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A Sexual Dimorphism in Intrathymic Sialylation Survey Is Revealed by the trans-Sialidase from *Trypanosoma cruzi*1

Juan Mucci,* Esteban Mocetti,† María Susana Leguizamón,‡ and Oscar Campetella2*

Sialylation is emerging as an important issue in developing thymocytes and is considered among the most significant cell surface modifications, although its physiologic relevance is far from being completely understood. It is regulated by the concerted expression of sialyl transferases along thymocyte development. After in vivo administration of trans-sialidase, a virulence factor from the American trypanosomatid *Trypanosoma cruzi* that directly transfers the sialyl residue among macromolecules, we found that the alteration of the sialylation pattern induces thymocyte apoptosis inside the “nurse cell complex.” This suggests a glycosylation survey in the development of the T cell compartment. In this study, we report that this thymocyte apoptosis mechanism requires the presence of androgens. No increment in apoptosis was recorded after trans-sialidase administration in females or in antiandrogen-treated, gonadectomized, or androgen receptor mutant male mice. The androgen receptor presence was required only in the thymic epithelial cells as determined by bone marrow chimeric mouse approaches. The presence of the CD43 surface mucin, a molecule with a still undefined function in thymocytes, was another absolute requirement. The trans-sialidase-induced apoptosis proceeds through the TNF-α receptor 1 death signaling leading to the activation of the caspase 3. Accordingly, the production of the cytokine was increased in thymocytes. The ability of males to delete thymocytes altered in their sialylation pattern reveals a sexual dimorphism in the glycosylation survey during the development of the T cell compartment that might be related to the known differences in the immune response among sexes. *The Journal of Immunology*, 2005, 174: 4545–4550.

Apoptosis in the thymus is an essential process associated with the maturation and selection of those thymocytes that finally render the peripheral T cell compartment. Because of this selection mechanism, autoimmunity-prone and unresponsive T cell clones are to be deleted. During the maturation process, the surface glycoconjugates found in the different subsets of thymocytes display distinctive sialylation patterns whose physiologic significance still remains largely unknown. These glycoconjugate decoration patterns constitute an issue of growing research interest and are due to the developementally regulated expression of sialyl transferases along the T cell maturation process (1, 2). It is hypothesized that the acquisition of the sialyl residue signals the trafficking of the thymocytes, given its ability to mask terminal sugars that works as potential binders to endogenous lectins and, in fact, the sialylation of the CD8 was shown critical in its binding affinity to the MHC (3–7).

The alteration of the surface sialylation leads to the in vivo depletion of the CD4+CD8+ double-positive (DP)3 thymocytes, then disclosing an apoptosis pathway associated with the glycosylation pattern as demonstrated by the treatment with trans-sialidase (TS) (8), an enzyme able to transfer terminal α2,3-linked sialyl residues directly among glycoconjugates. The TS is a virulence factor shed by the pathogen protozoon *Trypanosoma cruzi*, the agent of the Chagas’ disease, that is found in the blood of patients and infected animals. This enzyme survives in the bloodstream and provides a tool to in vivo exogenously alter the surface sialylation of cells. In the thymus, the TS initiate a process that leads to the apoptosis induction of DP thymocytes. It requires their further interplay with the thymic epithelial cells (TEC) that happens inside the so-called “nurse cell complex,” as no apoptosis is induced in TS-treated isolated cells (8). Sialy1 residue mobilization is required because an enzymatically inactive Tyr342His mutant TS does not induce apoptosis (8, 9), even when a specific lectin-like activity is retained by this molecule (10).

In this study, we describe that the sialyl residue mobilization by TS induces thymocyte apoptosis, surprisingly only in males, and then the requirement of androgens in this process was further explored. Sexual differences constitute an emerging issue in immunology as well as in other health-related areas beyond the reproductive organs. Sex hormones are involved in the immune system physiology and are suggested to be associated with the enhanced immune response in females, their resistance to viral infections and augmented autoimmunity contrasting with the predominant malignant diseases in males (11–13). They are also known to play a role in thymocyte development where an increment in plasmatic androgens induces apoptosis of these cells leading to strong thymic involution (14, 15). Accordingly, gonadectomized male animals develop larger thymus, an effect that can be prevented or reverted

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3 Abbreviations used in this paper: DP, double positive; AR, androgen receptor; KO, knockout; TEC, thymic epithelial cell; TNFR1, TNF-α receptor 1 (p55); TNFR2, TNF-α receptor 2 (p75); TS, trans-sialidase.
by androgen replacement therapy (11, 16, 17). Interestingly, androgen-induced thymocyte apoptosis is mediated through their interaction with the TEC (18) as happens with the TS (8).

By using the TS as an inducer of altered glycosylation patterns in vivo, we found that the thymocyte apoptosis observed after the sialyl residue mobilization requires of androgens, leading us to postulate the presence of a dimorphic glycosylation survey in the development of the T cell compartment that can be related to the observed differences in the immune response among sexes.

