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Costimulation of Host T Lymphocytes by a Trypanosomal trans-Sialidase: Involvement of CD43 Signaling

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Trans-sialidase is a membrane-bound and shed sialidase from Trypanosoma cruzi, the protozoan parasite responsible for Chagas disease (1, 2). We investigated the role of soluble trans-sialidase on host CD4+ T cell activation. Trans-sialidase activated naïve CD4+ T cells in vivo. Both enzymatically active and inactive recombinant trans-sialidases costimulated CD4+ T cell activation in vitro. Costimulation resulted in increased mitogen-activated protein kinase activation, proliferation, and cytokine synthesis. Furthermore, active and inactive trans-sialidases blocked activation-induced cell death in CD4+ T cells from T. cruzi-infected mice. By flow cytometry, inactive trans-sialidase bound the highly sialylated surface Ag CD43 on host CD4+ T cells. Both costimulatory and antiapoptotic effects of trans-sialidases required CD43 signaling. These results suggest that trans-sialidase family proteins are involved in exacerbated host T lymphocyte responses observed in T. cruzi infection. The Journal of Immunology, 2002, 168: 5192–5198.

Induction of T lymphocyte activation is crucial for host defense against infection by Trypanosoma cruzi, the intracellular protozoan parasite responsible for Chagas disease (1, 2). However, the parasite induces widespread polyclonal lymphocyte activation before the immune system mounts focused T cell responses, a condition that leads to immunopathology and represents a main obstacle for effective vaccination (3). In fact, exacerbated T cell activation before the immune system mounts focused T cell responses in vitro through activation of accessory cell function (15). However, the interaction of TS with CD4+ T cells from normal and T. cruzi-infected hosts was not investigated. In this study, we report that soluble T. cruzi recombinant TS (rTS) induces CD4+ T cell activation in vivo and costimulates naïve CD4+ T cells in vitro. In CD4+ T cells from infected hosts, TS exacerbates mitogenic responses and rescues T cells from activation-induced cell death (AICD). By flow cytometry, an enzymatically inactive mutant TS binds the highly sialylated mucin CD43 on the T cell surface, and CD43 engagement is required for costimulation and rescue from AICD. Therefore, T. cruzi TS is a natural CD43 ligand with T cell-activating properties and is a candidate molecule for induction of immunopathology during T. cruzi infection.

Materials and Methods

Mice and T. cruzi infection

Male BALB/c mice, ages 4–5 wk, were obtained from the Instituto Oswaldo Cruz (Instituto Oswaldo Cruz-Fundaçao Oswaldo Cruz, Rio de Janeiro, Brazil) animal facility. Mice were infected (10^7 parasites/0.1 ml s.c.) with chemically induced metacyclic trypomastigotes from T. cruzi clone Dm28c, obtained as described previously (16). Uninfected littermates were used as controls. CD43−/− and wild-type control mice (17) were a gift from Dr. M. Correa (Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil) and were infected with T. cruzi as above. Cells from infected mice were obtained 21–25 days after infection. All experiments were conducted according to protocols approved by the Committee on Ethics and Regulations of Animal Use of the Instituto de Biofísica Carlos Chagas Filho.

Native trans-sialidase

Dm28c T. cruzi metacyclic trypomastigotes were centrifuged, and the cell pellet was lysed at 4°C in 50 mM Tris-HCl, 2% Nonidet P-40, 0.1 mM PMSF, 5 µg/ml leupeptin, 0.1 mM iodoacetamide, and 0.1 mM EDTA. The lysate was processed by affinity chromatography on Sepharose 4B-Con A and eluted with α-methyl mannoside (0.1 M). The eluate was applied to a Mono Q (HR 10/10) column followed by a Mono S (HR 5/5) column (Pharmacia Biotech, Uppsala, Sweden), and the enzyme was eluted with a linear gradient of NaCl (0–1 M). The homogeneity of the enzyme was evaluated by 10% SDS-PAGE; it migrated as a band of ~60 kDa.
Recombinant TS

