Institut Pasteur
Microbes and Infection 15 (2013) 365–374
www.elsevier.com/locate/micinf

Original article

Trans-sialidase from *Trypanosoma cruzi* enhances the adhesion properties and fibronectin-driven migration of thymocytes

Ana Flávia F.R. Nardy [a], João Luiz da Silva Filho [b], Ana Rosa Pérez [c], Juliana de Meis [d], Désio Aurélio Farias-de-Oliveira [d], Luciana Penha [b], Isadora de Araújo Oliveira [b], Wagner B. Dias [b], Adriane R. Todeschini [b], Célio Geraldo Freire-de-Lima [b], Maria Bellio [a], Celso Caruso-Neves [b], Ana Acácia Pinheiro [b], Christina Maeda Takiya [b], Oscar Bottasso [c], Wilson Savino [d], Alexandre Morrot [a,*]

[a] *Institute of Microbiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*
[b] *Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*
[c] *Institute of Immunology, National University of Rosario, Rosario, Argentina*
[d] *Oswaldo Cruz Foundation, Rio de Janeiro, Brazil*

Received 13 September 2012; accepted 8 February 2013
Available online 24 February 2013

Abstract

In experimental *Trypanosoma cruzi* infections, severe thymic atrophy leads to release of activated $CD4^+CD8^+$ double-positive (DP) T cells to the periphery. In humans, activated DP T cells are found in the blood in association with severe cardiac forms of human chronic Chagas disease. The mechanisms underlying the premature thymocyte release during the chagasic thymic atrophy remain elusive. We tested whether the migratory properties of intrathymic thymocytes are modulated by the parasite *trans*-sialidase (TS). We found that TS affected the dynamics of thymocytes undergoing intrathymic maturation, and these changes were accompanied by an increase in the number of recent DP thymic emigrants in the peripheral lymphoid organs. We demonstrated that increased percentages of blood DP T cell subsets were associated with augmented antibody titers against TS in chagasic patients with chronic cardiomyopathy. *In vitro* studies showed that TS was able to activate the MAPK pathway and actin filament mobilization in thymocytes. These effects were correlated with its ability to modulate the adhesion of thymocytes to thymic epithelial cells and their migration toward extracellular matrix. These findings point to effects of TS that could influence the escape of immature thymocytes in Chagas disease.

Keywords: Chagas disease; *Trypanosoma cruzi*; *Trans*-sialidase; Thymocyte; Cell migration

1. Introduction

Trypanosoma cruzi is the parasite protozoan responsible for Chagas disease, which is an important public health burden in the Americas. According to the Pan American Health Organization, 20% of Latin America's population is at risk of infection [1]. The initial acute phase of the disease is characterized by numerous circulating parasites able to infect several tissues in the host, including skeletal muscle, lymphoid tissues, nervous tissues, and glands [2]. It progresses to an indefinite asymptomatic period with almost undetectable blood parasitemia [3,4]. It has been shown that the events occurring during this phase are essential for activation of the immune system during the late chronic phase of the disease [5].

In the murine model of *T. cruzi* infection, several changes are observed in lymphoid organs, including the thymus, where the parasitism occurs [6]. Our studies have revealed an intense and severe thymic atrophy in acutely infected animals, mainly

* Corresponding author.
E-mail address: morrot@micro.ufrj.br (A. Morrot).

1286-4579/$ - see front matter © 2013 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.micinf.2013.02.003

due to depletion of $CD4^+CD8^+$ double-positive (DP) thymocytes by apoptosis in the cortical area of the thymus [6,7]. These changes are paralleled by enhanced expression of extracellular matrix (ECM) molecules, such as laminin and fibronectin in the thymus, accompanied by phenotypic and functional alterations of thymic epithelial cells (TECs) [8].

Thymus atrophy is also associated with the release of these double-positive cells to the periphery, where they have been found to express potentially autoreactive TCRs and to acquire an activated phenotype, similar to what is described for effector/memory single-positive T cells [9,10]. Most importantly, we have recently demonstrated that increased percentages of peripheral blood subsets of DP cells exhibiting an activated $HLA\text{-}DR^+$ phenotype are associated with severe cardiac forms of human chronic Chagas disease [10]. These results suggest that *T. cruzi* infection alters the human thymus, which is already atrophic in adult individuals [11], in such a way as to promote an abnormal release of non-selected thymocytes [5,6].

To date, the mechanisms underlying the infection-induced thymic disturbance and the escape of immature thymocytes are not fully understood. However, an important role has been suggested for the *T. cruzi* surface protein known as *trans*-sialidase (TS) [12,13]. Since the protozoan is unable to synthesize sialic acid *de novo*, the glycosylphosphatidylinositol-anchored *trans*-sialidase allows the parasite to scavenge α-(2,3)-linked sialyl residues from exogenous glycoconjugates and to transfer them to mucine-like acceptors covering the parasite surface [14]. Changes in the sialylation profile of cell surface receptors by TS are able to induce apoptosis in cells of the immune system *in vivo*, including the thymus [15]. A detailed study using TS-treated animals revealed that these effects also resulted from apoptosis in the thymic nurse cell complex [16].

The aim of this work was to test whether TS alters the migratory and adhesive properties of thymocytes undergoing intrathymic differentiation. To that end, we analyzed whether the *T. cruzi trans*-sialidase plays a role in the premature release of double-positive $CD4^+CD8^+$ thymocytes to peripheral lymphoid organs.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Research Ethics Committee of Fiocruz (protocol CEUA-LW8/10). Protocols for animal and human studies were approved by the Institutional Ethical Committees in accordance with international guidelines. All animal experimentation was performed in accordance with the terms of the Brazilian guidelines for the animal welfare regulations.

2.2. Study population

Healthy volunteers and *T. cruzi* chronic chagasic patients were recruited from Chagas Unit, Hospital Provincial del Centenario de Rosario, UNR, Argentina. Subjects using any medication thought to affect immune functions were excluded from the study. The diagnosis was based on standard serological test, including indirect immunofluorescence and haemaglutination assay. All chronic-infected patients and non-infected individuals participated in serological tests to confirm the diagnosis for *T. cruzi* infection, with ages ranging from 30 to 64 years. The seropositive cases included fifteen cardiac chronic-infected patients presenting dilated cardiomyopathy diagnosed based in a detailed clinical examination, including electrocardiography (ECG) and chest X-ray. Additionally, we included fifteen chagasic patients without any cardiac alterations detected, being diagnosed as in the indeterminate form of the disease. Fifteen sex and age matched-controls were also included.

2.3. Animals and infection

Male BALB/c mice and C57BL/6 TLR4-deficient mice, aged 4–8 weeks, were obtained respectively from the Oswaldo Cruz Foundation animal facilities and Federal University of Rio de Janeiro. Acute *T. cruzi* infection was performed by inoculating the animals intraperitoneally with 10^2 blood-derived trypomastigote forms of the *Tulahuen* strain, after isolation from BALB/c mice. After 2 weeks of acute infection (in the peak of parasitemia), animals were killed and the organs to be studied were removed. Blood parasites were counted using Neubauer's chambers.

