Trypanosoma cruzi trans-Sialidase Prevents Elicitation of Th1 Cell Response via Interleukin 10 and Downregulates Th1 Effector Cells

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The *trans*-sialidases (TSs) from *Trypanosoma cruzi*, the agent of Chagas disease, are virulence factors shed to the bloodstream that induce strong alterations in the immune system. Here, we report that both enzymatically active TS (aTS) and its lectinlike isoform (iTS) disturb CD4 T cell physiology, inducing downregulation of Th1 cell functionality and *in vivo* cell expansion. By using ovalbumin-specific DO11.10 cells as tracers of clones developing the Th1 phenotype, we found that the infection induced significant amounts of gamma interferon (IFN-γ) but low levels of interleukin 2 (IL-2) and increased IL-4 production *in vivo*, in agreement with a mixed T helper response. The production of cytokines associated with the Th2 phenotype was prevented by passive transfer of anti-TS neutralizing antibodies. TSs also reduced the T cell receptor signaling as assayed by Zap-70 phosphorylation. TSs also reduced IL-2 and IFN-γ secretion, with a concomitant increase in IL-4 production and then an unbalancing of the CD4 T cell response toward the Th2 phenotype. This effect was prevented by using anti-IL-10 neutralizing antibodies or IL-10$^{-/-}$ antigen-presenting cells, supporting the subversion of this regulatory pathway. In support, TSs stimulated IL-10 secretion by antigen-presenting cells during their interaction with CD4 T cells. When polarized cells were stimulated in the presence of TSs, the secretion of IL-2 and IFN-γ was strongly downregulated in Th1 cells, while IL-2 production was upregulated in Th2 cells. Although the Th1 response is associated with host survival, it may simultaneously induce extensive damage to infected tissues. Thus, by delaying the elicitation of the Th1 response and limiting its effector properties, TSs restrain the cell response, supporting *T. cruzi* colonization and persistence while favoring host survival.

Chagas disease, the American trypanosomiasis, is a chronic, disabling parasitic disease caused by the flagellate protozoon *Trypanosoma cruzi*. With an estimated total of 100 million people at risk of infection, about 8 to 10 million infected, and around 40,000 new cases per year, Chagas disease represents a major health, social, and economic problem in Latin America (1). The acute stage of infection is characterized by several transient abnormalities in the physiology of the immune system, leading to declines in cellular responses to mitogens, parasite antigens, or irrelevant antigens (2–4) that favor parasite colonization (5). These findings are consistent with reductions in the proliferation and expression of interleukin 2 (IL-2) and its high-affinity receptor IL-2Rα (CD25) in murine T cells and human peripheral blood mononuclear cells (reviewed in reference 6). CD4 T (7) and B cell (8) apoptosis has been recorded, as well as alterations in the adhesion, migration, and antigen presentation of dendritic cells (9). Although the resolution of *T. cruzi* infection is strictly dependent on the Th1 response (10–12), both Th1 and Th2 clones are elicited during the course of infection (13). Therefore, the adequate counterbalancing of the Th1/Th2 cellular response becomes critical.

T. cruzi alters the immune system of the host in many ways, through several parasite components. One such virulence factor is the *trans*-sialidase (TS) that allows the sialylation of the membrane glycoconjugates of *T. cruzi* in terminal positions. However, TS is also shed and found in the bloodstream, and its systemic distribution induces modified host cell sialylation patterns (14, 15). These are associated with several abnormalities during early infection stages, including depletion of thymocytes (16), absence of germinal centers in secondary organs (17), and thrombocytopenia and erythropenia (18, 19), all of which can be prevented by the passive transfer of anti-TS neutralizing antibodies to infected

mice (17, 18, 20). TS also inhibits human lymphocyte proliferation involving IL-2 signaling (21). Accordingly, as the amount of shed enzyme increases, the virulence of the corresponding parasite strains also increases (22). Moreover, CD8 T cells from infected animals have been shown to be extra sialylated and then reduced in their ability to infiltrate tissues (23). Two TS isoforms are predicted in the parasite genome, the enzymatically active (aTS) isoform, which contains a Tyr$_{342}$ residue, and the catalytically inactive (iTS) isoform, which has His$_{342}$ instead (24). However, the iTS isoform is in fact a lectin, for it retains the ability to bind the substrate sugars (25, 26).

Due to the ability of TSs to manipulate the immune system, we decided to explore their possible effect on CD4 T cell responses.

Here, we describe for the first time that both virulence factors induced the nonprotective (10–13) Th2-like phenotype in naive T cells while downregulating elicitation of Th1 cells through the induction/expression of IL-10 during the antigen-presenting cell (APC)/T cell interplay. Moreover, both TS isoforms were associated with the parasite's ability to reduce IL-2Ra expression and IL-2 production by T cells. Our results clearly demonstrate that TSs manipulate the T CD4 response throughout their maturation stages to favor parasite survival and infection.

MATERIALS AND METHODS

Mice. The protocol of this study was approved by the Committee on the Ethics of Animal Experiments of the Universidad Nacional de San Martín (UNSAM), following the recommendations of the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (27). BALB/cJ C.Cg-Tg(DO11.10)10Dlo/J (DO11.10) mice, transgenic for a major histocompatibility complex class II (MHC-II)-restricted, rearranged T cell receptor specific for ovalbumin (TCR$_{OVA}$), and BALB/cJ IL-10$^{-/-}$ mice were obtained from The Jackson Laboratory and bred in our facilities. Male mice (60 to 90 days old) were used in all experiments.

TS purification. Recombinant TS proteins were expressed in *Escherichia coli* BL21 and purified to homogeneity by immobilized metal affinity chromatography through Ni^{2+}-charged Hi-Trap chelating columns (GE Healthcare) and ion-exchange chromatography (Mono Q; GE Healthcare) as described previously (14, 15), followed by passage through a polymyxin column (Pierce) for endotoxin depletion.