rTS and inactive TS (irTS) containing the C-terminal repeats were obtained from *Escherichia coli* MC1061 electrotransformed with plasmids containing either the wild-type TS insert, TSREP.C (18), or the inactive mutant TS insert bearing a Tyr^142→His^142 substitution, pTRFHeiAs (18). Bacteria were grown in supplemented terrific broth medium in the presence of 100 μg/ml ampicillin. When OD₆₀₀ reached an A₆₀₀ of 0.5–0.6, 30 μg/ml of β-nitroglutathione was added and incubation was continued overnight. Bacteria were lysed at 4°C in 20 mM Tris-HCl containing 2.0 mg/ml lysozyme, 2% Triton X-100, 0.1 mM PMSF, 5.0 μg/ml leupeptin, 1.0 μg/ml trypsin inhibitor, and 0.1 μM iodoacetamide. Both rTS and irTS contained a poly(His) tag and were purified as described by Buschiazzo et al. (19), modified by Todeschini et al. (8), using Ni²⁺ chelating chromatography on a HiLoad 16/60 Nickel-NTA affinity gel (Amersham Biosciences) eluted with imidazol gradient (20 mM Tris-HCl, 150 mM NaCl, 200 mM imidazol, pH 8). The eluates were applied to Mono Q and Mono S columns as described above. The homogeneity of the proteins was evaluated by 10% SDS-PAGE. rTS and irTS were stored in 20 mM Tris-HCl buffer (pH 7.4) at 4°C until use. For in vivo experiments and for cultures in the presence of dendritic cells (DCs), rTS and irTS were passed through an agarose-immobilized polymyxin B column (Sigma-Aldrich, St. Louis, MO) to obtain a LPS-free preparation. The LPS content of TS preparations was quantitated by the *Limulus* amoebocyte lysate assay (Charles River Endosafe, Charleston, SC) and was below detection. For flow cytometry (FCM) and Western blotting analyses, rTS was FITC or biotin conjugated as described elsewhere (20).

**TS activity measurements**

TS activity was assayed by incubating enzyme preparations in 5 mM calcium chloride buffer (pH 7.0) in the presence of 0.25 μM of 2,3-sialylactose and 0.25 μM of [α-D-glucose-1,4-C]lactose (6). After incubation at 37°C for 30 min, the reaction mixture was diluted with 1 ml of water and applied to a column containing 1 ml of Dowex 2X8 (acetate form) equilibrated with water. [α-D-Glucose-1,4-C]lactose was eluted by washing with 9 ml of distilled water, while sialylated [α-D-glucose-1,4-C]lactose was eluted with 3 ml of 0.8 M ammonium acetate. The sialylated product was quantitated by beta scintillation counting (Beckman LS 6500; Beckman Coulter, Fullerton, CA). The enzyme concentration was adjusted to 1 μM/μg protein (1 U was defined as the amount of enzyme required to catalyze the incorporation of 1 μmol of sialic acid into *N*-acyethyl lactosamine per minute).

**Antibodies**

Anti-CD43 mAb S7, anti-CD45 mAb 16A, PE-labeled anti-B7.2, anti-B220, anti-CD3, anti-MA-CI, anti-CD16/32 (2.4G2), FITC or PE-labeled anti-CD3 and anti-CD8 mAbs, and FITC-labeled anti-CD4 mAb were purchased from BD PharMingen (San Diego, CA). Anti-αβT细胞 mAb H57.597 (10% v/v culture supernatant), native or rTS (15 μg/μl/ml, anti-CD43 Fab, control Fab fragments (50 μg/ml), and anti-cruciferin mutant (15 μg/μl/ml, B. napus cv. cruentum) was purified from G strain epimastigotes as described elsewhere (23). For proliferation assays, cultures were incubated for 3 days at 37°C and 7% CO₂ in a humid atmosphere and pulsed with 0.5 μCi of tritiated thymidine ([³H]Tdr), 5.0 Ci/mmol; Sigma-Aldrich) 18 h before harvest. Cultures were terminated with a semiautomated cell-harvesting device and the amount of [³H]Tdr incorporated into DNA was assessed by liquid scintillation spectrometry. Results shown are means of triplicate wells.