2.4. *Recombinant* trans-*sialidase and specific-polyclonal antibodies*

Recombinant TS were obtained from *Escherichia coli* MC1061 electrotransformed with plasmids containing the wild-type TS insert, TSREP.C [17]. The recombinant protein contained a poly(His) tag for purification as described [18,19], and was passed through an agarose-immobilized polymyxin B column (Sigma–Aldrich, St. Louis, MO) to obtain an LPS-free preparation. To generate a polyclonal antibody against TS, 0.5 ml of the inactive enzyme containing C-terminal repeats (500 μg/ml) (obtained as described above) with 0.5 ml of complete Freund's adjuvant was used to immunize rabbits. Serum was separated by centrifugation (2500 × *g* for 10 min) and immunoglobulin purification was done by using a protein-A affinity column (Pierce Biotechnology, Inc. USA). The antiserum specificity was determined by immunoabsorbent assay (ELISA).

2.5. Trans-*sialidase intrathymic injection*

Mice were anaesthetized by intraperitoneal injection of 5 mg/ml of ketamine (Virbac S.A., Rio de Janeiro, Brazil) and 1 mg/ml of xylazine (Bayer Ltd, Rio de Janeiro, Brazil). The thoracic cavity was opened via a midline incision and 10 μg of TS or bovine serum albumin (BSA) was injected intrathymically (10 μl of solution at 0.5 mg/ml for each thymic lobe). Surgical clips (Becton, Dickinson and Company, USA) were used to close the thoracic cavity after intrathymic injections.

Animals were killed 5 days later and their thymocytes subsets evaluated by flow cytometry.

2.6. Cell adhesion and transmigration assay

The TEC line was plated onto 25 cm^3 culture flasks (Nunc, Rochester, NY) at a ratio of 7×10^4 cells/flask for 48 h. TECs and thymocytes were treated for 30 min with 10 μg/ml of recombinant TS. Thymocytes were then allowed to adhere to TEC cultures (50 thymocytes/TEC) for 30 min at 37 °C and for more 30 min at room temperature on a horizontal shaker (60 rpm). Nonadherent cells were gently washed out with PBS, and the number of adhered thymocytes per TEC was then determined by direct counting. Thymocyte migratory activity was assessed in the Transwell system. Briefly, 5 μm pore size Transwell plates (Costar; Corning) were coated with 10 μg/ml fibronectin (Chemicon International) or with BSA at the same concentration for 1 h at 37 °C and later blocked with 0.5% BSA-PBS for 45 min at 37 °C. Thymocytes were treated with 10 μg/ml of recombinant TS and then added in the upper chambers of the transwell chambers. After 3 h of incubation at 37 °C in a 5% CO_2 humidified atmosphere, migration was defined by counting the cells that migrated to the lower chambers by flow cytometer (FACSCalibur, BD).

2.7. Isolation of peripheral blood cells (PBMC), antibodies, flow cytometry and fluorescent-phalloidin staining

Heparinized whole blood was collected and diluted 1/1 with PBS before separated by density centrifugation on Ficoll-Hypaque (Sigma) for 30 min at 2000 rpm. All antibodies were purchased from BD Biosciences (CA, USA). For human T cell phenotyping, individual samples contained 1×10^6 living cells were pre-blocked with human AB serum for 15 min and stained at 4 °C for 30 min simultaneously with three colors using the following antibodies for FACS analysis: APC-labeled anti-CD3, APCCy7-labeled anti-CD4 and PECy7-labeled anti-CD8 antibodies (BD/PharMingen). For murine T cell phenotyping experiments, thymuses were minced, washed and resuspended in 5 μl of Fc receptor blocking solution (BD Pharmingen) for 15 min. The thymocytes were labeled with Alexa 488-labeled anti-CD4 (Caltag) and PercP-labeled anti-CD8 (BD Pharmingen) antibodies as described [20]. In order to verify which thymocytes subsets could be targets of TS, thymocytes were also incubated with TS-FITC conjugate obtained as described [21]. Analyzes were done after recording 25,000–50,000 events for each sample, using CELLQuest software (Becton Dickinson). Lymphocytes were gated based on forward and side scatter parameters, so as to avoid larger leukocytes such as macrophages and granulocytes. For fluorescent-phalloidin staining of actin filaments, thymocytes were left in starvation for 1 h previously to the TS treatment as described before. The cells were then fixed in paraformaldehyde 4% at 4 °C for 20 min and after permeabilization (Perm Wash − BD) were subsequently incubated with phalloidin-FITC (Sigma) and DAPI (Santa Cruz) for immunofluorescence analysis.

2.8. ELISA

Anti-TS antibodies were quantified in plasma samples from healthy ($n = 10$), indeterminate (IND, $n = 12$) or cardiac chronic ($n = 12$) chagasic patients by ELISA. Briefly, Nunc-Immuno microtiter plates (Roskilde, Denmark) were coated with 250 ng of TS lacking the immunodominant SAPA repeats tail (supplied by S. Revelli), blocking with 5% non-fat and aliquots of 150 μL/well from each patient were then added (by duplicate). Chagatest reactives (Wiener Lab, Rosario, Argentina) were used to detect specific antibodies. The plate was read at 450/545 nm using a Stat Fax 2100 plate reader (Awareness Technology, USA).

