The American Journal of Pathology, Vol. 170, No. 2, February 2007
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DOI: 10.2353/ajpath.2007.060389

Immunopathology and Infectious Disease

Altered Expression of Galectin-3 Induces Cortical Thymocyte Depletion and Premature Exit of Immature Thymocytes during *Trypanosoma cruzi* Infection

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During acute infection with *Trypanosoma cruzi*, the causative agent of Chagas' disease, the thymus undergoes intense atrophy followed by a premature escape of $CD4^+CD8^+$ immature cortical thymocytes. Here we report a pivotal role for the endogenous lectin galectin-3 in accelerating death of thymocytes and migration of these cells away from the thymus after *T. cruzi* infection. We observed a pronounced increase in galectin-3 expression that paralleled the extensive depletion of $CD4^+CD8^+$ immature thymocytes after infection. *In vitro*, recombinant galectin-3 induced increased levels of death in cortical immature thymocytes. Consistent with the role of galectin-3 in promoting cell death, thymuses from gal-$3^{-/-}$ mice did not show cortical thymocyte depletion after parasite infection *in vivo*. In addition, galectin-3 accelerated laminin-driven $CD4^+CD8^+$ thymocyte migration *in vitro* and *in vivo* induced exportation of $CD4^+CD8^+$ cells from the thymus to the peripheral compartment. Our findings provide evidence of a novel role for galectin-3 in the regulation of thymus physiology and identify a potential mechanism based on proteinglycan interactions in thymic atrophy associated with acute *T. cruzi* infection. ***(Am J Pathol 2007, 170:546–556; DOI: 10.2353/ajpath.2007.060389)***

Acute infection with *Trypanosoma cruzi*, the causative agent of Chagas' disease, is followed by strong activation of the immune system that influences the function of macrophages,[1] natural killer (NK) cells,[2] and B and T lymphocytes.[3,4] We have previously shown that the thymus is severely affected during acute infection with *T. cruzi* in mice and becomes atrophic because of the death and premature exit of cortical thymocytes.[5–7] Moreover, an increased expression of extracellular matrix (ECM) glycoproteins, such as laminin and fibronectin, and their integrin-type receptors occurs during thymic atrophy.[5,7]

The cortical population of thymic nurse cells (TNCs), lymphoepithelial complexes in which immature thymocytes undergo differentiation and occasionally die by apoptosis, is altered after infection. Specifically, i*n vitro* analysis of TNCs recovered from the thymus of infected mice showed that they are smaller in both size and number, have altered expression of ECM molecules,

Supported in part by The Oswaldo Cruz Foundation, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro, and Fundação de Amparo a Pesquisa do Estado de São Paulo.

Accepted for publication October 27, 2006.

Supplemental material for this article can be found on *http://ajp.amjpathol.org.*

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and release thymocytes faster than those from control animals.[8] In this regard, alterations in cell-cell and cell-ECM interactions in the thymic microenvironment after *T. cruzi* infection are accompanied by high numbers of immature $CD4^+CD8^+$ T cells found in lymph nodes of infected mice. Interestingly, some of these lymphocytes bear potentially autoreactive T-cell receptors (TCRs), probably representing cells that escaped from the normal processes of thymic selection.[9] The presence of these immature T cells in the periphery might contribute to the induction of chagasic cardiopathy through an autoimmune response.

Galectins are a family of β-galactoside-binding proteins highly conserved throughout animal evolution, which are present at different subcellular compartments.[10] These proteins modulate several biological processes, such as cell adhesion, migration, proliferation, and apoptosis.[11] Galectin-1, -3, and -9 are expressed in thymus, among which galectin-1 and -3 are present throughout all of the thymic parenchyma, being mainly produced by thymic epithelial cells (TECs).[12–15] Interestingly, recent evidence indicates that galectins can interact with ECM glycoproteins and modulate cell-cell interactions within the thymic microenvironment.[12,15] We have demonstrated that galectin-3 produced by thymic stromal cells can interfere with TEC/thymocyte adhesion, by acting as an antiadhesive molecule and modulating protein-carbohydrate interactions.[15]

It has become increasingly apparent that galectin-1 and -3 function as regulatory proteins, sometimes with opposite effects. Galectin-1 can induce apoptosis in immature thymocytes displaying the $CD3^{low}CD4^-CD8^-$ and $CD3^{low}CD4^{low}CD8^{low}$ phenotypes, an effect that parallels the processes of selection and thymocyte differentiation.[12,16] On the other hand, *in vitro* studies revealed that intracellular galectin-3 inhibits apoptosis induced by a wide variety of stimuli in activated T lymphocytes and tumor cells,[17,18] whereas extracellular galectin-3 promotes apoptosis when added exogenously to T cells.[19,20] Interestingly, recent data suggest that galectin-1 and -3 kill T cells by binding to them and engaging different cell surface glycoproteins.[20] In addition, these carbohydrate-binding proteins can trigger different cell death pathways, with or without caspase activation, in different cell types.[21–23]

Despite the increasing understanding of the effects of *T. cruzi* infection on thymus physiology, the mechanisms that control $CD4^+CD8^+$ cell depletion and the escape of immature thymocytes from the infected thymus remain unclear. In the present study, we have found evidence of a critical role of galectin-3 in thymic atrophy during the acute phase of *T. cruzi* infection. Combining *in vitro* and *in vivo* assays conducted in control and infected galectin-3-null mice, we found a clear association of this protein with physiological processes linked to thymocyte depletion, including increased susceptibility of cells to cell death and enhanced ability of cells to migrate away from the thymus.

Materials and Methods

Animals and Parasites

Male BALB/c mice (5 to 8 weeks old) were used in most experiments. Gal-$3^{-/-}$ mice in BALB/c and C57BL/6 genetic backgrounds were generated as previously described.[24] These mice were housed and cared for at the Animal Facilities of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Blood-derived *Trypanosoma cruzi* parasites (Colombian strain) were obtained from previously infected BALB/c mice. Trypomastigotes ($n = 10^5$) were inoculated intraperitoneally into mice. Mice were bled and sacrificed 18 and 21 days after infection. For selected *in vitro* experiments, parasites were also obtained from infected cultures of the VERO cell line.[5] Animals were handled according to the guidelines approved by the Oswaldo Cruz Foundation Ethics Committee for animal research.