**Proliferation and viability assays**

CD4^+^ T cells (2 × 10⁷ or 2 × 10⁸) were resuspended in culture medium containing 1% Nutridoma (Sar-Nutridoma; Boehringer Mannheim, Indianapolis, IN) instead of PBS. Cultures (0.2 ml or 1.0 ml) were established in 96- or 24-well vessels (Corning Glass Works, Corning, NY), respectively. All cultures, except those in the presence of DCs (5 × 10⁴ DCs/0.2 ml well), were treated with PMA (Sigma-Aldrich) at 0.5 ng/ml. The stimuli used were: anti-αβT细胞 mAb H57.597 (10% v/v culture supernatant), native or rTS (15 μg/μl/ml, anti-CD43 Fab, control Fab fragments (50 μg/ml), and anti-cruciferin mutant (15 μg/μl/ml, B. napus cv. cruentum) was purified from G strain epimastigotes as described elsewhere (23). For proliferation assays, cultures were incubated for 3 days at 37°C and 7% CO₂ in a humid atmosphere and pulsed with 0.5 μCi of tritiated thymidine ([³H]Tdr), 5.0 Ci/mmol; Sigma-Aldrich) 18 h before harvest. Cultures were terminated with a semiautomated cell-harvesting device and the amount of [³H]Tdr incorporated into DNA was assessed by liquid scintillation spectrometry. Results shown are means of triplicate wells. Highly purified CD4^+^ T cells were stimulated with a submitogenic dosage of anti-CD3 mAb 145-2C11 (1/5000 v/v dilution of ascites fluid) coated on 96-well flat-bottom wells in the presence or absence of TS, with or without PMA. Supernatants were collected after 48 h. Proliferation was measured in six sister cultures after 3 days. For viability assays, CD4^+^ T cells from T. crucifera-infected mice were cultured as above. After 20 h, cultures were dispersed and counted by trypan blue exclusion. Mean viable cell recovery in unstimulated (PMA alone) cultures was taken as reference. Percent cell loss (the reciprocal of remaining viable cells) was calculated for each individual well according to the formula: percent cell loss = 100 – (viable cell number in stimulated culture) / (viable cell number with PMA alone). Mean and SE of triplicate cultures are shown. Negative values of cell loss indicate lack of AICD and a block of spontaneous death; positive values indicate AICD.

**ELISA**

For cytokine production, CD4^+^ T cells (1 × 10⁹/1.0 ml) were stimulated as above. Levels of IFN-γ, TNF-α, IL-2, and IL-4 were determined in culture supernatants harvested 24 h (for IL-2) or 48 h after initiation and kept at −20°C until assayed. Cytokine levels were measured in sandwich ELISA using pairs of specific mAbs, one of which was biotinylated (BD PharMingen) and developed with streptavidin-alkaline phosphatase (BD PharMingen) and p-nitrophenylphosphate substrate.

**Western blotting and TS-labeling analyses**

For Western blotting, CD4^+^ T cells were obtained and cultured as above. After 4 h, 1 × 10⁷ cells were pooled, centrifuged, and washed twice in ice-cold PBS. The cell pellet was resuspended in 0.4 ml of ice-cold lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM NaCl, 1 mM p-chloromercuribenzenesulfonic acid, 1 mM PMSF, 5.0 μg/ml leupeptin, and 0.1 μM iodoacetamide, and incubated at 4°C for 1 h. The nuclear fraction was pelleted by centrifugation and the supernatant was referred to as cytosolic extract (24). The nuclear pellet was washed and resuspended in 0.1 ml of ice-cold lysis buffer supplemented with protease inhibitors plus 1% Triton X-100 and incubated at 4°C for 1 h (nuclear extract). Total protein was measured, 30 μg of protein/ lane was electrophoresed on 10% SDS-PAGE at 100 mV, and electrotansferred to a nitrocellulose membrane. After overnight incubation (5% milk in TBS and 0.2% Tween 20), the blot was reacted with anti-ERK-1/2 Ab,
followed by HRP-conjugated anti-goat IgG. The reaction was detected using ECL in Hyperfilm-ECL according to the manufacturer (Amersham, Arlington Heights, IL).