2.9. Western blotting analysis

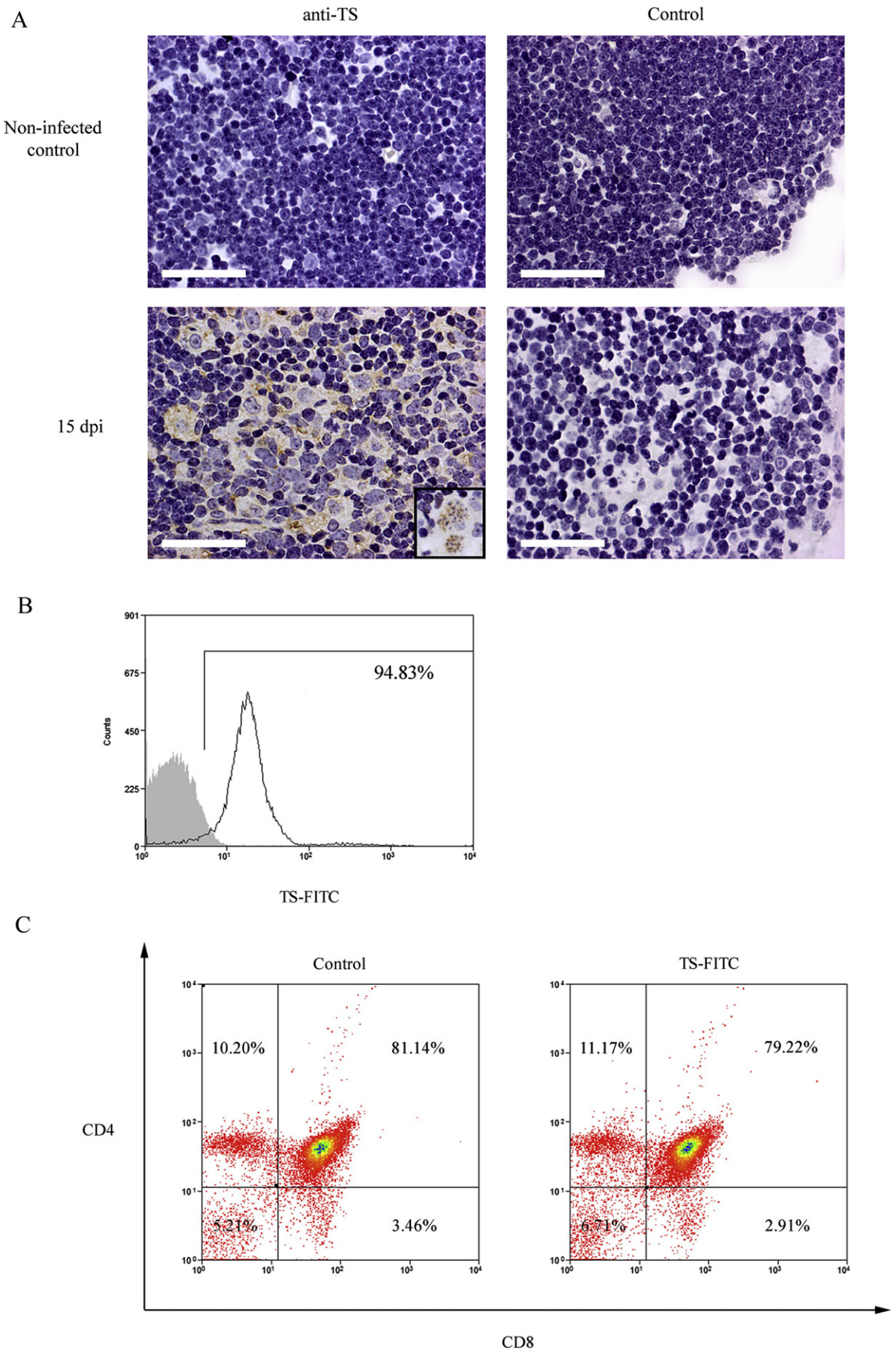
Thymocytes were left under starvation for 1 h. Later, they were treated with both forms of recombinant TS for 0, 15 and 30 min. Thymocytes were then lysed in RIPA lysis buffer. Lysates were centrifuged at $16{,}000 \times g$ for 10 min at 4 °C and the proteins present in the supernatants were solubilized in an SDS buffer for electrophoresis [10] and fractionated in SDS-PAGE 9%. The proteins were transferred to PVDF membranes (Trans-Blot system, Bio-Rad) and the membranes were incubated overnight with anti-p-Tyr, anti-p-ERK and anti-p-JNK (Cell Signaling), followed by horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies IgG for ECL quimioluminescence reaction (Amersham-Pharmacia).

2.10. Tissue preparation and histochemistry

Thymuses from TS-injected mice and from infected animals (15 days post-infection) were fixed in paraformaldehyde and process for paraffin wax sectioning. Endogenous peroxidase was blocked with 3% H_2O_2. The slides were blocked with a solution containing 8% non-fat dried milk in PBS-BSA/10%, Tween 0.05%, Triton 0.1%, normal goat serum 20% and normal mice serum 20%. The paraffin sections, 5 μm thick, were labeled with anti-TS polyclonal antibody (diluted in PBS/BSA 3%, Tween 0.05%, Triton 0.1%, normal goat serum 10% and normal rabbit serum 10%), overnight at 4 °C in a humidified chamber. The sections were washed with PBS-Tween 0.2% and incubated with anti-rabbit IgG polymer (for anti-TS labeled sections) diluted in PBS (NICHEREI, Japan).

2.11. Statistical analysis

Statistical analyzes were performed with GraphPad Prism 4 software, using one-way ANOVA test. Results were expressed as mean ± standard error (S.E.). Differences between control vs. treated group were considered statistically significant when $P < 0.05$.


A
anti-TS
Control
Non-infected control
15 dpi
B
94.83%
Counts
TS-FITC
C
Control
TS-FITC
10.20%
81.14%
5.21%
3.46%
11.17%
79.22%
6.71%
2.91%
CD4
CD8

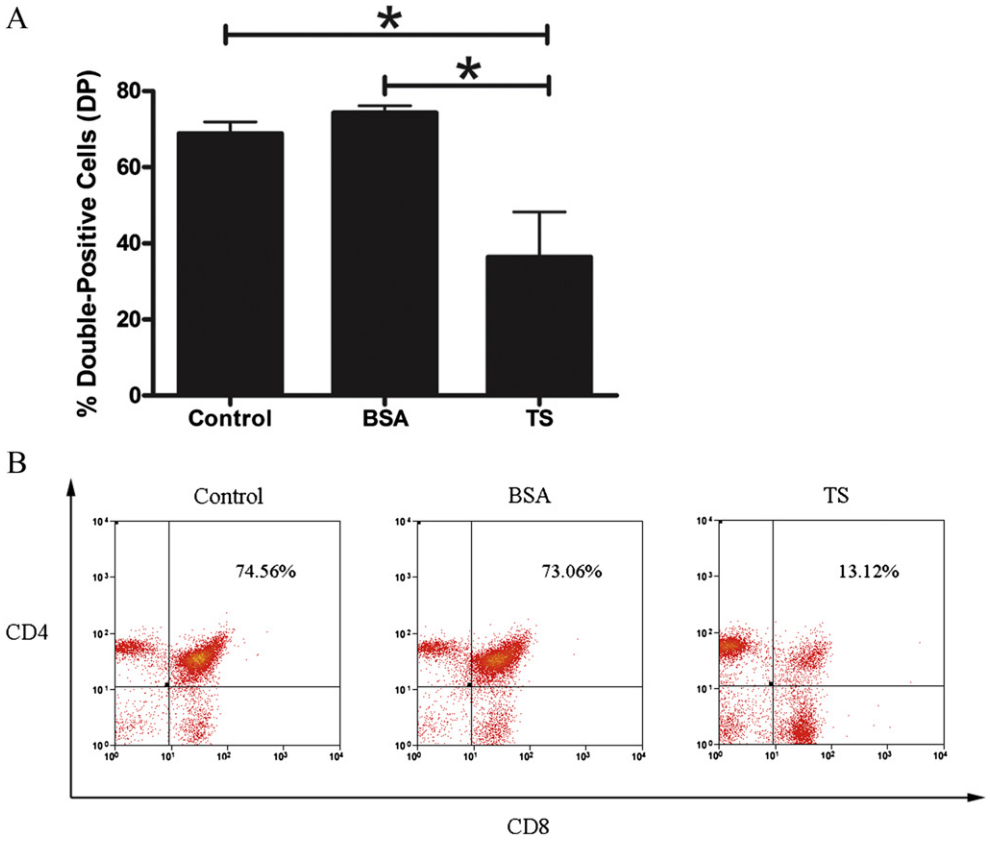


Fig. 2. Intrathymic injection of *T. cruzi trans*-sialidase induces thymocyte depletion. BALB/c mice were injected with 10 μg of TS, or alternatively PBS or BSA as control. After 5 days, thymuses were collected and thymocytes were stained with anti-CD4 and anti-CD8 mAbs prior to flow cytometry analysis. The percentages of double-positive $CD4^+CD8^+$ T cells are represented by (A) histograms and (B) dot-plot analysis. Data are representative of three independent experiments, and are means ± S.Ds. of two independent experiments ($n = 6$ mice per group). $^*P < 0.05$.