***In vivo* assays.** BALB/cJ mice received 2×10^7 splenocytes from the DO11.10 mice intravenously (i.v.). Twenty-four hours later, the animals were injected with 300 μg of an ovalbumin peptide comprising residues 323 to 339 (OVA$_{323-339}$) (Genscript) in phosphate-buffered saline (PBS) emulsified in complete Freund's adjuvant and distributed among three different sites of the back (28). Control animals were injected with PBS in complete Freund's adjuvant. Inguinal and axillar ganglia were removed 6 days after TS administration (1 μg in PBS intraperitoneally [i.p.]), and TCR$_{OVA}$ cells were quantified with fluorescein-labeled anti-TCR$_{OVA}$ monoclonal antibody (MAb) KJ1-26 from eBioscience. To test the functionality of antigen-specific T cells, BALB/cJ mice received 2×10^7 splenocytes i.v. from DO11.10 animals and 5 μg OVA i.p. in PBS at day zero. At days +1, +3, and +5, animals received 5 μg of either aTS or iTS i.p. At day +7, splenocytes were cultured for 72 h with 1 μg of OVA peptide, and supernatants tested for cytokines by enzyme-linked immunosorbent assay (ELISA) (Biolegend). In another set of assays, BALB/cJ mice were infected (100 bloodstream-form parasites of the RA strain) and then received 2×10^7 splenocytes from DO11.10 animals i.p. and 5 μg of OVA subcutaneously on day +7 postinoculation (p.i.). A group of animals received 3 μg of purified anti-TS monoclonal antibody (neutralizing titer of over 1:15,000) (17, 18) by i.p. passive transfer every 2 days (four doses total), starting 1 day before the splenocyte transfer. Remnant TS-neutralizing activity in blood was confirmed before every antibody injection. Splenocytes were tested with OVA peptide as described above on day +13 p.i.

CD4 T cell purification and CD8 T cell depletion. Splenocyte suspensions in RPMI 1640 plus 10% fetal bovine serum (FBS) (Gibco/BRL) were depleted of red cells with Tris-buffered ammonium chloride. CD4$^+$ T cells were purified with anti-CD4 (L3T4) microbeads followed by passage through an MS column (Miltenyi Biotech, Germany) according to the manufacturer's instructions. The typical purity was over 95%. CD8 T cells were depleted using anti-CD8 (Ly-2) beads (Myltenyi); the amount of CD8 cells remaining was less than 2%.

Proliferation assays and CD25 detection. For proliferation assays, 1×10^5 splenocytes from DO11.10 mice or 2×10^5 purified CD4 T cells plus 0.2×10^5 A20 cells (as APC) were plated in RPMI 1640 plus 10% FBS in 96-well flat-bottom tissue culture plates (Orange Scientific, Belgium). Cells were stimulated with 2 μg/ml OVA peptide for 48 h, and 1 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear) was added 18 h before

harvesting. For CD25 detection, 1×10^6 splenocytes were incubated with 1 μg/ml OVA peptide and *T. cruzi* trypomastigotes (CL strain) or TSs for 48 h. Then, cells were labeled with fluorescein–KJ1-26 and phycoerythrin (PE)–anti-CD25 antibody (PC61.5) (eBioscience). Cells were analyzed in a CyFlow space cytometer (Partec, Germany).

Intracellular signaling. For Zap-70 activation, splenocytes were seeded onto 96-well flat-bottom plates and activated with 1 μg/ml OVA peptide with or without 5 μg/ml TSs for 5 min. Cells (1×10^6 splenocytes) were fixed with 4% *p*-formaldehyde and then permeabilized with cold methanol. Labeling was performed with PE–anti-phospho-Zap-70 (Y319)–Syk (Y352) antibody (BD Bioscience). To test for IL-2 production after Ca^{2+} signaling activation, splenocytes from DO11.10 mice were incubated for 24 h in the presence of 1 μg/ml OVA peptide and phorbol 12-myristate 13-acetate (PMA) (50 ng/ml)–ionomycin (500 ng/ml) (Sigma) with or without 5 μg/ml TSs.

Cytokine detection. For cytokine secretion assays, splenocytes from DO11.10 mice were cultured (5×10^6/ml) in 96-well U-bottom plates and stimulated with 5 μg/ml OVA peptide with or without 5 μg/ml TSs for 3 days in RPMI 1640 plus 10% FBS in a humidified incubator with 5% CO$_2$ at 37°C. The cytokine concentrations in culture supernatants were assayed by ELISA using paired antibodies (Biolegend). For intracytoplasmic cytometry, cells were incubated with PMA (50 ng/ml)–ionomycin (500 ng/ml) (Sigma) for 6 h and Golgi-stop (Biolegend) for 4 h prior to staining with PE–anti-IL-4 antibody (clone 11B11) or PE–anti-gamma interferon (IFN-γ) antibody (clone XMG1.2) (eBioscience).

T helper differentiation. T helper differentiation was performed as described in reference 29. Briefly, CD8 T cell-depleted splenocytes from DO11.10 mice (5×10^5/ml) were activated in 96-well U-bottom plates with 1 μg/ml OVA peptide. Anti-IL-4 antibody (clone 11B11, 5 μg/ml) plus IL-12 (10 ng/ml) were added to create Th1-polarizing conditions. For Th2 conditions, anti-IL-12 antibody (clone C17.8, 5 μg/ml) plus IL-4 (4 ng/ml) were added. For both conditions, 10 ng/ml IL-2 was added at day 4. Cells were used at day 8 of culture. All reagents were from eBioscience. The effect of TSs on Th1/Th2 elicitation was assayed by adding TSs throughout the differentiation process (5 μg/ml), together with the inducers. The effect of TSs on already polarized Th cells was assayed by adding them at 5 μg/ml in the antigen presentation assay.