FCM analysis of TS binding to lymphocytes

For TS-binding studies, splenic naive CD4⁺ T cells were washed in sorting buffer (containing 2% BSA instead of FBS) and incubated with Fe block (10 μg/ml), followed by addition of 50 μg/ml unlabeled irTS, anti-CD45 mAb S7, or anti-CD45 mAb 16A. After 30 min at 4°C, FITC-conjugated irTS (10 μg/ml) was added for 30 min at 4°C. Cells were then washed and resuspended in sorting buffer containing 2% paraformaldehyde. Lymphocytes were gated by forward and side scatter parameters, and 10,000 cells were analyzed on a FACSCalibur system using CellQuest software.

Statistics

Data were analyzed by Student’s t test for independent samples using a SigmaPlot for Windows (version 4.01) package. Differences with a p value of <0.05 were considered to be significant.

Results

Trypanosomal TS activates host CD4⁺ T cells in vivo

To examine whether T. cruzi TS activates host CD4⁺ T cells in vivo, rTS emulsified in IFA was injected into the footpads of naive BALB/c mice. After 5 days, cell number in lymph nodes draining TS was 2.6-fold higher than in control nodes draining PBS-IFA (data not shown). Injection of rTS induced T cell activation in vivo, as measured by an increase in percentage and absolute number of CD4⁺ T cells expressing a CD44high phenotype, compared with control T cells from nodes draining PBS-IFA (Fig. 1). Furthermore, injection of T. cruzi mucin emulsified in IFA did not result in enlarged lymph nodes (data not shown), and CD4⁺ T cells from draining lymph nodes did not express enhanced levels of CD44 (Fig. 1).

Trypanosomal TS costimulates host naive CD4⁺ T cell activation

The mechanism of T cell stimulation by TS was investigated in vitro using naive BALB/c splenic CD4⁺ T lymphocytes (containing around 10% endogenous APCs) as responder cells. Both native and rTS failed to induce T cell mitogenesis. However, in the absence of PMA as costimulus, both native and rTS induced polyclonal CD4⁺ T lymphocyte activation in vitro, as measured by a dose-dependent increase of T cell proliferation (Fig. 2A). The costimogenic effect of rTS, but not of anti-TCR mAb, was selectively eliminated by adding the soluble TS substrate N-acetyllactosamine (Fig. 2B). At suboptimal (3 μg/well), but not optimal, concentrations, TS (both native and recombinant) and anti-TCR mAb synergized to induce CD4⁺ T cell proliferation (Fig. 2C). In addition, rTS also costimulated CD4⁺ T cells from T. cruzi-infected mice (data not shown). An enzymatically inactive form of TS (irTS), containing a Tyr³⁴²→His³⁴² substitution (18), induced naive T cells

FIGURE 1. T. cruzi TS activates CD4⁺ T cells in vivo. Naive mice were injected with saline (dotted line), T. cruzi mucin (thin line), or T. cruzi rTS (thick line), emulsified in IFA, in the hind footpads. Draining lymph node cells were stained 5 days later. CD4⁺ cells were gated and analyzed for CD44 expression by FCM.