3. Results

3.1. Immunohistochemical evidence that parasite trans-*sialidase antigens are present in the* T. cruzi*-infected thymus and bind to thymocyte surfaces*

Intense thymic atrophy is a hallmark of the acute phase of experimental Chagas disease. This phenomenon is evident by 10 days post-infection after intraperitoneal injection of 10^2 trypomastigotes into BALB/c mice. The severe thymic atrophy has been shown to be at least in part due to the activity of the parasite-derived *trans*-sialidase [16]. We tested for the parasite *trans*-sialidase in the thymus of infected animals using a specific-polyclonal antibody and detected reactivity 15 days post-infection, the peak of parasitemia. As shown in Fig. 1A, the cortical and medullary regions of the thymus of infected mice stained strongly with the polyclonal anti-TS antibody. This staining profile indicates that the *trans*-sialidase depots are present near the parasite nests in the thymic tissue surrounding the thymocytes.

These results raise the possibility that *trans*-sialidase binds to the surfaces of differentiating thymocytes. This would have an important effect on the biology of these cells as the parasite enzyme has a lectin binding site besides its *trans*-sialidase activity [17,22], which could promote signal transduction by interacting with surface signaling receptors. Following this line of reasoning, we decided to test whether the *trans*-sialidase derived from *T. cruzi* was able to bind to the thymocyte surface. Using FACS analysis we showed that FITC-labeled recombinant parasite *trans*-sialidase bound to the surface of thymocytes (Fig. 1B) obtained from normal thymus, with a staining profile not restricted to any particular subset of cells (Fig. 1C).

Fig. 1. Immunohistochemical evidence of *trans*-sialidase in thymus *in acute T. cruzi* infection and its binding to thymocyte cells. BALB/c mice were injected intraperitoneally with 10^2 blood-derived trypomastigote forms of the *Tuluahuen* strain. At 15 days p.i., animals were killed and thymuses were removed for (A) immuno-histochemistry analysis with a polyclonal antibody against *T. cruzi trans*-sialidase. Thymus sections from non-infected control mice were labeled with polyclonal anti-TS and streptavidin (upper left panel), or streptavidin only (upper right panel). The insert is a higher magnification of an amastigote niche stained for TS. Bars = 40 μm. Data are representative of two independent experiments using six mice per group. To detect direct binding of TS to thymocyte surface, cells were incubated for 30 min with TS conjugated to FITC and co-stained with anti-CD4 and anti-CD8 mAbs prior to flow cytometry. Panel (B) depicts representative histograms of TS-$FITC^+$ cells (line graph), compared with control thymocytes (solid gray). (C) Dot-plots show the percentages of double-positive $CD4^+CD8^+$ cells gated on TS-$FITC^+$ cells. Data are representative of three independent experiments.

3.2. *Intrathymic injection of* trans-*sialidase promotes thymic atrophy and premature release of double-positive CD4$^+$CD8$^+$ thymocytes to peripheral lymphoid organs*

There is evidence that the *T. cruzi trans*-sialidase affects nurse cell complexes and leads to apoptosis of thymocytes undergoing differentiation inside the nurse cells. Importantly, this phenomenon depends on the α-(2,3) sialyltransferase activity of the *trans*-sialidase since it is not observed with the inactive enzyme [16].

To see whether the *trans*-sialyltransferase plays a direct role in the depletion of double-positive CD4$^+$CD8$^+$ thymocytes *in vivo*, we injected mice intrathymically with 10 μg of recombinant *trans*-sialidase. Five days later, thymuses were collected and viable cells were counted by trypan blue exclusion, and the percentages of the various thymocyte subsets were assessed by FACS analysis. As described for acute *T. cruzi* infection, we found a significant reduction of the proportion of DP T cells in the TS-injected mice as compared to controls injected with PBS or the non-related BSA protein (Fig. 2). Similar results were observed in TLR4-deficient mice, thus excluding the possible effect of any contaminating LPS in the *trans*-sialidase preparation (Supplementary Fig. 1).

Thymic atrophy in *T. cruzi* infections is correlated with an increase in the population size of extrathymic immature thymocytes [6,7]. We next asked whether the parasite enzyme actually plays a role in the premature release of double-positive CD4$^+$CD8$^+$ thymocytes to peripheral lymphoid organs in chronic chagasic patients, and we obtained evidence of a possible relationship between the presence of the *trans*-sialidase as measured immunologically (Fig. 3A) and the frequency of the peripheral blood DP cell subset (Fig. 3B) in cross-sectional studies of patients with the indeterminate or cardiac clinical forms of Chagas disease. There was a gradual enhancement of the antibody titer against the parasite enzyme as the percentage of DP cells in the peripheral blood of patients increased (Fig. 3).

Given this result, we next tested directly whether the presence of the parasite *trans*-sialidase in the thymic microenvironment interferes with thymopoiesis and leads to release of undifferentiated thymocyte cells to the periphery. BALB/c naïve mice were intrathymically injected with FITC and *T. cruzi trans*-sialidase (10 μg), and 24 h later FITC-labeled cells appearing in the spleen were analyzed. We observed an increase in the size of splenic CD4$^+$CD8$^+$ RTE population after thymic injection of the *trans*-sialidase, with 30–40% of the recent thymic emigrants (RTEs) representing DP cells (Fig. 4). These results show that the increase in the peripheral DP T cell