Reagents

The apoptosis detection kit and murine recombinant galectin-1 and -3 were obtained from R&D Systems (Minneapolis, MN). Fetal bovine serum and culture medium were purchased from Hyclone Laboratories (South Logan, UT). Penicillin and streptomycin were obtained from Life Technologies, Inc. (Gaithersburg, MD), whereas collagenase A, dispase, and DNase I were from Boehringer-Mannheim (Mannheim, Germany). Formaldehyde and paraformaldehyde were from Merck (Rio de Janeiro, Brazil). All other reagents were purchased from Sigma (St. Louis, MO), except those used for Northern blotting (below).

Antibodies

Rabbit anti-galectin-1 immune serum was obtained as described.[11] Goat anti-human galectin-1 antibody was purchased from R&D Systems. Anti-CD8/Cy-chrome, anti-CD4/phycoerithrin (PE), and mouse anti-human galectin-3 (clone B2C10) monoclonal antibodies (mAb) were purchased from Pharmingen/Becton Dickinson (San Diego, CA). Biotinylated rat anti-mouse galectin-3 mAb (clone M3/38) was purchased from Cedarlane Laboratories (Hornby, ON, Canada). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Biosys (Compiègne, France). Anti-mouse laminin and fibronectin sera were obtained from Novotec (St. Martin-La-Garenne, France). Rabbit pan anti-cytokeratin was obtained from Sigma. Alexa 543-conjugated goat anti-mouse IgG, Alexa 488-conjugated goat anti-rabbit IgG, and Alexa 633-conjugated streptavidin were purchased from Molecular Probes (Eugene, OR). Rhodamine-conjugated anti-goat IgG was from Santa Cruz Biotechnology (Santa Cruz, CA).

Tissues and Cell Cultures

Thymuses were obtained from either control or infected BALB/c mice at the peak of parasitemia and immediately

frozen in liquid nitrogen. The thymic epithelial cell line IT-76M1 was provided by Dr. T. Itoh (Tohoku University, Sendai, Japan) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For *in vitro* infections, IT-76M1 cells were plated onto sterile glass slides (10^5 cells/slide) and cultured for 24 hours. Parasites obtained from infected VERO cultures were added to these cells, in a ratio of 50 parasites/cell, for an additional period of 48 hours. Cultures were then fixed in 4% buffered paraformaldehyde for 5 minutes and processed for immunocytochemistry. TNCs, isolated from thymuses of control or infected mice as previously described,[25,26] were plated onto sterile glass slides and cultured for 35 to 60 hours. Adhered TNCs were then washed with phosphate-buffered saline (PBS), fixed as mentioned above and processed for immunocytochemistry.

Immunofluorescence and Flow Cytometry

Cryostat sections (5 μm) were prepared from frozen thymuses and then fixed with cold acetone for further processing, essentially as described elsewhere.[5] Paraformaldehyde-fixed TNC cultures and the TEC line were subjected to immunofluorescence as described.[27] Staining with appropriate dilutions of anti-galectin-1 or anti-galectin-3 antibodies was performed for 1 hour, followed by incubation with the corresponding secondary antibodies. For double labeling, additional incubations with rabbit anti-laminin or anti-fibronectin anti-sera were performed. The samples were analyzed using a laser confocal microscope (LSM 410; Zeiss, Jena, Germany).

To characterize the presence of galectins on thymocyte surface, living cells obtained by mechanical tearing of the thymus were labeled with anti-galectin-1 sera or biotinylated anti-galectin-3 mAb for 1 hour, followed by the appropriate secondary Ab and then incubated with an anti-CD4-PE plus anti-CD8-Cy for 40 minutes, washed, and fixed in 1% formaldehyde. To evaluate the intracellular expression of galectin-1 and galectin-3, the thymocytes were labeled with anti-CD4-PE plus anti-CD8-Cy for 40 minutes, washed and fixed in 1% formaldehyde. Then, the cells were washed with PBS/bovine serum albumin 0.5%, permeabilized with PBS/saponin 0.05% for 20 minutes, labeled with anti-galectin-1 and biotinylated anti-galectin-3 for 40 minutes and followed by the appropriate secondary Ab. Samples were immediately analyzed using a flow cytometer (FACScalibur, Becton Dickinson). A minimum of 10,000 up to 500,000 cells were analyzed depending on the type of assay.

Northern Blotting

Murine galectin-1 and human galectin-3 cDNA were generated by the reverse transcription-polymerase chain reaction (RT-PCR). The primers used were 5′-TCTCAAACCTGGGGAATGTC-3′ (sense) and 5′-CTTGATGGTCAGGTCAGC-3′ (anti-sense) for galectin-1 and 5′-CCTGGAGCTTATCCTGGTCA-3′ (sense) and 5′-GTCACCACTGATCCCCAGTT-3′ (anti-sense) for galectin-3.

Cell Adhesion Assay

The TEC line was plated onto eight-well culture chambers (Nunc, Rochester, NY) at a ratio of 10^4 cells/well for 24 hours. Before each experiment, TECs were treated for 30 minutes with an anti-galectin-3 mAb or control Ig. Thymocytes were then allowed to adhere to TEC cultures (50 thymocytes/TEC) for 1 hour. Nonadherent cells were gently washed out with PBS, and the remaining cells were subsequently fixed in ethanol and stained with Giemsa (Merck). The number of adhered thymocytes per TEC was then determined by direct counting. Data were expressed as an association index,[28] calculated as follows:

$$\text{Adhesion Index (AI)} = (\text{TEC with bound thymocytes})/\text{Total TEC number} \times (\text{thymocytes bound to TEC})/\text{Total TEC number} \times 100$$

Evaluation of Thymocyte Release from TNC Complexes

Freshly isolated TNC complexes were plated onto flat-bottom 96-well tissue culture dishes (7.5×10^3 TNCs/well) and cultured for 24 hours. After this period, these cells were treated with mouse recombinant galectin-3 at 5 μg/ml (R&D Systems). To investigate the role of carbohydrate recognition in galectin-3-mediated thymocyte release, cells were treated with galectin-3 plus lactose or melibiose (10 mmol/L). Controls included cells treated with bovine serum albumin (5 μg/ml) or untreated cultures. After 12 hours of incubation, TNCs were fixed in methanol for 5 minutes, labeled with Toluidine blue (0.05% in water). The dye was then solubilized in a fixed volume of ethanol, and the absorbance of the solution was determined using a spectrophotometer (595 nm). Absorbance values were used as an estimation of thymocyte release in cell cultures.

Thymocyte Migration Assay

Transwell membranes (Costar, New York, NY) were treated with 10 μg/ml laminin (Sigma) for 2 hours and then blocked with 0.5% bovine serum albumin for 30 minutes. Thymocytes were incubated with mouse recombinant galectin-1 (1 μg/ml) or galectin-3 (5 μg/ml) for 30 minutes at 37°C. Alternatively, galectin-1 or -3 was incubated with 0.1 mol/L lactose for 30 minutes at 37°C and applied to the cells. Treated thymocytes were added to the upper chamber of the transwell device, at a ratio of 2.5×10^6 cells/chamber, and allowed to migrate for 4 hours. Cells that migrated through the membranes were counted. The phenotype of both migrating and nonmigrating cells was determined by flow cytometry as described above.