FIGURE 2. T. cruzi TS activates CD4⁺ T cells in vitro. A. Dose-dependent induction of mitogenesis in naive splenic CD4⁺ T cells by rTS in the presence of PMA. Dosage of rTS refers to micrograms of rTS added per well (0.2 ml). Proliferation was measured by [³H]TdtR uptake after 3 days in culture. All experiments in this figure were done in the presence of PMA as costimulus. Results are mean and SE of triplicate cultures. B. Activation by rTS is reversed by a soluble TS substrate. CD4⁺ T cells were treated with optimal doses of the indicated stimuli in the absence (○) or presence (+) of the soluble TS substrate N-acetyllactosamine (1 mM). Lactosamine blocked the response to TS (p < 0.01), but not to anti-TCR (NS). C. Synergism between TCR and rTS stimulation. Naive CD4⁺ T cells were treated with suboptimal doses of anti-TCR mAb, rTS, or both, and mitogenesis was evaluated 3 days later by [³H]TdtR uptake. D. An inactive TS mutant activates T cells. Naive CD4⁺ T cells were stimulated with native (n), recombinant (r), inactive mutant recombinant (ir) TS, or with T. cruzi mucin (muc), all at 3 μg/well, and proliferation was measured. E. TNF-α production elicited by TS. CD4⁺ T cells were treated with optimal doses of rTS or irTS in the absence or presence of anti-TCR and with or without 1 mM N-acetyllactosamine. After 48 h, supernatants were harvested and assayed for levels of TNF-α by sandwich ELISA.
cell activation in the presence of PMA (Fig. 2D), suggesting that sialic acid transfer is not required for T cell costimulation. Under the same conditions, a GPI anchor-containing mucin fraction from *T. cruzi* did not induce T cell mitogenesis (Fig. 2D). Addition of rTS to naive CD4^+^ T cells increased IL-2 secretion driven by TCR ligation (data not shown). Although TCR stimulation induced little or no secretion of TNF-α by T cells, costimulation by rTS induced TNF-α secretion (Fig. 2E). Secretion of TNF-α was eliminated by soluble TS substrate N-acetylectosamine (Fig. 2E). To investigate whether exposure of CD4^+^ T cells to TS affected mitogenic intracellular signaling, we tested by immunoblotting the nuclear translocation of MAP kinases ERK-1 and ERK-2 from CD4^+^ T cell extracts (Fig. 3). Both rTS and TCR ligation induced nuclear translocation of ERK-1/2 in naive CD4^+^ T lymphocytes (Fig. 3, lanes 2 and 3), compared with treatment with PMA alone (Fig. 3, lane 1). A synergism between rTS and TCR signaling was seen with nuclear MAP kinase translocation (Fig. 3, lane 4).

As TS activates naive CD4^+^ T cells in vivo, but requires phorbol ester to activate APC-depleted T cells in vitro, it was possible that potent APCs, such as DCs, were necessary for induction of T cell activation in the absence of phorbol ester. To investigate this possibility, we stimulated splenic naive CD4^+^ T cells with TS in the presence of purified splenic DCs and in the absence of serum and phorbol ester. Syngeneic splenic DCs induced significant activation of CD4^+^ T cells in the absence of exogenous stimuli, a phenomenon known as the syngeneic MLR (25). Both rTS and irTS increased CD4^+^ T cell mitogenesis induced by syngeneic DCs, while *T. cruzi* mucin had no effect (Fig. 4). These results indicate that TS costimulates autoreactive T cell responses induced by syngeneic DCs.

A recent study suggested that TS costimulates T cell responses indirectly through effects on macrophages and B cells (15). To investigate whether TS could interact directly with T cells, highly purified CD4^+^ T cells were stimulated with a low density of plate-bound anti-CD3, an accessory cell-independent stimulus. Soluble irTS markedly increased T cell proliferation, while *T. cruzi* mucin had no effect (Fig. 5A). In addition, soluble irTS potently increased production of the cytokine IL-4 by purified T cells (Fig. 5B). Although these effects were exacerbated by PMA (Fig. 5), costimulatory effects were also seen in the absence of PMA (data not shown). Addition of irTS induced cell spreading in CD3-stimulated purified T cells (data not shown). These results indicate that TS can act directly on T cells.

**FIGURE 3.** *T. cruzi* TS induces ERK-1/2 nuclear translocation in CD4^+^ T cells. Naive splenic CD4^+^ T cells were stimulated with: PMA alone (lane 1), PMA plus anti-TCR (lane 2), rTS (lane 3), or anti-TCR plus rTS (lane 4) for 4 h. Cytosolic (A) and nuclear (B) extracts were obtained, equal protein amounts were resolved by SDS-PAGE, electrotransferred, and blotted with an anti-ERK-1/2 polyclonal Ab.

**FIGURE 4.** *T. cruzi* TS costimulates T cell responses to syngeneic DCs. Naive splenic CD4^+^ T cells (2 × 10^5_) were cultured with purified splenic DCs (2 × 10^5_), and also with: *T. cruzi* mucin (muc), inactive recombinant (ir), active recombinant TS (rTS), all at 3 μg/well, plus control or anti-CD43 Fab fragments at 10 μg/well. All cultures were done in the absence of any costimulus. Proliferation was measured after 48 h in culture. Results are mean and SE of triplicate cultures. Both rTS (p < 0.05) and irTS (p < 0.01) activated T cells; anti-CD43 Fab (p < 0.05), but not control Fab (NS) blocked the response to TS.