BMM. Bone marrow cells (2.5×10^6) from BALB/cJ or BALB/cJ IL-10$^{-/-}$ mice were plated onto 10-cm-diameter culture dishes with 10 ml of macrophage-differentiating medium (RPMI 1640 with 10% FBS and 30% conditioned medium from macrophage colony-stimulating factor-transfected L929 cells) in a humidified incubator with 5% CO$_2$ at 37°C. At day 3, 10 ml of differentiating medium was added, and bone marrow-derived macrophages (BMM) were used 4 days later. BMM (1×10^5) were used as APC with the same number of purified DO11.10 CD4 T cells.

Statistical analysis. Analysis of variance (ANOVA) with Bonferroni's correction was used for statistical analysis.

RESULTS

The TSs from *T. cruzi* limit CD4 T cell expansion *in vivo* and modify their elicited phenotype. To explore the ability of TSs to modulate CD4 T cell response, the antigen-specific model DO11.10 (transgenic TCR$_{OVA}$ on CD4 T cells) was selected. First, the ability of TS to affect clonal expansion of CD4 T cells *in vivo* was tested with this model. For this purpose, BALB/cJ mice received T cells from DO11.10 animals by passive transfer and were challenged with the cognate OVA peptide (28). The administration of TSs reduced the OVA antigen-specific T cell number found in the draining ganglia (Fig. 1A). Having found that TSs affected T CD4 cell expansion after antigen-specific stimulation, the ability of TSs to modulate the CD4 T cell response was tested in another set of assays. To investigate the cytokine profile induced, splenocytes from DO11.10 animals were transferred to BALB/cJ mice,
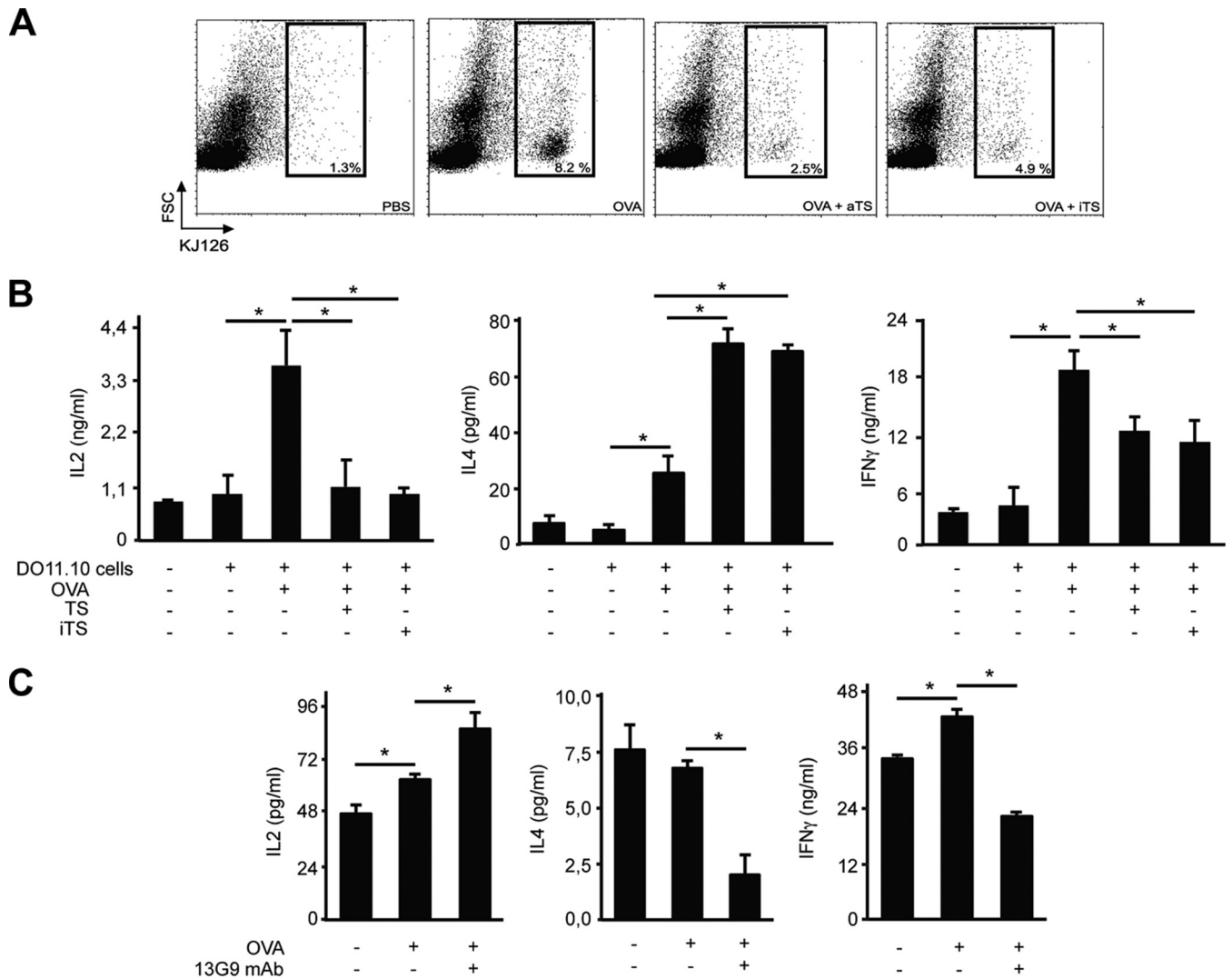
FIG 1 *trans*-sialidases reduce expansion of CD4 T cells and favor the Th2 phenotype. (A) BALB/cJ mice received DO11.10 splenocytes by passive transfer. Animals were challenged with the OVA$_{323-339}$ peptide injected into their backs, and TSs were administered. DO11.10 T cells in axillary and inguinal ganglia were then quantified using anti-TCR$_{OVA}$ MAb (KJ1-26). FSC, forward scatter. (B) Mice that received DO11.10 splenocytes were immunized with OVA and treated with three doses of aTS or iTS, as indicated by + or − signs. Splenocytes were taken at day +7 and cultured with the OVA peptide, and interleukins were evaluated. (C) Mice were infected and, 5 days later, received DO11.10 splenocytes and were immunized with OVA. Anti-TS neutralizing monoclonal antibodies (clone 13G9) were passively transferred to one group of animals. Splenocytes were challenged with the OVA peptide, and interleukins were assayed. There were at least 5 animals in each group. Results are expressed as means ± standard errors of the means (SEM). *, $P < 0.05$.