Cell Death Assay

Thymocytes obtained from both C57BL/6 wild-type and gal-3$^{-/-}$ mice were treated with mouse recombinant galectin-3 (50 μg/ml). Alternatively, galectin-3 plus 0.1 mol/L lactose was applied to the cells. Thymocytes treated with 100 nmol/L hydrocortisone hemisuccinate sodium were used as a positive control for apoptosis. After 12 hours, thymocytes were then recovered and labeled with annexin V and propidium iodide using the Annexin V detection kit, according to the protocol suggested by the manufacturer (R&D Systems), and analyzed by flow cytometry.

Statistical Analysis

The one-way analysis of variance and the Newman-Keuls post test were used for comparison purposes. *P* values less than 0.05 were considered statistically significant.

Results

Differential Expression of Galectin-1 and -3 in the Thymus of Normal and T. cruzi-Infected Mice

Since recent evidence indicates the presence of galectin-1 and -3 in TECs,[12,13,15] we first investigated the expression and localization of these proteins in the thymus of control and infected mice. Although galectin-1 was detected throughout all of the thymic cortex and medulla (Figure 1A), galectin-3 was predominantly found in the medulla and in scattered cells in the cortex (Figure 1C). After *T. cruzi* infection, thymic atrophy was accompanied by a striking accumulation of galectin-3 in both the medulla and the cortex (Figure 1D), an effect that was associated with *de novo* expression of galectin-3, as judged by Northern blot analysis (Figure 1G, middle). In contrast, galectin-1 staining profile remained unchanged on infection (Figure 1B), and there was a slight decrease in the mRNA levels (Figure 1G, left).

Because galectin-3 binds laminin through lectin-carbohydrate interactions,[29] we next examined the co-localization of these proteins (Figure 1, E and F). Double-staining experiments demonstrated the co-localization (blue) of laminin and galectin-3 labeling in both thymic cortex and medulla (Figure 1E). In the thymic cortex, some laminin staining (green) was not detected in galectin-3-positive areas (red) (Figure 1E). In infected thymus, an increase in the co-localization of laminin and galectin-3 staining was detected in thymic cortical and medullary regions (Figure 1F). Cytokeratin staining showed a decrease in the extension of the cortical region in infected animals when compared to control mice, reflecting thymic atrophy. The increased labeling for galectin-3 found in both the cortical and medullary regions of the infected thymus showed many points of co-localization with cytokeratin staining (yellow; Supplemental Figure 1, see *http://ajp.amjpathol.org*).

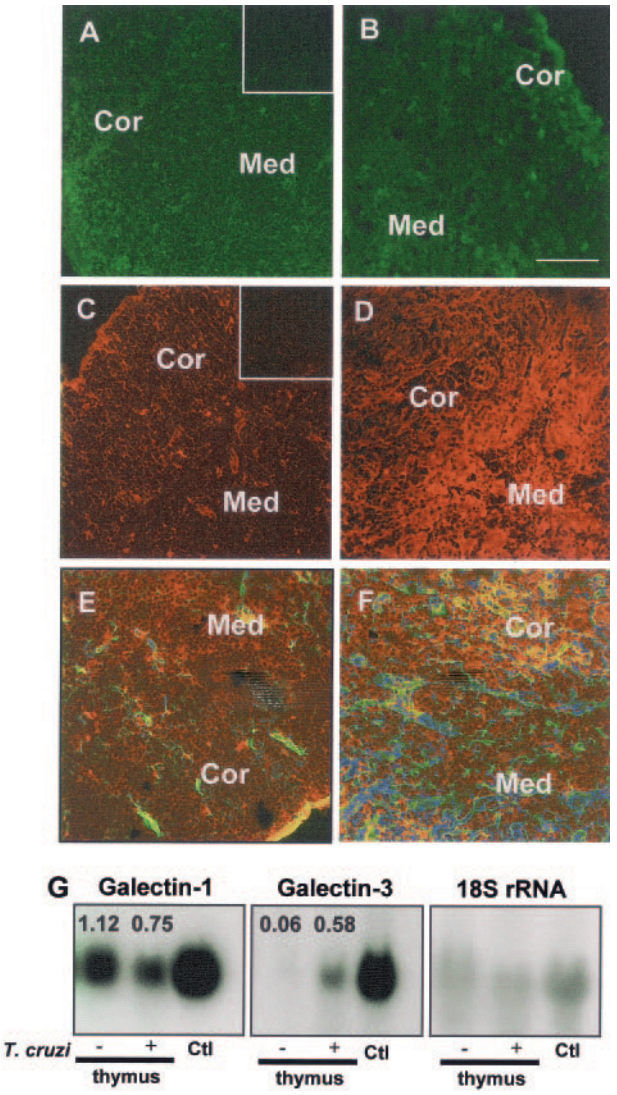


Figure 1. Regulated expression of galectin-1 and -3 in *T. cruzi*-infected mice. Galectin-1 shows the same staining profile in the thymus of control (**A**) and infected (**B**) mice. In contrast, the presence of galectin-3 is increased in the thymus of infected mice (**D**) as compared to controls (**C**). Double labeling of galectin-3 (red) and laminin (green) in control (**E**) and infected (**F**) thymus showed that both proteins accumulate on infection. Blue staining in **E** and **F** shows co-localization of these proteins. (Cor, cortex; Med, medulla). **Inserts** represent respective isotype-matched primary antibodies with similar secondary antibodies used for staining of galectin-1 and -3. Controls using secondary antibody alone did not show any background staining. **G:** The expression of galectin-1 (left) and galectin-3 (middle) mRNA in the thymus of control (−) and infected mice (+) by Northern blot analysis. Melanoma cells were used as positive controls (Ctrl). Control 18S ribosomal RNA is shown at the right. Values were obtained by densitometry after normalization with 18S rRNA. Data are representative of one independent experiment of three. Scale bar = 50 μm.