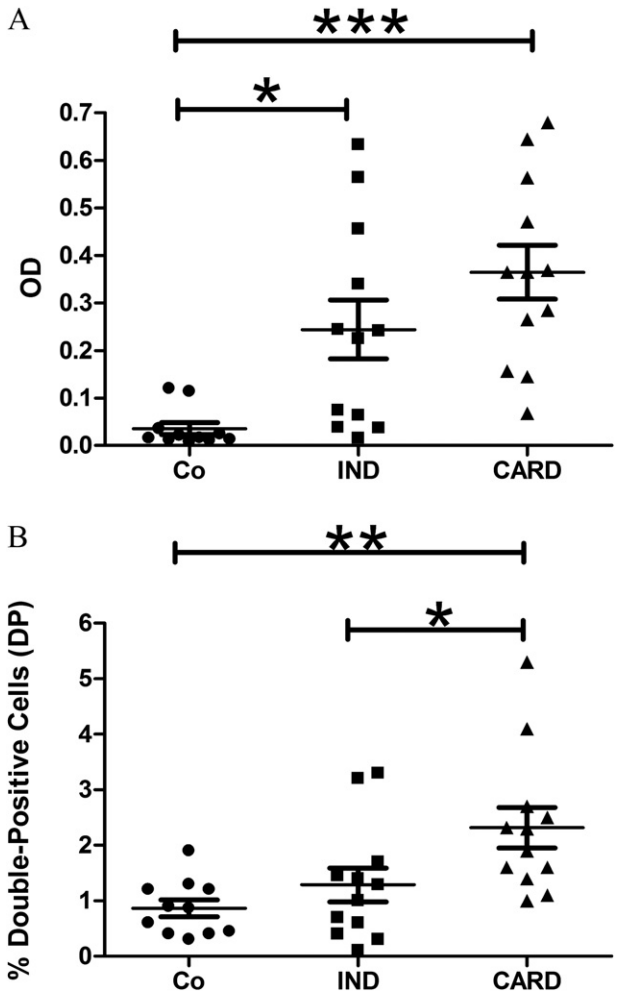


Fig. 3. Anti-TS antibody titers and frequency of the peripheral blood CD4$^+$CD8$^+$ T cell subset in chronic chagasic patients. (A) Distribution of anti-TS Ab in sera from non-infected individuals (NI), and patients with the indeterminate (IND) and cardiac (CARD) forms of Chagas disease analyzed by ELISA. Data are means ± S.Ds. (B) Peripheral whole blood was analyzed by four-color flow cytometry for expression of the CD3, CD4, CD8 markers. The percentages of CD4$^+$CD8$^+$ cells on CD3$^+$ T lymphocytes are indicated for each histogram. Each point represents one subject. $p < 0.05$ ($n = 15$ individuals per IND and CARD group; $n = 12$ individuals per NI group).

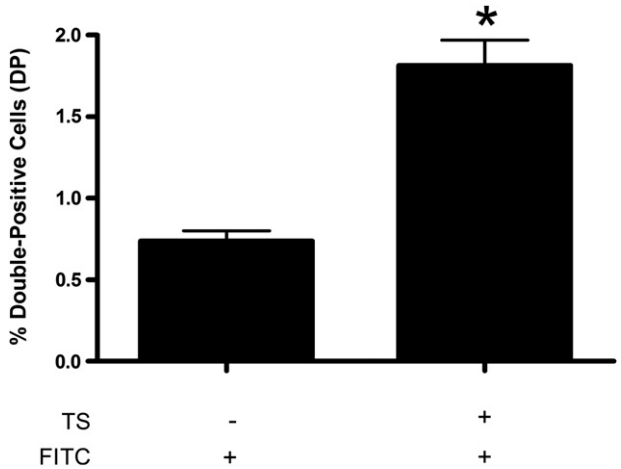


Fig. 4. Intrathymic injection of *T. cruzi trans*-sialidase promotes the premature release of CD4$^+$CD8$^+$ double-positive T cells to the periphery. BALB/c mice were intrathymically injected with an FITC solution containing 10 μg of TS, or PBS only. After 24 h, spleen cells were stained with anti-CD4 and anti-CD8 mAbs prior to flow cytometer analyzes. Histograms represent the percentages of CD4$^+$CD8$^+$ T cells among recent thymic emigrants (RTEs) obtained from the spleen. Data are means ± S.D. ($n = 6$ mice per group). *$P < 0.05$.

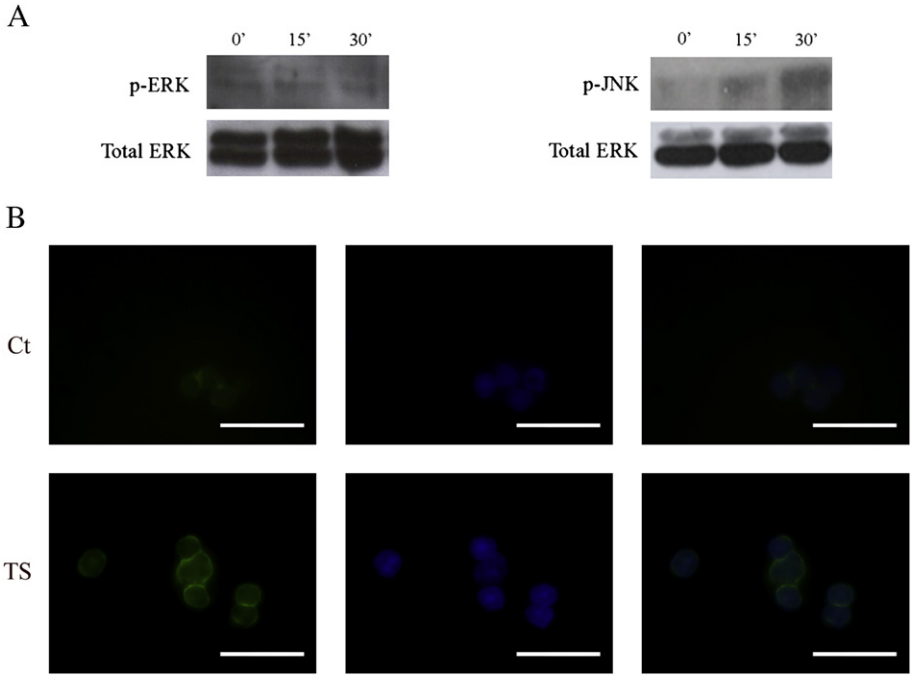


Fig. 5. *T. cruzi trans*-sialidase activates the JNK MAPK pathway and actin rearrangement in thymocytes. Thymocytes were treated with *T. cruzi* TS for Western blotting analysis probing with (A) anti-p-ERK and anti-p-JNK. In order to characterize actin filaments the cells were incubated with phalloidin-FITC (B) for 20 min after treatment with TS (left panels); DAPI was used for nuclear staining (middle panels); merged images (right panels). The results are representative of three independent experiments.

subset after intrathymic treatment with *T. cruzi trans*-sialidase derives from thymocyte subpopulations that leave the thymus.

3.3. Activation of JNK MAP kinases and actin rearrangement in trans*-sialidase-treated thymocytes*

As the *trans*-sialidase has been shown to activate the mitogen-activated protein kinase (MAPK) cascade in other cell types [23], we tested whether the association of the *T. cruzi trans*-sialidase with thymocytes also activated this important signaling pathway involved in cell migration. To this end, we first approached the activation of the classical MAPK by western blot analysis with anti-phospho-MAP Kinase1/2 (ERK1/2) and anti-phospho JNK monoclonal antibodies. We observed no change in the profile of ERK phosphorylation (Fig. 5A), but a marked increase in JNK phosphorylation (Fig. 5A).