and then the BALB/cJ mice were immunized with OVA and injected with 3 doses of TS or iTS every 2 days. TS treatment resulted in decreased IL-2 and IFN-γ but enhanced IL-4 production (Fig. 1B). Upon cognate antigen stimulation, DO11.10 T cells express a Th1 phenotype with a high ratio of IFN-γ to IL-4. Therefore, this outcome indicates that TS shifted the cytokine secretion profile of an antigen-specific T cell response. To test whether the TSs shed during *T. cruzi* infection actually mediate this effect, DO11.10 splenocytes were transferred to infected BALB/cJ mice at day +7 p.i., and then the mice were immunized with OVA and an anti-TS neutralizing monoclonal antibody was administered (18, 19). In agreement with the results shown in Fig. 1B, cells taken from infected animals displayed reduced IL-2 and IFN-γ production, while IL-4 was increased. Conversely, the animals in which TS activity was prevented by the transfer of neutralizing antibodies

retained the ability to produce IL-2 and significant amounts of IFN-γ but had reduced IL-4 levels (Fig. 1C). Taken together, these results strongly support the idea that TSs shed by the parasite delineate CD4 T cell physiology *in vivo*.

TS downregulates IL-2/IL-2Rα expression and hampers CD4 T cell activation. It is known that *T. cruzi* infection decreases the proliferation of T lymphocytes and downregulates both IL-2 production and the expression of CD25, the IL-2Rα subunit (30). Taking into account the finding that TS limited CD4 T cell clonal expansion *in vivo* (Fig. 1A), we next analyzed the ability of TS to directly modulate IL-2 secretion and cell proliferation *in vitro*. We found that TS isoforms diminished the CD4 T cell proliferation and IL-2 secretion of purified DO11.10 CD4 T cells incubated with the cognate OVA peptide and with A20 cells as APC (Fig. 2A). In agreement with this, the Zap-70 phosphorylation signaling de-
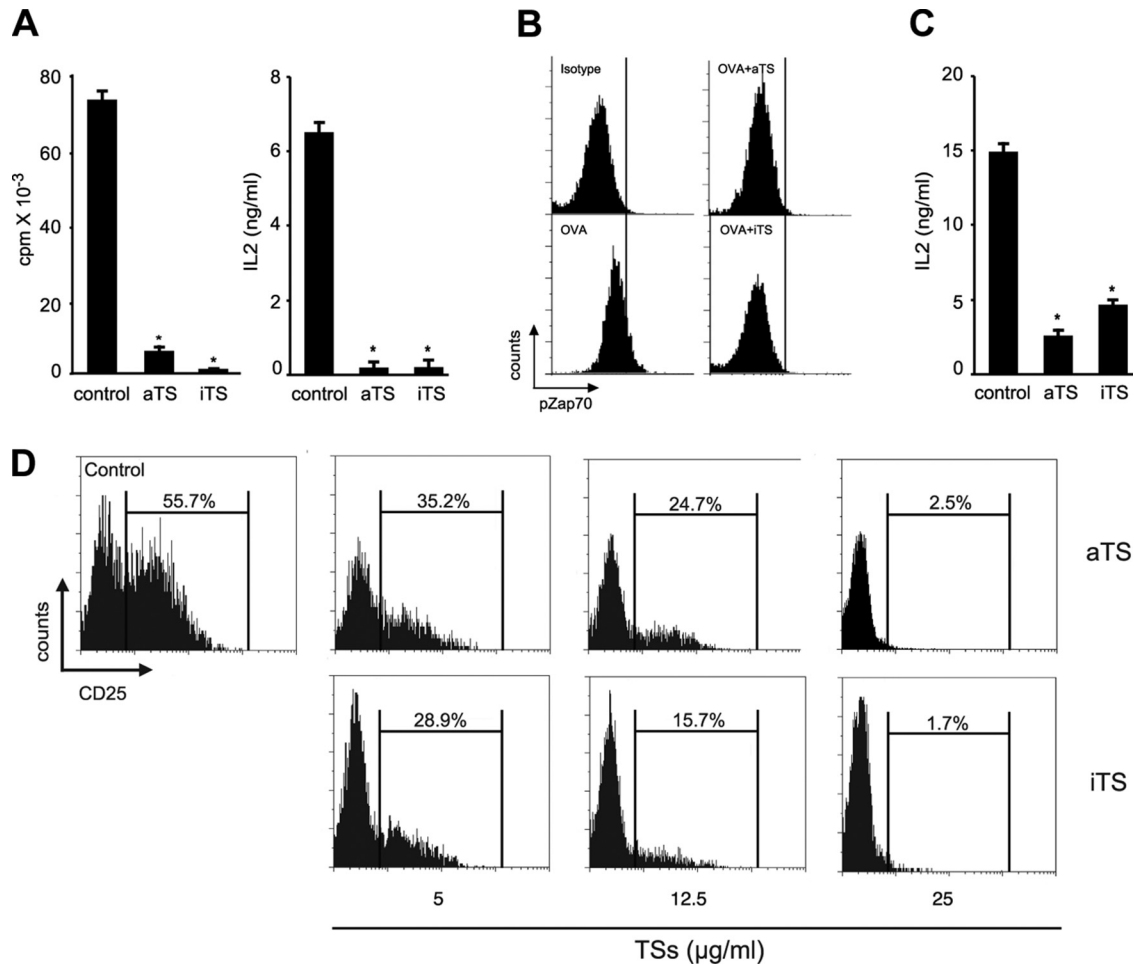
FIG 2 TS downregulates proliferation, activation, and CD25 expression of CD4 T cells. (A) CD4 T cells (2×10^5) from DO11.10 mice were cultured for 48 h with the OVA peptide in the presence of TSs and of mitomycin-treated 0.2×10^5 A20 cells as APC (10:1 ratio). Results are expressed as means ± SEM of replicate values from three independent cultures. cpm, counts per minute; *, $P < 0.01$. (B) Zap-70 phosphorylation was tested by flow cytometry. (C) TSs inhibited IL-2 production by PMA-ionomycin-OVA stimuli in DO11.10 splenocytes cultured for 24 h. *, $P < 0.01$. (D) DO11.10 splenocytes (1×10^6) were cultured for 48 h with 1 μg/ml OVA peptide in the presence of (top) 0.5×10^6, 2.5×10^6, or 5×10^6 trypomastigotes/ml (32) or 5, 12.5 or 25 μg/ml TSs (bottom). CD25 expression was assayed in CD4 T cells gated with anti-TCR$_{OVA}$ monoclonal antibody (KJ1-26 MAb). Results representative of three assays are shown.