The next issue we addressed was the modulation of these endogenous lectins in cellular models resembling the thymic microenvironment, namely TEC lines and TNCs. Although IT-76M1 cells did not express galectin-1 (data not shown), this TEC line expressed substantial amounts of galectin-3, and its expression was up-regulated after *in vitro T. cruzi* infection (Figure 2, A and B). Furthermore, TNCs expressed low levels of galectin-1, and its expression was not modulated after infection (Figure 2, C and D), whereas galectin-3 expression in-

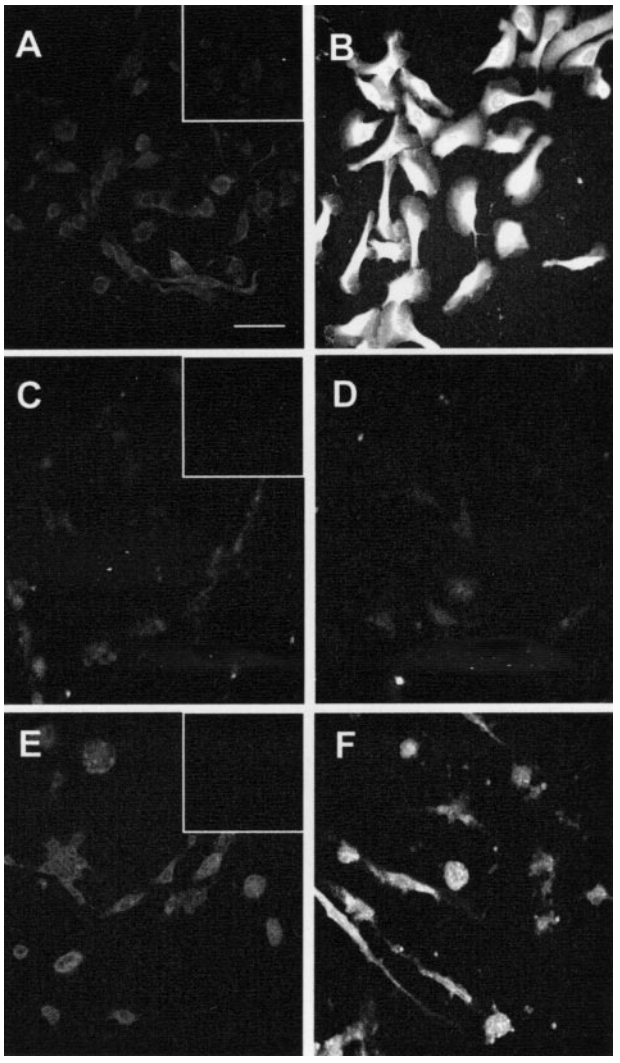


Figure 2. Characterization of galectin-1 and -3 in thymic epithelial cells after *T. cruzi* infection. As compared to control cultures (**A**), the presence of galectin-3 was strongly up-regulated in the IT-76M1 cells after *in vitro T. cruzi* infection (**B**). Staining of TNC cultures isolated from control and infected mice showed that the presence of galectin-1 was not affected on infection (**D**), as compared with TNCs from control animals (**C**). In contrast, galectin-3 was increased in TNC cultures obtained from infected mice (**F**) versus those from controls (**E**). **Inserts** represent isotype-matched controls for immunostaining with the anti-galectin-1 and anti-galectin-3 antibodies. Scale bar = 10 μm.

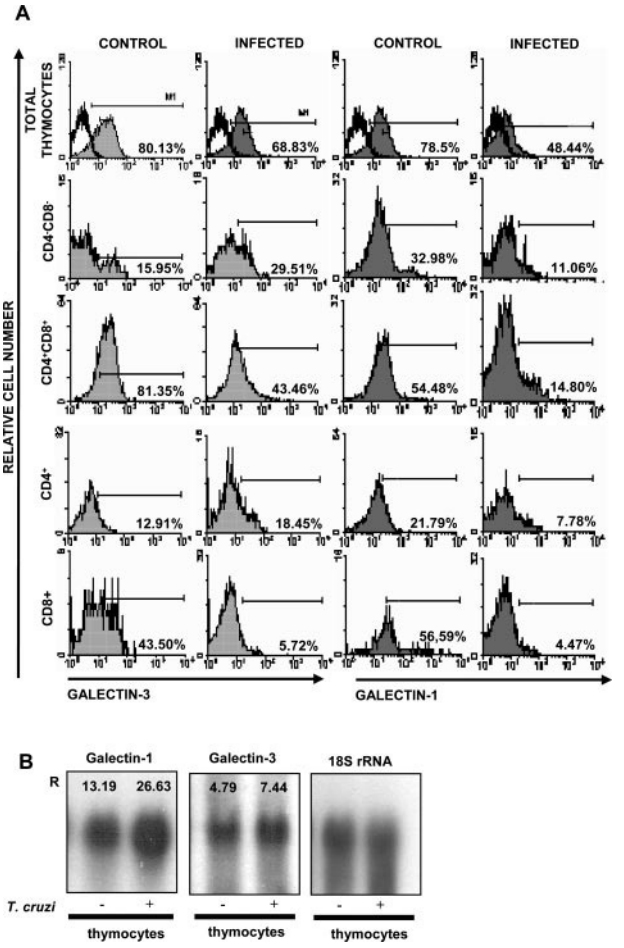


Figure 3. Characterization of galectin-1 and -3 in thymocytes from control and *T. cruzi*-infected mice. **A:** Flow cytometry analysis of galectin-1 (right) and galectin-3 (left). A clear decrease of galectin-1 and -3 was found on the surface of thymocytes obtained from infected mice when compared to control mice. $CD4^+CD8^+$ cells are the main cells expressing galectin-1 and -3. **B:** Expression of galectin-1 (left) and galectin-3 (middle) in thymocytes of control (−) and infected mice (+), assessed by Northern blot analysis. Control 18S ribosomal RNA is shown (right). Values were obtained by densitometry after normalization against 18S rRNA. Data are representative of one independent experiment of three.

creased in infected TNCs (Figure 2F). These results were similar to those found in the normal and infected thymus, indicating that comparable mechanisms may be operating regarding the control of galectin-1 and -3 expression in models resembling the thymic epithelial microenvironment. These data demonstrate that the expression of galectin-3 is up-regulated in thymus from *T. cruzi* infected mice and suggest that the main cellular component involved in this modulation is represented by thymic epithelial cells although the participation of thymocytes in this process cannot be discarded.