The JNK pathway plays crucial roles in cell adhesion and migration, processes finely coordinated with remodeling of the actin cytoskeleton [24,25]. Since adhesive and migratory responses are critical features of intrathymic T cell differentiation and the export of mature T lymphocytes from the thymus to peripheral tissues, we examined whether the events initiated by *T. cruzi trans*-sialidase in thymocytes lead to actin filament rearrangements. Using FITC-labeled phalloidin, which binds to F-actin in the form of filaments or oligomers, we detected polymerization of actin filaments in thymocytes upon treatment with the parasite *trans*-sialidase; as shown in Fig. 5B, the actin filaments in thymocytes treated with *trans*-sialidase have a more homogenous distribution than in untreated thymocytes in which the filaments seem to be concentrated in the region of contact between cells.

3.4. T. cruzi trans*-sialidase modulates the adhesive behavior of thymocytes and increases their migration toward extracellular matrix (ECM) molecules*

Since our findings indicated that the *T. cruzi trans*-sialidase promotes actin remodeling in thymocytes, an important feature of cell adhesion and migratory responses, we next investigated a possible effect of the parasite enzyme on thymocytes-thymic epithelial cell (TEC) interactions during thymocyte development. For this purpose, either thymocytes or TECs were treated with the *T. cruzi* enzyme and then co-cultured in the adhesion assay for 30 min at 37 °C. When the thymocytes were incubated with *trans*-sialidase, we found a significant increase in the numbers of adhering thymocytes, but this effect was not observed when instead the TECs were pretreated with TS enzyme (Fig. 6A).

Since adhesion processes are required for thymocyte migration during intrathymic development [26], we next determined whether the *T. cruzi* enzyme affected the migration of thymocytes toward the extracellular matrix. When we measured thymocyte migration in transwell chambers precoated with fibronectin, we found an increase in the number of migrating cells if the thymocytes were first treated with the parasite *trans*-sialidase (Fig. 6B). This treatment did not lead to enhanced expression of fibronectin receptors on the thymocytes, as we found no effect on the FACS staining profile of VLA-4, VLA-5 and VLA-6 (data not shown). These findings

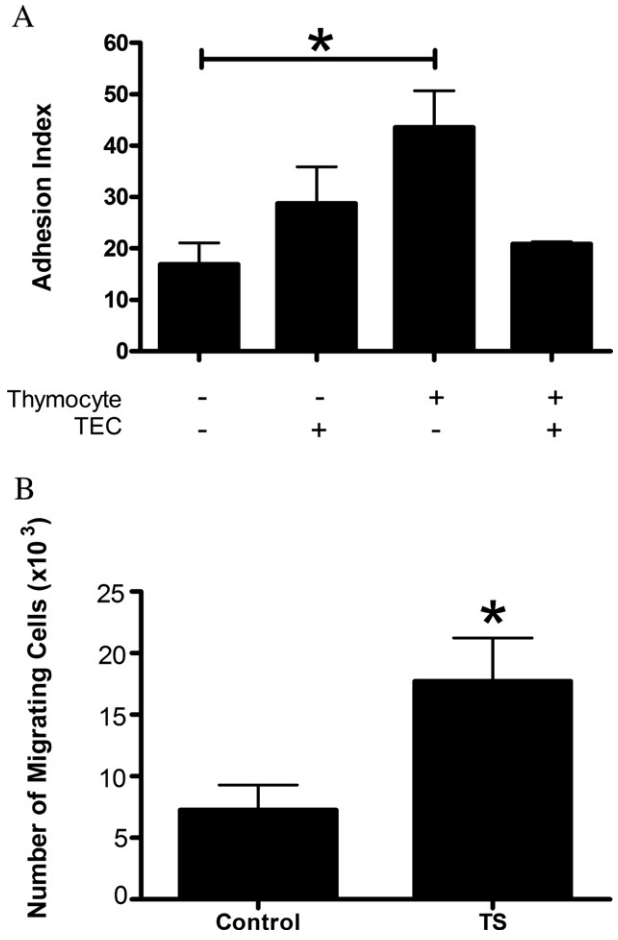


Fig. 6. *T. cruzi trans*-sialidase modulates adhesion and fibronectin-driven migration of thymocytes. Thymocytes and/or TEC epithelial IT-76M1 cells from BALB/c mice were incubated with 10 μg of TS for 30 min at 37 °C. Following treatment, thymocytes were allowed to adhere to TEC cultures for a further 30 min. The plots show the association index for (A) TS treatment. Data are means with S.E.M. of triplicate measurements of two representative experiments and are expressed as association indexes calculated as follows: Adhesion Index (AI) = (TEC with bound thymocytes)/Total TEC number × (thymocytes bound to TEC)/(Total TEC number) × 100. (B) Migratory responses were assessed in transwell chambers by adding thymocytes to the upper chambers coated with fibronectin or BSA after TS treatment for 30 min at 37 °C. The cells were allowed to migrate for 3 h, and the number of cells migrating toward fibronectin was assessed by subtracting the average number of non-treated migrating cells obtained in wells coated with BSA. Cells were counted in a flow cytometer. Data shown are mean with S.E.M. of five representative experiments. *$P < 0.05$.

indicate that the *T. cruzi trans*-sialidase is able to modulate ECM-mediated thymocyte migration, probably via signaling processes that alter integrin affinity ('activation').

4. Discussion

Thymic atrophy is a feature of several infectious diseases characterized by developmental disturbance of the intrathymic thymocyte subpopulations and export of immature T cell [27]. In Chagas disease, it has been demonstrated that molecules that are shed by the *T. cruzi* contribute to the thymic disturbances seen during infection. This is the case for the parasite-derived *trans*-sialidase which promotes the intrathymic T cell death by apoptosis induction in the nurse cell complex [16]. Nevertheless, a lack of a suitable explanation for the underlying mechanisms responsible for the premature release of immature thymocytes in *T. cruzi* infection still remains.

In the present work we aimed to investigate the possible role of the *T. cruzi trans*-sialidase in modifying the migratory responses of intrathymic thymocytes. We detected TS antigens in the thymuses of acutely infected mice. Furthermore, we showed that the parasite enzyme binds to the thymocyte surface and injection of 10 μg of TS induced intense thymic atrophy with thymocyte depletion, mainly of $CD4^+CD8^+$ cells, similar to what occurs in the acute phase of experimental *T. cruzi* infection. Although 10 μg is the amount produced by about 10^9 parasites [28], it is reasonable to suppose that an adequate level of TS could be reached as a result of the cumulative effect of the TS produced during the acute phase of infection, in which the parasitemia peaks at about 10^6–10^7 parasites/ml of blood in BALB/c mice in our experimental model [29]. Moreover, it has been shown that the activity of exogenous TS-injected intravenously into uninfected BALB/c mice decays rapidly, probably as a result of breakdown, whereas TS activity stays high in infected mice throughout the parasitemia [28].