creased when antigen presentation occurred in the presence of aTS or iTS (Fig. 2B). These results reinforce the notion that TS was actually limiting T cell expansion *in vivo* by reducing the TCR activation state.

In his pioneering studies, F. Kierszenbaum described an as-yet-uncharacterized parasite-shed product named trypanosomal immunosuppressor factor (TIF) (30) that inhibits T cell proliferation even under PMA-ionomycin stimuli (31). Shed TSs could be associated with this parasite activity; therefore we tested IL-2 production in PMA-ionomycin-OVA-stimulated splenocytes. Remarkably, we found that IL-2 production was inhibited by TSs (Fig. 2C). No difference was observed in the immunological synapses in the presence of either TS as tested by the number of T cell/APC complexes (not shown), strongly supporting the idea that TSs did not inhibit physical interaction of the APC and T cells during the antigen-presenting process but instead hampered the activation of the T cells by inhibiting the intracytoplasmic signaling.

It was also reported that the mere addition of trypomastigotes (32) is enough to induce lower IL-2 production and T cell prolif-

eration through TIF (30). Given that we found that TS shed by the parasite during infection was able to modulate CD4 T cell functionality (Fig. 1C), we then tested whether shed TS also affected IL-2Rα downregulation and IL-2 secretion. To test this hypothesis, we followed the conditions originally used by Kierszenbaum's laboratory (32) and the expected amount of shed TS per parasite concentration (22). In the first case, DO11.10 splenocytes were activated with the cognate peptide and cocultured with purified trypomastigotes, which were able to downregulate CD25 (IL-2Rα) expression on T cells (Fig. 2D, top). Both TS isoforms were able *per se* to reduce, in a dose-dependent manner (Fig. 2D, bottom), CD25 expression to levels similar to those induced by the addition of equivalent number of parasites.

TS turns the response toward the Th2 phenotype and downregulates IFN-γ secretion by Th1 cells. The Th1 elicitation response and its effector molecules, such as IL-12 and IFN-γ, have been associated with the outcome of infection control (10–12). Together with its IL-2/IL-2Rα alterations described above, *T. cruzi* is also known to inhibit IFN-γ secretion in an *in vitro* system (33), consistent with our findings reported in Fig. 1. The involvement of
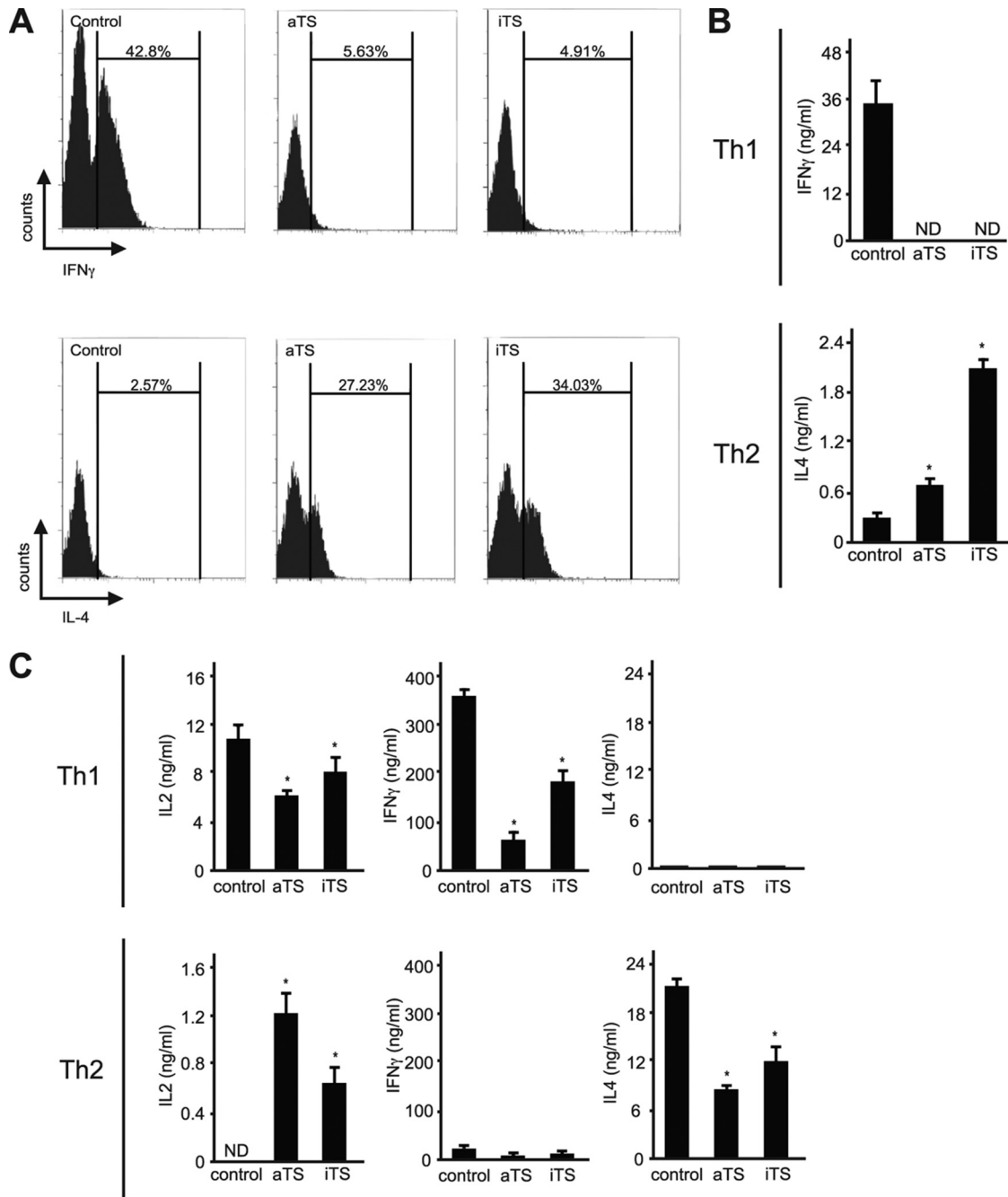
FIG 3 TS inhibits Th1 elicitation and effector function. (A) Intracytoplasmic IFN-γ (top) and IL-4 (bottom) labeling of DO11.10 splenocytes cultured for 72 h with the OVA peptide in the presence of TSs. PMA-ionomycin was added 6 h before harvesting of culture. Note the reduction of IFN-γ expression and concomitant increase in IL-4. (B) Splenocytes from DO11.10 mice depleted of CD8 cells were induced for Th1/Th2 differentiation under standard conditions (30) but in the presence of TSs from the beginning. Note the deviation from the Th1 to the Th2 phenotype. OVA peptide was used as the antigen. The levels of IL-4 and IFN-γ in culture supernatants were determined by ELISA. *, $P < 0.01$. (C) Splenocytes from DO11.10 mice depleted of CD8 cells were induced for Th1/Th2 differentiation (30) in the absence of TSs. Th1-/Th2-polarized cells were then treated with TSs during the antigen-presenting process. Levels of cytokines in supernatants were determined by ELISA. Note the reduction in Th1 function with concomitant increase of Th2 activity. Results are expressed as means ± SEM of replicate values from three independent cultures. *, $P < 0.03$; ND, not detected.