To gain insight about the expression of galectin-1 and -3 in thymocytes during the parasite infection, we next investigated the presence of cell surface-associated galectin-1 and -3 in different thymocyte subsets by flow cytometry. As shown in Figure 3A, a significant decrease in the number of galectin-1-positive and galectin-3-positive thymocytes was found in the total population on infection. Remarkably, $CD4^+CD8^+$ cells were found to be the major subpopulation presenting galectin-3 whereas galectin-1 was found mainly in $CD4^+CD8^+$ and $CD8^+$ cells. Moreover, thymocytes from infected mice expressed substantial levels of galectin-1 and -3 transcripts, as demonstrated by Northern blot analysis (Figure 3B). These results indicated that the expression of galectin-1 and -3 mRNA is up-regulated in thymocytes after *T. cruzi* infection, although proteins do not accumulate on the surface of these cells. To clarify this point, we performed intracellular staining for both galectin-1 and -3 in thymocytes and found that the expression of galectin-3 was increased in the cytoplasm of thymocytes from infected mice, especially in $CD4^+CD8^+$ cells. Comparatively, the intracellular expression of galectin-1 did not

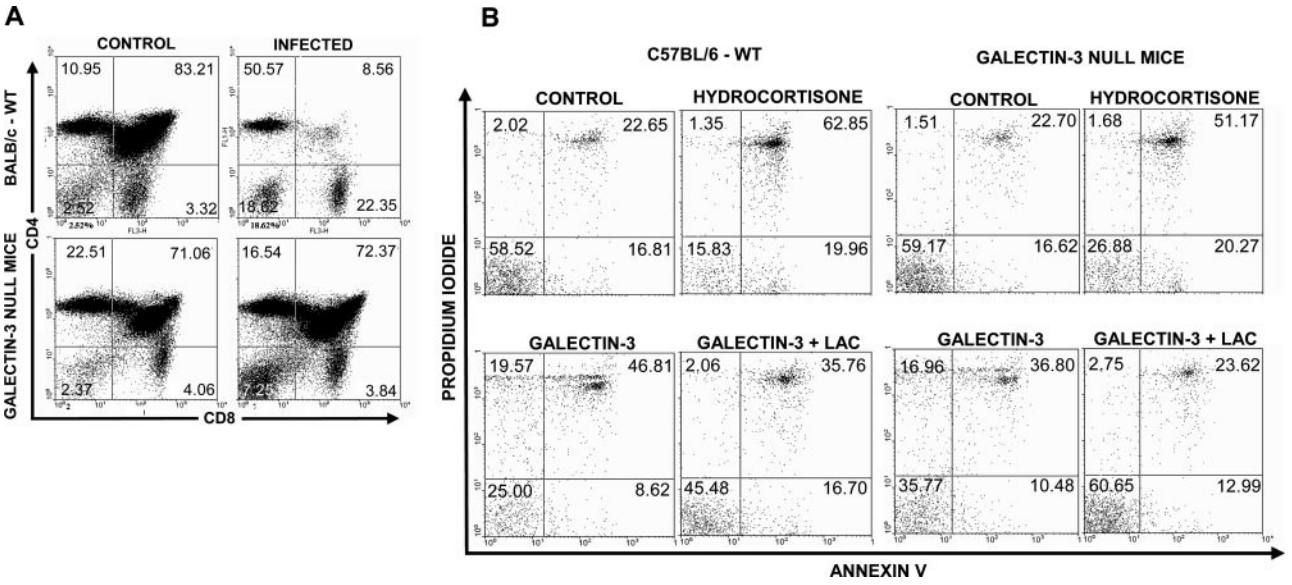


Figure 4. Characterization of thymocyte death after *T. cruzi* infection. **A:** CD4/CD8-defined thymocyte subsets of BALB/c wild-type and gal-3$^{-/-}$ mice were analyzed by flow cytometry. Values represent the percentages of each CD4/CD8-defined subset. Data depict representative profiles of five independent experiments. **B:** Flow cytometry analysis of *in vitro* thymocyte death was determined by annexin V and propidium iodide staining using wild-type and galectin-3$^{-/-}$ mice in the C57BL/6 background. Data show representative profiles of seven independent experiments.

show a significant increase in thymocytes on infection (Supplemental Figure 2, see *http://ajp.amjpathol.org*).

Infection of gal-3$^{-/-}$ Mice Is Not Accompanied by Thymic Atrophy

We have previously demonstrated that, on acute *T. cruzi* infection, a striking thymic atrophy takes place in different mouse strains.[5] The C57BL/6 mice are resistant to *T. cruzi* infection, when compared to BALB/c mice. Both mouse strains show a parasitemia peak within 21 days after infection. However, few C57BL/6 mice die from infection, whereas BALB/c mice show high mortality. Interestingly, gal-3$^{-/-}$ mice of both genetic backgrounds showed higher susceptibility to infection, exhibiting a parasitemia peak within 16 to 18 days and a 100% mortality rate (data not shown).

Interestingly, wild-type *T. cruzi*-infected mice of both C57BL/6 and BALB/c genetic backgrounds exhibited significant thymocyte depletion (absolute number of thymocytes) when compared to the respective control mice. Strikingly, this difference was not apparent between control and infected gal-3$^{-/-}$ mice. When thymocyte subsets from infected wild-type animals were analyzed by flow cytometry, a significant decrease in the relative amounts of $CD4^+CD8^+$ double-positive cells, together with an increase of either $CD4^+$ and $CD8^+$ single-positive cells, was observed, as shown in Figure 4A (top) for BALB/c mice. This observation could be attributable to the $CD4^+CD8^+$ double-positive cell depletion during infection, because no significant changes in absolute cell number of $CD4^+$ or $CD8^+$ single-positive cells were detected between thymus from control and infected mice. Interestingly, when gal-3$^{-/-}$ mice were infected, the expected depletion of $CD4^+CD8^+$ double-positive cells did not occur (Figure 4A, bottom).

Comparable data on C57BL/6 wild-type and gal-3$^{-/-}$ mice are shown in Table 1. C57BL/6 Gal-3$^{-/-}$ mice showed a significant decrease in the absolute number of the total thymocyte population when compared to wild-type mice. It related to a smaller absolute number of $CD4^+CD8^+$ thymocytes present in the thymus of gal-3$^{-/-}$ mice. Moreover, C57BL/6 gal-3$^{-/-}$ mice also did not show thymic atrophy on infection. Our results obtained

Table 1. Absolute Cell Number of Different Thymocyte Subsets from Wild-Type (C57BL/6) and Galectin-3-Null Mutant Mice in the Absence or Presence of Infection with the *T. cruzi* Protozoan Parasite

	gal-3$^{+/+}$ control	gal-3$^{+/+}$ infected	gal-3$^{-/-}$control	gal-3$^{-/-}$ infected
Total	1.82 ± 0.62*†	0.64 ± 0.07†	0.85 ± 0.39*	0.96 ± 0.36
$CD4^-CD8^-$	0.18 ± 0.16	0.06 ± 0.02	0.10 ± 0.10	0.11 ± 0.11
$CD4^+CD8^+$	1.42 ± 0.5*†	0.15 ± 0.13†	0.59 ± 0.32*	0.50 ± 0.11
$CD4^+$	0.19 ± 0.06	0.28 ± 0.13	0.11 ± 0.05	0.12 ± 0.22
$CD8^+$	0.09 ± 0.04	0.12 ± 0.05	0.12 ± 0.22	0.20 ± 0.11

In each experimental condition five thymuses were analyzed. Values are represented as mean ± SD ($\times 10^8$ cells) of one of three independent experiments.