**FIGURE 5.** *T. cruzi* TS costimulates highly purified CD4^+^ T cells. Highly purified naive splenic CD4^+^ T cells (> 97% CD4^+^ cells) were cultured with a low density of anti-CD3 mAb 2C11 in the presence of PMA and in the presence or absence of irTS or *T. cruzi* mucin (MUC). A Proliferation was assessed by [^3]H-Tdr uptake after 3 days in culture. B, IL-4 content was measured by sandwich ELISA in culture supernatants collected after 48 h. Results are mean and SE of triplicate cultures.

An inactive trypanosomal trans-sialidase binds CD34 on T cells

CD34 and CD45 are mucins that express high amounts of sialic acid and are abundantly expressed by T cells (26, 27). These surface Ags could potentially interact with TS. Since irTS costimulates T cells and since irTS acts like a lectin on RBC (18), we used FITC-labeled irTS and FCM to investigate the interaction of TS with host T cells. Naive splenic CD4^+^ T cells were subjected to different pretreatments and subsequently stained with FITC-irTS. Binding of FITC-irTS to CD4^+^ T cells was prevented by 96% with pretreatment with excess (50 μg/ml) unlabeled irTS (Fig. 6). Although pretreatment with excess (50 μg/ml) anti-CD45 mAb 16A gave only marginal inhibition of FITC-irTS binding (17% reduction in median channel fluorescence), pretreatment with anti-CD34 mAb S7 blocked FITC-irTS binding almost completely, i.e., by 90% (Fig. 6). In agreement with these data, both irTS and anti-CD34 mAb S7 bound the same 115-kDa protein band on CD4^+^ T cell extracts expected for CD43, while anti-CD45 mAb 16A recognized a single 190-kDa band expected for the CD45 isoform expressed by splenic CD4^+^ T cells (data not shown). These results suggest that *T. cruzi* irTS binds CD34.

Costimulatory effects of trypanosomal trans-sialidases are dependent on CD34

To investigate whether CD43 is involved in the costimulatory effects of TS on CD4^+^ T cells, we made monovalent anti-CD43 Fab fragments to act as specific antagonists of CD43 engagement. We
tested Fab fragments from anti-CD43 mAb S7 and from a rat isotype control mAb (D7; IgG2a) that binds Ly-6A/E molecules on T cells (21). Addition of anti-CD43 Fab, but not control Fab fragment, completely blocked costimulatory effects of *T. cruzi* rTS on naive CD4⁺/H11001 T cells in the absence (Fig. 7A) or in the presence of TCR stimulation (Fig. 7B). We also tested the effect of anti-CD43 Fab on responses induced against syngeneic DCs. Anti-CD43 Fab had no effect on syngeneic T cell mitogenesis induced by DCs alone (data not shown), but blocked the costimulatory increase elicited by rTS by 76%, while control Fab had no effect (Fig. 4). These results suggest that CD43 is involved in TS-mediated costimulation.

CD4⁺ T cells from *T. cruzi* infection undergo intense AICD (28). Using infected mice, we investigated the effect of *T. cruzi* TS on T cell apoptosis. Exposure of T cells to rTS or irTS failed to induce apoptosis and also rescued T cells from AICD induced by TCR engagement (Fig. 7C). We investigated whether CD43 engagement was involved in rescue from apoptosis. Addition of anti-CD43 Fab, but not control Fab, blocked the rescue mediated by rTS (Fig. 7C). Addition of an equimolar dosage of a GPI anchor-containing *T. cruzi* mucin had no effect on TCR-induced T cell death (data not shown). Moreover, exposure to intact anti-CD43, but not to anti-CD45 mAb, mimicked the effect of TS and rescued T cells from AICD (Fig. 7C). Finally, we used CD43⁻/⁻ mice (17) infected with *T. cruzi* to confirm the pivotal role of CD43 on TS effects. CD4⁺ T cells from both wild-type and CD43⁻/⁻ mice underwent AICD following TCR stimulation (Fig. 7D). Treatment with rTS rescued wild-type (Fig. 7D, left), but not CD43⁻/⁻ T cells from TCR-induced death (Fig. 7D, right). These results suggest that CD43 expression is required for antiapoptotic effects of rTS on CD4⁺ T cells.