TS in the crucial Th1/Th2 decision making was then tested. When DO11.10 cells are stimulated with OVA peptide, they spontaneously acquire the Th1-like phenotype, as evaluated by their IFN-γ/IL-4 expression pattern (Fig. 3A). However, the addition of aTS or iTS induced a notable decrease in IFN-γ and a simultaneous increase in IL-4 production (Fig. 3A). This finding is consistent with the results of the *in vivo* assays reported in Fig. 1 and further supports the finding that TSs diverted the elicited response to Th2. We then analyzed whether TSs are able to modulate cell functionality even under strongly polarizing conditions. TSs decreased
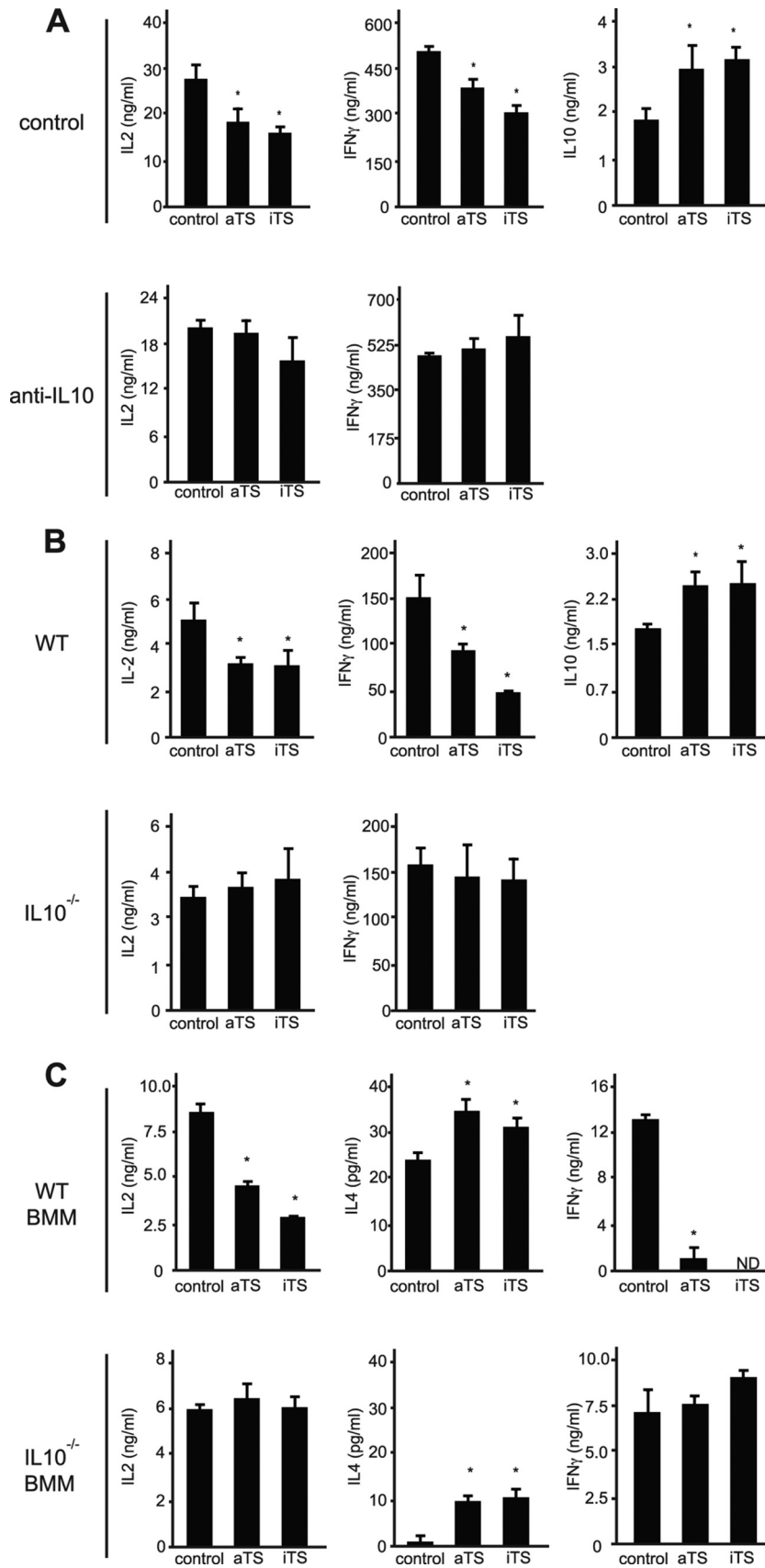
IFN-γ production even when IL-12 and neutralizing anti-IL-4 antibodies were included ([Fig. 3B]). In contrast, TSs increased IL-4 production under Th2-inducing conditions, i.e., anti-IL-12 neutralizing antibodies plus IL-4. No increase in apoptosis was observed (not shown). These findings demonstrated that TSs *per se* prompted T cells to acquire the nonprotective Th2 phenotype even under conditions strongly favoring Th1 differentiation.