* Total and $CD4^+CD8^+$ thymocytes from control wild-type and gal-3-null mutant mice were analyzed (*$P < 0.001$).

†After *T. cruzi* infection, depletion of total and $CD4^+CD8^+$ thymocytes was observed in wild-type mice but not in galectin-3-null mutant mice (**$P < 0.001$).

with two different strains indicate that the thymus of gal-$3^{-/-}$ mice present alteration on its physiology in normal conditions. However, thymus cellularity in gal-$3^{-/-}$ mice is not altered during the *T. cruzi* infectious process.

Galectin-3 Promotes Death of Thymocytes

Taken together, these results suggest that the accumulation of galectin-3 in the thymus of infected mice may be associated with the depletion of $CD4^+CD8^+$ thymocytes. This observation prompted us to investigate whether the exposure of thymocytes to galectin-3 would induce thymocyte death. No significant differences were observed regarding spontaneous cell death during the course of the experiments (Figure 4B). However, treatment of thymocytes from either wild-type or gal-$3^{-/-}$ mice with galectin-3 for 12 hours induced cell apoptosis, an effect that was inhibited by 0.1 mol/L lactose. Interestingly, thymocytes treated with galectin-3 showed a significant increase in the frequency of cells stained with propidium iodide. As a positive control of thymocyte death, cells were also treated with hydrocortisone (Figure 4B). Thymocytes from gal-$3^{-/-}$ mice showed resistance to hydrocortisone-and galectin-3-induced cell death as compared to wild-type mice, suggesting that the presence of galectin-3 may increase their susceptibility to cell death. These results indicate that galectin-3 plays a key role in thymocyte apoptosis and thymic depletion after *T. cruzi* infection.

Galectin-3 Favors the Escape of Immature Thymocytes from the Thymus

We have previously shown the presence of immature $CD4^+CD8^+$ T cells in peripheral lymphoid organs of *T. cruzi*-infected mice at the peak of parasitemia.[7] These immature $CD4^+CD8^+$ T cells are thymus-derived because they were absent in peripheral lymphoid organs of infected thymectomized mice.[7] To investigate if galectin-3 participates in the premature escape of these cells to the periphery, we compared the presence of immature and mature T-cell subsets in the subcutaneous lymph nodes of gal-$3^{-/-}$ and wild-type mice after *T. cruzi* infection. As expected, an increased number of immature $CD4^+CD8^+$ T cells was observed in the subcutaneous lymph nodes of infected wild-type mice (Figure 5). Interestingly, this subpopulation was not observed when gal-$3^{-/-}$ mice were infected with the parasite (Figure 5). These data suggest a putative role for galectin-3 in the exportation of immature $CD4^+CD8^+$ double-positive thymocytes to peripheral lymphoid organs secondary to *T. cruzi* infection.

Galectin-3 Modulates Migration of Thymocytes

Recently, it was demonstrated that galectin-3 modulates the migration of peripheral blood monocytes/macrophages.[30] This effect may be a possible consequence of the deadhesive property of galectin-3. Thus, in an at-

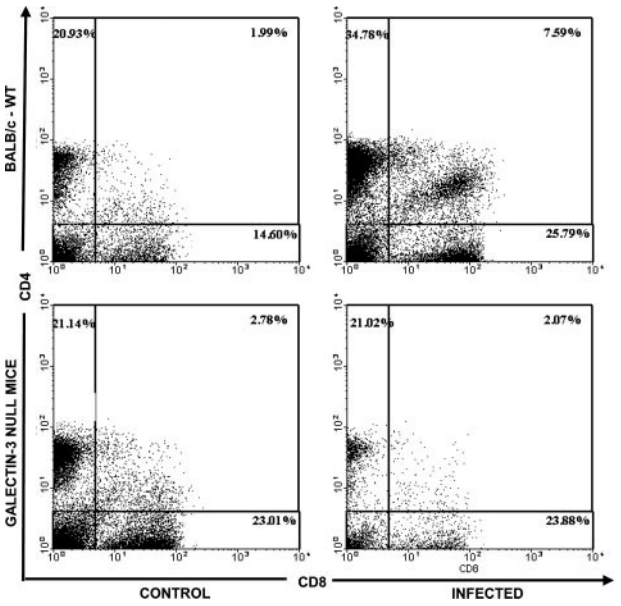


Figure 5. Galectin-3 plays a key role in the escape of immature thymocytes from the thymus. Flow cytometry analysis of CD4/CD8-defined lymphocyte subsets corresponding to wild-type or galectin-3-null mutant mice in subcutaneous lymph nodes under normal conditions (left) or after infection with *T. cruzi* (right). Data show representative profiles of two independent experiments.

tempt to explain the involvement of galectin-3 with the premature escape of immature $CD4^+CD8^+$ thymocytes to peripheral lymphoid organs secondary to *T. cruzi* infection, we decided to analyze thymocyte capability to migrate using different experimental models.

Initially, we evaluated the effects of galectin-1 and -3 on the migratory response of thymocytes toward laminin. Thymocytes from both control and infected mice were assayed for migration using transwell chambers containing polycarbonate filters coated with laminin-1. Thymocytes were preincubated with either galectin-1 or -3 in the presence or absence of lactose. When thymocytes from control and infected mice were treated with galectin-1, these cells aggregated and adhered to laminin, inhibiting the cell migration (data not shown). In contrast, galectin-3 enhanced migration of thymocytes toward laminin by as much as 10-fold (Figure 6A). The effect of galectin-3 on thymocytes obtained from infected animals was even more pronounced (Figure 6A). In both cases, galectin-3-induced migration was inhibited by the addition of 0.1 mol/L lactose.

