presence of an Ag-specific CD4 IFN-γ response. In addition, the results demonstrate the development of an anergic IFN-γ response during *Trypanosoma cruzi* infection. To our knowledge the development of an anergic CD4 IFN-γ response during an infection has not been previously described.

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**References**


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