The next step where TSs might be involved in altering the Th1 cell functionality is during the effector phase of the immune response. To investigate this, Th1/Th2 cells that had been fully differentiated *in vitro* were tested. Remarkably, TSs decreased the production of IL-2 and IFN-γ on Th1 cells without any detectable increase in IL-4 ([Fig. 3C]). In contrast, IL-2 secretion was increased in Th2 cells, while IFN-γ secretion was not and IL-4 remained at high values ([Fig. 3C]). Again, no increment in apoptosis was recorded in these cultures (not shown). Therefore, these results indicate that TSs hamper Th1 effector properties but stimulate Th2 cells.

TS-induced deviation to a Th2 response is dependent on IL-10. IL-10 is an anti-inflammatory cytokine that forces the response toward the Th2 phenotype. It is capable of decreasing the proliferation of CD4 T cells and their IL-2 and IFN-γ secretion and of blocking the Th1 response (34–36). Increased resistance to *T. cruzi* infection and high levels of release of Th1 cytokines like IL-12, tumor necrosis factor, and IFN-γ are observed in mice that have received anti-IL-10 neutralizing antibodies by passive transfer and in IL-10$^{-/-}$ mouse models (13, 37–39). In agreement with these findings, *T. cruzi* antigens modulate dendritic cells, leading them to secrete IL-10 and then to a dwindling of the immune response (40). Hence, an interesting hypothesis that might provide a plausible explanation for the findings reported here is that IL-10 became involved in the TS-induced immune regulation process. We found that TS induced the secretion of IL-10 in peptide-stimulated DO11.10 splenocytes ([Fig. 4A], top). Moreover, the addition of anti-IL-10 neutralizing antibodies prevented the unbalancing of IL-2/IFN-γ secretion induced by TS ([Fig. 4A], bottom). These results suggest that IL-10 was actually involved in the TS modulation of the CD4 T cell antigen-specific response. Next, splenocytes from BALB/cJ or BALB/cJ IL-10$^{-/-}$ mice as sources of APC were assayed with purified CD4 T cells from DO11.10 animals. TSs were unable to depress IL-2/IFN-γ secretion when IL-10$^{-/-}$ APC were assayed ([Fig. 4B]), strongly supporting the idea that the TSs-induced mechanism involves the IL-10 pathway. To further test this hypothesis, BMM were tested as APC. In agreement with the results described above, when CD4 T cells from DO11.10 mice were cultured with IL-10$^{-/-}$ BMM in the presence of TSs and peptide, no variation in IL-2/IFN-γ secretion was observed ([Fig. 4C]). Therefore, these results strongly imply the requirement of IL-10 production by APC as the mechanism governing the T helper response modulation by TSs. The possibility that TS directly induces IL-10 production on BMM was tested. However, IL-10 was not found in culture supernatants treated with TSs

(not shown), suggesting that the interaction with T cells during the antigen-presenting process is strictly required.

DISCUSSION

By following a parasite-unrelated, antigen-specific single clone response, we found that TSs interfered with the CD4 T cell response at all stages. During the elicitation of the CD4 response, these virulence factors diverted the immune response toward the Th2 phenotype *in vivo* and reduced the number of antigen-specific T cells in the draining ganglia ([Fig. 1]). TSs reproduced the observed imbalance of the immune response against *T. cruzi* by reducing CD4 T cell proliferation and decreasing IL-2 secretion, IL-2Rα expression, TCR signaling, and inhibition of IFN-γ secretion (33). On the other hand, TSs also inhibited effector CD4 T cell properties. The parasite replicates intracellularly and, after transformation to trypomastigotes, sheds large amounts of TSs into the host cell cytoplasm (41) that are released *in situ* after parasites are freed. Therefore, when T cells reach infected tissues, they will find high local concentrations of TSs (41) that cannot be titrated by the circulating antibodies. Thus, the TSs will restrain the already elicited protective Th1 cells, with a dual benefit for the parasite by favoring its persistence in replication nests while preventing extensive damage to the infected tissue. This is consistent with the reported inhibitory activity of aTS on the CD8 T cell compartment (23).

In contrast to thymocyte apoptosis, where enzymatic activity and sialylation are required (14, 20), a lectinlike binding signal, which is not associated with remodeling of the lymphocyte sialylation pattern, was involved in the events reported here. Sialylation and lectin activities lead to similar outcomes, and no differences were observed when both TSs were assayed simultaneously. Although iTS works as a lectinlike molecule, it retains a residual sialidase activity (42) that might release it from the target cell to eventually attack another cell. Other authors, working at higher concentrations and requiring phorbol esters, reported a costimulatory activity through CD43 for this lectinlike activity (43). However, TS seems to follow diverse pathways to modulate the immune system, for it is known to also stimulate T cells independently of the CD43 or CD40/CD40L pathways (44) and to activate APCs that lead to IL-6 induction, thus fueling T cell proliferation (44). In fact, when tested as an antigen, either emulsified with adjuvants (45) or in DNA vaccines (46), TS induces protective immunity that is independent of its enzymatic or lectin properties, stressing the relevance of this virulence factor.