Under the experimental conditions described above, migrating thymocytes were harvested in the lower chamber of the transwell plates, analyzed for CD4 and CD8 expression, and compared with nonmigrating cells, which remained in the upper compartment. As expected, total thymocytes from uninfected mice were mostly $CD4^+CD8^+$ thymocytes (Figure 6B, total cells). In the meantime, $CD4^+$ and $CD8^+$ cells were enriched in the upper compartment, whereas most of the thymocytes harvested in the lower chamber were $CD4^+CD8^+$ cells, indicating that galectin-3 preferentially induces migration of immature thymocytes. When thymocytes from infected mice were analyzed, we did not observe such migratory

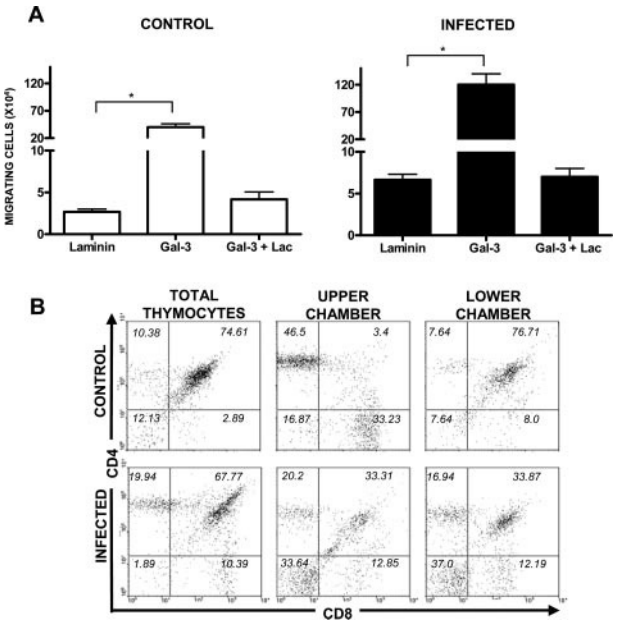


Figure 6. Galectin-3 enhances thymocyte migration through laminin. **A:** Thymocytes from control or infected mice were treated with recombinant galectin-3 or recombinant galectin-3 plus lactose and plated onto transwell membranes previously treated with laminin. The number of migrating thymocytes was counted. Data are represented as mean ± SD. $*P < 0.001$. Statistical significance was determined by analysis of variance. **B:** CD4/CD8-defined thymocyte subpopulations from control and infected mice treated with galectin-3, before (total thymocytes, left) and after (middle and right) migration in transwell chambers. Cells were analyzed by flow cytometry. Data shown are representative of five independent experiments.

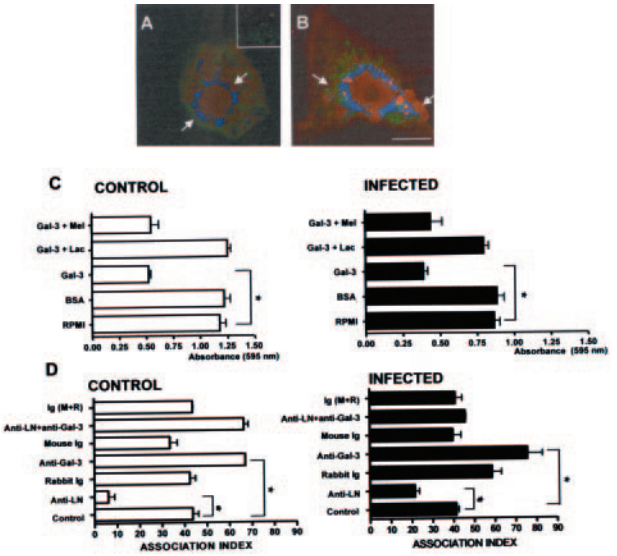


Figure 7. Galectin-3 modulates functional interactions between TECs and thymocytes. **A:** Analysis of TNC from control mice showed that galectin-3 (red) co-localizes with laminin (green), being distributed as a halo around thymocytes (negatively seen as black holes). At this level, the TNC complex displayed a honeycomb-like structure. The blue spots show regions of co-localization. **Insert** represents isotype-matched controls for immunostaining with the anti-galectin-3 and anti-laminin antibodies. **B:** In TNC from infected mice a strong co-localization is observed, with thymocytes highly stained for galectin-3. **Arrows** indicate thymocyte localization within the TNC complexes. **C:** *In vitro* analysis of thymocyte release from TNC complexes isolated from control and infected mice. TNCs were treated with either recombinant galectin-3 alone or galectin-3 plus lactose or melibiose and analyzed by spectrophotometry. $*P < 0.001$. Statistical significance was determined by analysis of variance. **D:** Thymocyte association with the IT-76M1 cells. IT-76M1 cell monolayer was treated with anti-galectin-3 monoclonal antibody and/or anti-laminin (LN) polyclonal antibody. Data are represented as mean ± SD of one of five independent experiments. $*P < 0.001$. Scale bar = 10 μm.

preference between thymocyte subpopulations. It may be related with an increased migratory potential observed in untreated thymocytes from infected mice when compared to untreated thymocytes from control mice (Figure 6A).

We have previously demonstrated that galectin-3 acts as a deadhesion molecule by disrupting thymocyte adhesion to thymic epithelial cells.[15] In cultured TNCs, we observed that both galectin-3 and laminin were found in regions of interactions between thymocytes and TECs (Figure 7A). This overlap was present in TNCs obtained from infected mice, but, as shown in Figure 7B, galectin-3 was involving the thymocytes, suggesting its participation in thymocyte exit from these cells. The dynamics of thymocyte release from TNCs may be modulated by exogenous factors, including ECM proteins and antibodies recognizing either ECM or their receptors.[28,31] We have exploited this model to determine whether galectin-3 could modulate thymocyte-TEC interactions within the TNCs. Lymphoepithelial complexes obtained from either control or infected mice were cultured and exposed to specific stimuli for 12 hours. Then, cells were fixed, and thymocyte release was assessed by toluidine blue staining. Absorbance was significantly higher for control TNCs than for TNCs from infected animals, indicating that thymocyte release was faster in infected mice. This result was confirmed by direct counting of cells remaining in the TNCs (data not shown). Addition of exogenous galectin-3 resulted in increased levels of thymocyte release, as determined by the lower absorbance. Lactose, which inhibits the lectin activity of galectin-3, but not melibiose, which does not bind galectin-3, was capable of blocking galectin-3-mediated thymocyte release, indicating that exogenous galectin-3 exerts its modulatory effect through protein-glycan interactions (Figure 7C). Interestingly, spontaneous thymocyte release from TNCs isolated from gal-$3^{-/-}$ mice was significantly slower than in wild-type mice (D.M.S. Villa-Verde, R.F. Schneider, R.F. Samico, E. Silva-Monteiro, W. Savino, unpublished observations).