**FIGURE 6.** *T. cruzi* irTS binds CD43 on host CD4⁺ T cells. Naive splenic CD4⁺ T cells were pretreated as indicated with medium or with excess (50 μg/ml) irTS, anti-CD43, or anti-CD45 mAbs. Cells were subsequently stained with FITC-labeled irTS and PE-labeled anti-CD4, electronically gated for CD4 staining, and analyzed by FCM.

**FIGURE 7.** Immunopotentiating effects of *T. cruzi* TS are mediated by CD43 signaling. A and B, T cell mitogenesis. Naive splenic CD4⁺ T cells were stimulated in the absence (A) or presence (B) of suboptimal doses of anti-TCR mAb, with or without rTS, as indicated. Monovalent anti-CD43 or control Fab fragments were included (10 μg/well). All cultures were treated with PMA. Proliferation assessed after 3 days in culture. Results are mean and SE of triplicate cultures. Anti-CD43 Fab (p < 0.05), but not control Fab (NS) blocked TS effects. C and D, Rescue from activation-induced apoptosis. Splenic CD4⁺ T cells from *T. cruzi*-infected BALB mice (C) were treated or not with anti-TCR mAb in the presence of PMA and the following reagents: rTS, irTS, rTS plus anti-CD43 Fab, rTS plus control Fab, intact anti-CD43 mAb S7 (10 μg/ml), and intact anti-CD45 mAb 16A (10 μg/ml). After 20 h, viable cell counts were determined and percent cell loss was calculated, taking unstimulated cell recovery (PMA alone) as reference. Note that TS completely blocked TCR-induced AICD (p < 0.01); anti-CD43 Fab (p < 0.05), but not control Fab (NS), prevented this TS effect. Anti-CD43, but not anti-CD45 mAb, also blocked TCR-induced T cell death. In addition, CD4⁺ T cells from *T. cruzi*-infected wild-type or CD43⁻/⁻ mice (D) were treated (+) or not (−) with anti-TCR in the absence or presence of rTS, as indicated. All cultures received PMA. After 20 h, percent cell loss was determined. Results are mean and SE of triplicate cultures. TS rescued wild-type (p < 0.01), but not CD43⁻/⁻ T cells (NS) from AICD.
Discussion

CD43 is an abundant mucin expressed on hemopoietic cells, with ubiquitous physiological relevance in cell-cell interactions (26). High surface density, pronounced length of protruding molecules, and abundance of sialic acid residues α2,3 linked to βGalp in O-linked sialyloligosaccharides (26) makes CD43 a candidate receptor for T. cruzi TS. Cross-linkage of CD43 induces DC maturation (29) and transduces costimulatory signals to T cells synergizing with TCR stimulation (30). Signaling through CD43 induces Vav tyrosine phosphorylation, activation of the MAP kinase cascade, Ca²⁺ mobilization, and increased IL-2 secretion (31). Costimulation by CD43 could result from release of inhibitory signals imposed on TCR. It has been described that CD43 inhibits TCR signaling at the immunological synapse, and active exclusion to a distal site is required to increase cytokine production (32). We report here that injection of T. cruzi TS activates host CD4⁺ T cells in vivo and demonstrate that TS binds and costimulates T cells through interaction with CD43. It is possible that other viral and bacterial sialidases share biological properties with T. cruzi TS. Whether immunopotentiating and mitogenic effects of Vibrio cholerae sialidase (33) and influenza A virus particles are also mediated by interaction with CD43 should be investigated.

CD43 is a neutrophil receptor for influenza A particles, partially responsible for virus-induced neutrophil deactivation (34). CD43 was also implicated in macrophage invasion by Mycobacterium tuberculosis (35).