Recently, it was possible to identify by mass spectrometry in mouse thymocytes several cell surface acceptors of sialyl residues in TS-catalyzed reactions [30]. Changes in the sialylation profile of the cell surface receptors caused by TS induced apoptosis in cells of the immune system *in vivo*, including the thymus [15]. A detailed study of TS-treated animals revealed that these effects were also a result of apoptosis in the thymic nurse cell complexes [16]. The release of undifferentiated $CD4^+CD8^+$ cells also accounts for thymic atrophy [6,7,31]. Here we investigated whether the *trans*-sialidase could also modulate the migratory responses of thymocytes, which might account for the abnormal $CD4^+CD8^+$ release from the thymus during infection.

Thymocyte migration is a crucial event in the intrathymic differentiation of T cells [26]. It comprises several steps such as entrance of bone marrow-derived precursors into the thymus, traffic of immature thymocytes within the cortex and from the cortex to the medulla, and finally, exit of these cells from the organ [26,27]. All these processes require first the adhesion and then de-adhesion of thymocytes from ECM compounds onto TEC or microenvironmental components [27,32]. Interestingly, we found that intrathymic *trans*-sialidase treatment increased the premature release of $CD4^+CD8^+$ cells during thymic atrophy. We found an increased proportion of $CD4^+CD8^+$ recent thymic emigrant (RTE) T cells migrating to the peripheral lymphoid organs in the murine model. In humans, our findings indicate a possible relationship between the presence of *trans*-sialidase enzyme and the size of the peripheral blood DP cell subset in chronic chagasic patients in the cardiac clinical forms of Chagas disease.

We showed that the parasite enzyme is able to activate the JNK MAPK pathway in thymocytes. JNK plays a role in regulating the cell migration of a broad range of cells [24,25]. Cell adhesion and migration require cytoskeleton structures such as those formed by the actin network and cytoskeleton-

associated proteins such as the actin-binding proteins, which are known to be JNK substrates [24,25,33]. In fact, our findings indicate that the cell signaling events promoted by the *trans*-sialidase result in mobilization of actin filaments. These signaling events are activated in the interactions between TEC and thymocytes, a required process for migratory dynamics of thymocytes during the intrathymic development [34]. Here we demonstrated that the *T. cruzi* TS increased thymocyte adhesion to TEC. Interestingly, it should be pointed out that in order to migrate, thymocytes must adhere to their microenvironment and afterward de-adhere [26]. In this regard, molecules with adhesive and de-adhesive properties may exert an important function in modulating the migration [26,35]. In fact, our findings indicated that *in vitro* thymocyte treatment with *T. cruzi* TS resulted in an increased cell migration toward fibronectin, indicating a role for this enzyme on this phenomenon.

In conclusion, our results show that *T. cruzi trans*-sialidase induced thymic atrophy affects the dynamics of intrathymic thymocytes resulting in an increase in the number of $CD4^+8^+$ double-positive recent thymic emigrants in the spleen. Furthermore, we showed that TS is able to activate in thymocytes MAPK JNK signaling and modulate thymocyte adhesion to TECs and their migration toward the extracellular matrix. These findings point to the possible involvement of parasite-derived TS in the abnormal thymocyte trafficking within the thymus of acutely *T. cruzi*-infected animals, which could influence the escape of immature thymocytes in Chagas disease. We also found an increased frequency of DP T cells with high antibody titles against the *T. cruzi* TS enzyme in chronic patients with the cardiac form of Chagas disease. The presence of peripheral activated DP cells with potentially autoreactive TCR may contribute to the immunopathological events found in this disease. Our data support the potential utility of chemotherapy approaches directed against the parasite TS in Chagas disease.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2013.02.003.

References

[1] V.H.M. Aguilar, F. Abad-Franch, V.J. Racines, C.A. Paucar, Epidemiology of Chagas disease in Ecuador. A brief review, Mem. Inst. Oswaldo Cruz 94 (1999) 387–393.

[2] J.R. Coura, J. Borges-Pereira, Chagas disease: 100 years after its discovery. A systemic review, Acta Tropic 115 (2010) 5–13.

[3] W. Savino, D.M. Villa-Verde, D.A. Mendes-da-Cruz, E. Silva-Monteiro, A.R. Perez, M. del Pilar Aoki, O. Bottasso, N. Guiñazú, S.D. Silva-Barbosa, S. Gea, Cytokines and cell adhesion receptors in the regulation of immunity to *Trypanosoma cruzi*, Cytokine Growth Factor Rev. 18 (2007) 107–124.

[4] R. Devera, O. Fernandes, J.R. Coura, Should *Trypanosoma cruzi* be called "cruzi" complex? A review of the parasite diversity and the potential of selecting population after *in vitro* culturing and mice infection, Mem. Inst. Oswaldo Cruz 98 (2003) 1–12.

[5] C.R. Marinho, M.R. D'Imperio Lima, M.G. Grisotto, J.M. Alvarez, Influence of acute-phase parasite load on pathology, parasitism, and activation of the immune system at the late chronic phase of Chagas' disease, Infect. Immun. 67 (1999) 308–318.

[6] W. Savino, M.C. Leite-de-Moraes, M. Hontebeyrie-Joskowicz, M. Dardenne, Studies on the thymus in Chagas' disease. I. Changes in the thymic microenvironment in mice acutely infected with *Trypanosoma cruzi*, Eur. J. Immunol. 19 (1989) 1727–1733.

[7] M.C. Leite-de-Moraes, M. Hontebeyrie-Joskowicz, M. Dardenne, W. Savino, Modulation of thymocyte subsets during acute and chronic phases of experimental *Trypanosoma cruzi* infection, Immunology 77 (1992) 95–98.

[8] V. Cotta-de-Almeida, A.L. Bertho, D.M.S. Villa-Verde, W. Savino, Phenotypic and functional alterations of thymic nurse cells following acute *Trypanosoma cruzi* infection, Clin. Immunol. Immunopathol. 82 (1997) 125–132.

[9] D.A. Mendes-da-Cruz, J. De-Meis, V. Cotta-de-Almeida, W. Savino, Experimental *Trypanosoma cruzi* infection alters the shaping of the central and peripheral T-cell repertoire, Microbes Infect. 5 (2003) 824–831.

[10] A. Morrot, E. Terra-Granado, A.R. Pérez, S.D. Silva-Barbosa, N. Milicevic, D.A. Farias-de-Oliveira, L.R. Berbert, J. de-Meis, C. Maeda Thakiya, J. Beloscar, X. Wang, V. Kont, P. Peterson, O. Botasso, W. Savino, Chagasic thymic atrophy does not affect thymocyte negative selection but results in the export of $CD4^+CD8^+$ T cells in severe forms of human disease, PLoS Negl. Trop. Dis. 5 (8) (2011) e1268.

[11] J.G. Simpson, E.S. Gray, J. Swanson Beck, Age involution in the normal human adult, Clin. Exp. Immunol. 19 (1975) 261–265.