Our results demonstrate that galectin-3 modulates thymocytes' interactions with laminin or TECs, inducing their migration. Thus, we decided to analyze the involvement of galectin-3 in the interactions between thymocytes with TEC line and laminin. Thymocytes from either control or infected mice were cultured with the TEC line in the presence of either anti-laminin or anti-galectin-3 antibodies or with both antibodies (Figure 7D). Although the anti-laminin antibody inhibited thymocyte-TEC adhesion, the anti-galectin-3 antibody (clone B2C10) enhanced this interaction. Remarkably, the positive modulatory effect of the anti-galectin-3 antibody significantly counteracted the inhibitory effect of anti-laminin treatment in the cells, leading to similar thymocyte-TEC adhesion values found in the untreated infected cells. Our data clearly showed that galectin-3

negatively modulates the adhesion of thymocytes with components of the thymic microenvironment.

Discussion

A variety of proteins, such as ECM components, chemokines, and matrix metalloproteinases, have been reported to be important modulators of the interactions between thymocytes and the thymic microenvironment.[32] Moreover, the thymus can be influenced by extrinsic factors such as infections with protozoan parasites, including *T. cruzi*.[5–9] Acute *T. cruzi* infection leads to a pronounced depletion of cortical thymocytes and to the escape of a significant number of immature cells to the periphery.[7] The participation of ECM components and chemokines in these phenomena has been suggested.[32] Here we provide evidence for the contribution of galectin-3 to different events potentially associated with thymocyte depletion in *T. cruzi*-infected mice, namely cell death and increased cell migration.

Previous observations demonstrated the presence of galectin-1 and -3 in the normal mouse thymus.[12,13,15] After *T. cruzi* infection, there is an accumulation of galectin-3 (but not galectin-1) in both cortical and medullary regions of the thymus. Northern blot analysis showed that galectin-3 accumulation was associated with *de novo* expression of this protein; we observed a 10-fold increase in the relative amount of galectin-3 mRNA in thymuses from infected mice, whereas galectin-1 expression decreased.

We report here that galectin-3 is mainly found in the medulla and in scattered cells in the cortex. On *T. cruzi* infection, we found a marked increase of galectin-3 immunoreactivity in both cortical and medullary compartments. Thymocytes harvested from both normal and infected mice displayed galectin-3 on the cell surface. More interestingly, thymocytes from infected animals expressed high levels of galectin-3 mRNA. Thus, not only TECs, but also thymocytes, show increased galectin-3 expression under specific conditions, such as *T. cruzi* infection. It is remarkable that most of the cell surface galectin-3-positive cells corresponded to the $CD4^+CD8^+$ double-positive thymocytes. This result was confirmed using an engineered probe that allowed the *in situ* identification of cells bearing galectin-3 ligands (F.H.M. Melo and R. Chammas, unpublished observations). Interestingly, increased galectin-3 expression was also observed on B cells from *T. cruzi*-infected mice, and this expression correlated with the ability of this protein to favor a memory B-cell phenotype.[33]

Two models of thymic epithelial cells, the IT-76M1 TEC line[34–36] and primary cultures of the TNCs,[25] were used in this study to examine galectin accumulation on infection with *T. cruzi.* In both cases, galectin-3 (but not galectin-1) accumulated after exposure to the parasite. The consistent accumulation of galectin-3 in the thymus of infected animals together with our previous observations that galectin-3 may act as a deadhesion molecule within the thymic microenvironment,[15] prompted us to investigate the potential role of this carbohydrate-binding protein in thymocyte cortical depletion in *T. cruzi*-infected mice.

Here we provide evidence showing that galectin-3 induces a thymocyte migratory response toward laminin and that this response is even greater in thymocytes from *T. cruzi*-infected mice. In addition, this molecule is involved in the premature exit of immature $CD4^+CD8^+$ double-positive T cells to peripheral lymphoid organs. Further, prolonged exposure of thymocytes to galectin-3 results in extensive cell death. These should be discussed in terms of the differential effects of intracellular and extracellular galectin-3 and the effects of this protein on different cell types. For example, it has been demonstrated that overexpression of galectin-3 in human Jurkat T-cell line and human breast carcinoma cells can protect the cells from apoptosis; in the latter, the effect has been associated with improved cell adhesion properties.[37] In addition, galectin-3 can act as an adhesion molecule during neutrophil extravasation in response to infection.[38]

The mechanisms leading to severe thymic atrophy with massive thymocyte death in acutely *T. cruzi*-infected animals are not completely understood. We have previously shown that thymic atrophy was not dependent on high levels of glucocorticoids because this effect was still observed in adrenalectomized mice.[39] More recently, we demonstrated that it was not dependent on the Fas or perforin cell death pathways because a massive thymocyte loss occurred in *T. cruzi*-infected Fas-L or perforin-null mice.[40] Furthermore, a recent study suggested a role for extracellular ATP in triggering thymocyte death after *T. cruzi* infection because this molecule induced an increase in plasma membrane permeabilization and cell death in $CD4^+CD8^+$ double-positive thymocytes from infected mice during thymic atrophy.[41] Interestingly, in the specific TNCs niche, cell death secondary to *T. cruzi* infection was found to be associated with the effects of a *T. cruzi* transialidase, which regulates sialic acid mobilization.[42] The preferential binding of galectin-3 to thymocyte subsets seems to be also involved in this event. In this regard, the fact that galectin-3 binds preferentially to $CD4^+CD8^+$ thymocytes might explain the depletion of cortical cells in wild-type mice. This is consistent with data obtained from gal-$3^{-/-}$ mice. These animals did not show a significant thymic atrophy after acute infection or changes in the percentage of $CD4^+CD8^+$ double-positive thymocytes. Moreover, because galectin-3 also favors thymocyte migration, it is likely that a small fraction of thymocyte depletion after infection may be attributable to an increased number of cells leaving this organ. Thus, it is conceivable that galectin-3 might not only disrupt thymocyte adhesion but also be responsible for promoting a migratory phenotype. In addition, this protein can trigger high levels of thymocyte apoptosis. In this regard, prolonged exposure to galectin-3 would dictate a run-or-die sentence, unless cells homed into a galectin-3-deprived microenvironment. Although the fate of the immature thymocytes that escape to the periphery is still primarily unknown, it seems likely that some T cells with prohibited TCRs would survive and might be involved in the pathogenesis of autoimmune-related lesions such as those found in Chagas' disease.[9]

In summary, our observations provide evidence of novel extracellular functions for galectin-3, including regulation of thymocyte death and modulation of thymocyte migration. These regulatory functions are clearly involved in thymic atrophy after acute *T. cruzi* infection. Taken together, our data provide important clues for a better understanding of the pathophysiology of experimental Chagas' disease, with potential implications for the future development of novel therapeutic strategies.

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