Molecular modeling studies demonstrated that TS folds into two structurally distinct domains: 1) a β-propeller fold expressing sialic acid and βGalp binding sites, where both the hydrolysis and transfer reactions take place; and 2) a C-terminal lectin-like domain which is probably involved in carbohydrate recognition (36). Our data demonstrated that both TS and an enzymatically inactive TS mutant costimulate T cells, indicating that sialic acid transfer is not required for the immunopotentiating effects of TS and, instead, rTS and irTS may be acting as a lectin. Recent studies indicate that irTS displays a βGalp binding site and thus it behaves like a lectin (18). Using nuclear magnetic resonance techniques, we recently found that TS binds sialic acid residues α2,3 linked to galactose.⁵ It has been suggested that TS could form trimers in solution (37). Cross-linkage of CD43 by TS trimers could explain delivery of activating signals to T cells, since CD43 associates with tyrosine kinases (38) and CD43 engagement initiates signal-transducing cascades in T cells (31). On the other hand, CD43 inhibits TCR signaling at sites of interaction with APCs and exclusion of CD43 to a site distal to the synapse is required for optimal T cell responses (32). Therefore, it is also possible that interaction with TS increases CD43 exclusion from the synapse amplifying T cell responses. However, TS binding to CD43 protects T cells from TCR-induced apoptosis. This result suggests that signals delivered by CD43 itself must be involved in protection. Furthermore, our data indicated that rTS and irTS transduce signals directly to T cells in the absence of APCs. TS and its enzymatically inactive catalytic domain also protect the PC12 neuronal cell line from apoptosis (39). Several ligands have been proposed for CD43, including ICAM-1 (40), galectin-1 (41), class I MHC (42), E-selectin (43), and macrophage sialoadhesin (44). Our studies suggest that TS is a microbial ligand for CD43, potentially involved in pathological effects of the infection.

Although CD43 plays a pivotal role, the molecular basis of TS-induced T lymphocyte activation needs to be further elucidated.

First, the requirement of PMA for costimulation by TS suggests that CD43 must be primed, perhaps by phosphorylation, to signal. It has been described that phorbol ester increases CD43 phosphorylation (38). Second, TS costimulated T cell activation by syngeneic DCs, suggesting that TS could exacerbate homeostatic syngeneic T cell-DC interactions in vivo. In support of this possibility, CD43 cross-linkage also increases interactions of DCs with syngeneic T cells (45). However, other potential explanations for the immunostimulatory effect of TS remain. Since generation of T cell clones specific for TS has been described (46), processing of TS could create multiple TS epitopes, stimulating a large number of T cells. Recently, Gao and Pereira (15) suggested that TS costimulates T cells indirectly through effects on accessory cells. Although we cannot discard that TS increases the costimulatory activity of APCs, we have provided evidence that TS binds to T cells and costimulates highly purified CD4⁺ T cells in the absence of APCs. TS induces purified CD4⁺ T cells to spread over anti-CD3-coated surfaces, which could be the basis for increased TCR engagement and costimulated responses. Different from the latter study (15), we found that increased T cell responses, as well as rescue from apoptosis, depended on CD43. We have used a different cloned rTS and higher doses of the enzyme, which could explain the differences.

Our data raise the possibility that TS and other noncatalytic members of the TS multigene family could be the parasite molecules responsible for the polyclonal T cell activation in T. cruzi infection. Polyclonally activated CD4⁺ T cells from T. cruzi infection undergo AICD by apoptosis (28). Here, we have demonstrated that TS rescues T cells from AICD through a mechanism dependent on CD43 engagement. AICD plays an important role in down-regulation of peripheral T cell responses (47). Rescue from AICD could amplify the host response to T. cruzi infection and increase cytokine production, e.g., TNF-α and IL-4. It is not surprising that a costimulatory molecule like TS protects T cells against apoptosis and up-regulates cytokine production. However, it is a paradox that TS also plays a virulence role in infection (12). One possibility would be that TS, like superantigens, rapidly induces T cell anergy following immunostimulation. Alternatively, TS could promote T cell apoptosis (14) or anergy indirectly, e.g., through increased NO production.

In summary, we found that T. cruzi TS binds and activates host CD4⁺ T lymphocytes in vivo and in vitro. If these findings can be extended to human cells, TS becomes a candidate molecule to be targeted to block immunopathology and to improve efficacy of vaccination in Chagas disease.

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