[12] J.O. Previato, A.F.B. Andrade, M.C.V. Pessolani, L. Mendonça-Previato, Incorporation of sialic acid into *Trypanosoma cruzi* macromolecules; a proposal for a new metabolic route, Mol. Biochem. Parasitol. 16 (1985) 85–96.

[13] S. Schenkman, M.S. Jiang, G.W. Hart, V. Nussenzweig, A novel cell surface *trans*-sialidase specific of *Trypanosoma cruzi* generates a stage -specific epitope required for invasion of mammalian cells, Cell 65 (1991) 1117–1125.

[14] S. Schenkman, L. Pontes de Carvalho, V. Nussenzweig, *Trypanosoma cruzi trans*-sialidase and neuraminidase activities can be mediated by the same enzymes, J. Exp. Med. 75 (1992) 567–575.

[15] M.S. Leguizamon, E. Mocetti, H. Garcia Rivello, P. Argibay, O. Campetella, *Trans*-sialidase from *Trypanosoma cruzi* induces apoptosis in cells from the immune system in vivo, J. Infect. Dis. 180 (1999) 1398–1402.

[16] J. Mucci, A. Hidalgo, E. Mocetti, P.F. Argibay, M.S. Leguizamon, O. Campetella, Thymocyte depletion in *Trypanosoma cruzi* infection is mediated by *trans*-sialidase-induced apoptosis on nurse cells complex, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 3896–3901.

[17] C.L. Cremona, O. Campetella, D.O. Sanchez, A.C.C. Frasch, Enzymically inactive members of *trans*-sialidase family from *Trypanosoma cruzi* display β-galactose binding activity, Glycobiology 9 (6) (1999) 581–587.

[18] A. Buschiazzo, A.C.C. Frasch, O. Campetella, Medium scale production and purification to homogeneity of a recombinant *trans*-sialidase from Trypanosoma cruzi, Cell. Mol. Biol. 42 (5) (1996) 703–710.

[19] T.R. Todeschini, L. Mendonça-Previato, J.O. Previato, A. Varki, H. van Halbeek, *Trans*-sialidase from *Trypanosoma cruzi* catalyzes sialoside hydrolysis with retention of configuration, Glycobiology 10 (2000) 213–221.

[20] D.A. Mendes-da-Cruz, J.S. Silva, V. Cotta-de-Almeida, W. Savino, Altered thymocyte migration during experimental acute *Trypanosoma cruzi* infection: combined role of fibronectin and the chemokines CXCL12 and CCL4, Eur. J. Immunol. 36 (2006) 1486−1493.

[21] N. Baumgarth, M. Roederer, A practical approach to multicolor flow cytometry for immunophenotyping, J. Immunol. Methods 243 (2000) 77−97.

[22] A.R. Todeschini, M.F. Girard, J.M. Wieruszeski, M.P. Nunes, G.A. DosReis, L. Mendonça-Previato, J.O. Previato, *Trans*-sialidase from *Trypanosoma cruzi* binds host T-lymphocytes in a lectin manner, J. Biol. Chem. 277 (2002) 45962−45968.

[23] M.V. Chuenkova, M.A. Pereira, The *T. cruzi trans*-sialidase induces PC12 cell differentiation via MAPK/ERK pathway, Neuroreport 17 (2001) 3715−3718.

[24] C. Huang, K. Jacobson, M.D. Schaller, MAP kinases and cell migration, J. Cell. Sci. 117 (2004) 4619−4628.

[25] B. Björkblom, A. Padzik, H. Mohammad, N. Westerlund, E. Komulainen, P. Hollos, L. Parviainen, A.C. Papageorgiou, K. Iljin, O. Kallioniemi, M. Kallajoki, M.J. Courtney, M. Mågård, P. James, E.T. Coffey, c-Jun N-terminal kinase phosphorylation of MARCKSL1 determines actin stability and migration in neurons and in cancer cells, Mol. Cell. Biol. 32 (2012) 3513−3526.

[26] W. Savino, D.A. Mendes-da-Cruz, S. Smaniotto, E. Silva-Monteiro, D.M.S. Villa-Verde, Molecular mechanisms governing thymocyte migration: combined role of chemokines and extracellular matrix, J. Leukoc. Biol. 75 (2004) 951−961.

[27] W. Savino, The thymus is a common target in infectious diseases, PLoS Pathog. 2 (6) (2006) e262.

[28] L. Freire-de-Lima, F. Alisson-Silva, S.T. Carvalho, C.M. Takiya, M.M. Rodrigues, G.A. DosReis, L. Mendonça-Previato, J.O. Previato, A.R. Todeschini, *Trypanosoma cruzi* subverts host cell sialylation and may compromise antigen-specific CD8+ T cell responses, J. Biol. Chem. 285 (2010) 13388−13396.

[29] V. Cotta-de-Almeida, A. Bonomo, D.A. Mendes-da-Cruz, I. Riederer, J. de Meis, K.R.F. Lima-Quaresma, A. Vieira-de-Abreu, D.M.S. Villa-Verde, W. Savino, *Trypanosoma cruzi* infection modulates intrathymic contents of extracellular matrix ligands and receptors and alters thymocyte migration, Eur. J. Immunol. 32 (2003) 2329−2338.

[30] R.P. Muiá, H. Yu, J.A. Prescher, U. Hellman, X. Chen, C.R. Bertozzi, O. Campetella, Identification of glycoproteins targeted by *Trypanosoma cruzi trans*-sialidase, a virulence factor that disturbs lymphocyte glycosylation, Glycobiology 20 (7) (2010) 833−842.

[31] J. de Meis, A. Morrot, D.A. Farias-de-Oliveira, D.M.S. Villa-Verde, W. Savino, Differential regional immune response in Chagas disease, PLoS Negl. Trop. Dis. 3 (7) (2009) e417.

[32] S.R. Dalmau, C.L. Freitas, W. Savino, High expression of fibronectin receptors and L-selectin as a hallmark of early steps of thymocyte differentiation: lessons from sublethally irradiated mice, Blood 93 (1999) 974−990.

[33] I.M. Otto, T. Raabe, U.E.E. Rennefahrt, P. Bork, U.R. Rapp, E. Kerkhoff, The p150-Spir protein provides a link between c-Jun N-terminal kinase function and actin reorganization, Curr. Biol. 10 (2000) 345−348.

[34] K. Utsumi, M. Sawada, S. Narumyia, J. Nagamine, T. Sakata, S. Iwagami, Y. Kita, H. Teraokaq, H. Hirasno, M. Ogata, T. Hamaoka, H. Fujiwara, Adhesion of immature thymocytes thymic stromal cells through fibronectin molecules and its significance for the induction of thymocyte differentiation, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 5685−5689.

[35] W. Savino, D.A. Mendes-da-Cruz, J.S. Silva, M. Dardenne, V. Cotta-de-Almeida, Intrathymic T cell migration: a combinatorial interplay of extracellular matrix and chemokines? Trends Immunol. 22 (2002) 305−313.