TSs are trypomastigote-shed virulence factors that might be related to the yet-uncharacterized TIF protein described by F. Kierszenbaum from his pioneering assays with trypomastigote-conditioned medium (7). The findings reported here show the ability of TSs to downregulate IL-2 production, IL-2Rα expression, and T cell proliferation, even under PMA-ionomycin stimuli (31), providing support for TIF/TS having parallel biologic activities. Similarly to the systemically distributed TS (18, 19, 21), TIF is

FIG 4 TSs induce the fate of CD4 T cells via IL-10. (A) Splenocytes (1×10^6/well) from DO11.10 mice were cultured without (top) or with (bottom) anti-IL-10 neutralizing antibodies in the presence of TSs plus the OVA peptide. IL-4 was tested for but not detected under these conditions. (B) CD4 T cells (0.2×10^6/well) from DO11.10 mice were cultured with splenocytes (0.8×10^6/well) from BALB/cJ mice (wild type [WT] top) or BALB/cJ IL-10$^{-/-}$ mice (IL-10$^{-/-}$, bottom) as APC in the presence of TSs plus the OVA peptide. (C) Purified CD4 T cells (0.1×10^6/well) from DO11.10 mice were cultured with 0.1×10^6 BMM from BALB/cJ mice (WT, top) or BALB/cJ IL-10$^{-/-}$ mice ($10^{-/-}$, bottom) as APC in the presence of TSs plus OVA peptide. Levels of cytokines were determined by ELISA. Results are expressed as means ± SEM of replicate values from three independent cultures. *, $P < 0.03$; ND, not detected.

mainly active during the acute phase of the infection, because neutralizing antibodies are elicited in chronic infection in patients and mice (42).

Several characterized TSs target molecules on lymphocytes that are related to regulatory or adhesion properties, CD45 being the major one (15). Recently, we showed that aTS but not iTS pushes isolated B cells to produce IL-17 through the activation of the CD45 pathway and sialyl pattern modification (47). T cells might also be modulated through a lectin pathway, as galectins activate T cells through CD45 (48), an effect that might be mimicked by the lectinlike activity of TSs. Unlike B or CD8 T cells, where a direct effect can be observed, thymocytes and CD4 T cells require an interaction with other cell type(s) to be affected by TSs. Unlike CD4 T cells, the lectinlike activity of TS seems not to play a major role in B cells, CD8 T cells, or thymocytes. Interestingly, in a recent study on 38 isolates collected throughout the area of endemicity, we found that iTS is absent from the *T. cruzi* discrete typing units TcI, TcIII, and TcIV that usually correspond to less aggressive isolates, suggesting a link between iTS and virulence (49).

Accurate decision making regarding the immune response strategy and subsequent effector actions is of vital importance to the resolution of an infectious process. As with *T. cruzi* infection, Th1 cells are essential for resistance against intracellular pathogens, such as *Leishmania major*, *Toxoplasma gondii*, or *Mycobacterium tuberculosis*, whereas Th2 cells are associated with the defense against parasitic nematodes. During *T. cruzi* infection, Th1 responses are protective (10–12), and IFN-γ is the main mediator involved, including for the activation of CD8 T cells (50). In contrast, Th2 seems not only to be nonprotective but also to worsen the infection outcome (13). When IL-10 production is prevented either by anti-IL-10 neutralizing antibodies or by using IL-10$^{-/-}$ mouse models (13, 37–39), the production of Th1 cytokines is increased. However, in spite of the control of parasitemia that allows the host to survive the acute stage of infection, the elicited immune response proves unable to prevent parasite colonization and evolution to the chronic phase of the disease, thus indicating only a partial success. Moreover, a CD4 T cell exhaustion process is observed during the progression of the infection, which might lead to worsening of the associated disease (51, 52). In line with these findings, parasite-shed vesicles containing several components, including TS, make naive mice more susceptible to infection due to increased production of IL-4 and IL-10 (53). Interestingly, it is also suggested that IL-10 production during *T. cruzi* infection prevents extensive pathogenic damage by the Th1 response (38). In this context, an adequate counterbalance between Th1 cells that are crucial to survive the infection and Th2 cells that seem to play a significant role both in preventing extensive damage and Th1 cell exhaustion (54) will tune the immune response. The results reported here support this view, because we found that IL-2, IL-4, and IFN-γ modulation depends on IL-10 secretion induced by TSs during the APC/T cell interplay. The concerted action of the interplay of these cytokines will delay the induction of a protective Th1 response and dampen these cells once elicited, thus favoring parasite persistence and host colonization. Notably, it was recently reported that TS upregulates the expression of monocyte chemotactic protein 1 (MCP-1/CCL2) and fractalkine (CX3CL1), which are associated with reduction of inflammation and enhancement of tissue repair (55). Thus, TS seems to be able to manipulate both the innate and adaptive immune responses to

control the damage to infected tissue caused by the protective Th1 response. Perhaps the only defined virulence factor already known to modify the Th phenotype is the T2 RNase/omega-1 from *Schistosoma mansoni* eggs (56, 57). This protein shifts the immune response toward Th2, increasing IL-4 and IL-10 and decreasing CD4 T cell proliferation and IL-2 and IFN-γ secretion, similar to the findings reported here for TSs. Thus, TSs emerge as molecules shed from the parasite that can modulate the T cell response during the entire life of lymphocytes, from thymus to periphery and from maturation to effector activities, taking advantage of the regulatory properties of the immune system and leading to a breakdown of the immune response that favors the survival of the parasite in the host.

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