Materials and Methods
trans-sialidase
Recombinant TS was expressed in Escherichia coli DH5α (19) induced with isopropyl β-D-thiogalactopyranoside (Sigma-Aldrich) and purified by homogenization through Ni²⁺ charged Hi-Trap Chelating followed by MonoQ columns (Amerham Biosciences) as described (20). TS was i.v. administered following a three-dose (1 mg) schedule given on days 0, +2, and +4 followed by thymus collection on day +7. A single 1-μg or 10-μg dose 18 h before collection was also assayed.

Mice
Breeding pairs of C3H/HeJ, C57BL/6J, and B6-Ago1-/-Cg-Elda1-/-+/+ ArTfm mice were obtained from The Jackson Laboratory and bred in our facilities. Hemizygous Tfm/Y males express an aberrant androgen receptor (AR) where an early stop codon renders a truncated inactive protein. B6.CDA34ull mice backcrossed eight times to C57BL/6J were gently provided by Dr. A. I. Sperling (University of Chicago, Chicago, IL). C57BL/6-Tnfrsf1aΔmIc/mice lacking the TNF-α receptor 1 (p55) (TNFR1), B6.129S2-Tnfrsf1bΔmMm/mice lacking the TNF-α receptor 2 (p75) (TNFR2), and B6.129S-Tnfrsf1aΔmMm/Tnfrsf1bΔmMm mice lacking both receptors were originally obtained from The Jackson Laboratory and were a gift of Dr. F. Pitossi (Instituto Leloir, Buenos Aires, Argentina). Except otherwise indicated, 60- to 90-day-old mice were used. All assays were performed following local regulations and under the approval of the animal use committee of our institute.

Immunofluorescence assays
After RBC lysis, thymocytes were washed with cold staining buffer (PBS plus 2% PBS and 0.1% NaN₃) and pelleted at 1–2 × 10⁶ viable cells/tube. Cells were resuspended in 50 μl of buffer plus 1–2 μg of a rat mAb anti-mouse CD16/CD32 (clone 2.4G2) (Fc Block). After 30-min incubation on ice, 1 μg of each R-PE conjugated rat anti-mouse CD4 (clone H129.19) or FITC-conjugated rat anti-mouse CD8α (clone 53-6.7) mAbs were added and incubated 30 min more. Isotype-matched labeled mAbs were used as control. All mAbs were from BD Pharmingen. Fluorescence was assayed with a flow cytometer (Ortho Cytoron Absolute; Ortho Diagnostics).

Chimeric bone marrow mice construction
Recipients were lethally irradiated (9 Gy) in a Gammaccell 220 at the facilities of the Comisión Nacional de Energía Atómica and 24 h later, 5 × 10⁶ bone marrow cells were given i.v. from the desired donor mice. T cells were previously removed from bone marrow cell suspensions by adsorption with Dynabeads Mouse pan T (Thy1.2) (Dynal). Animals were allowed to replenish the thymus for at least 20 days.

Testosterone and flutamide treatment
Pellets containing testosterone (7.5 mg/pellet, 60-day release, blood level reached 3–4 ng/ml reported as normal (21, 22)), flutamide (25 mg/pellet, 21-day release, effective dose 1.19 mg/day), or placebo where obtained from Innovative Research of America. Flutamide is a nonsteroidal androgen that mediates the inhibition of the uptake and/or nuclear binding of testosterone and dihydrotestosterone. Pellets were s.c. implanted with a trochar following the instructions of the manufacturer.

Gonadectomy
Mice (30- to 45-day-old) were gonadectomized under chloral hydrate anesthesia and left to recover for at least 2 wk before further assay.

Histology
Organs were collected, fixed in 4% p-formaldehyde in PBS, and embedded in paraffin. Slices were stained by H&E, and the number of apoptotic bodies by field (×40) was determined. To highlight cells undergoing apoptosis, unstained sections mounted in silanized slides were subjected to TUNEL procedure by using the ApopTag kit for immunoperoxidase staining from Intergen, followed by light hematoxylin counterstaining.

Expression of TNF-α
Thymocytes were obtained 6 h after in vivo TS administration, fixed with 2% p-formaldehyde in PBS and spread on poly-l-lysine (Sigma-Aldrich)-coated cover glass. Specimens were incubated with 5% normal goat serum/2% BSA in PBS for 30 min. Then 1 μg of rat anti-mouse TNF-α mAb (clone G281-2626; DB Pharmingen) diluted in 50 μl of blocking solution was added and, after 30 min, washed with PBS. Alexa Fluor 488 goat anti-rat IgG (Molecular Probes) was then added (2 μg/ml) in blocking solution. Fluorescence was assayed with a flow cytometer (Ortho Cytron Absolute; Ortho Diagnostics) and by microscopy.

Caspase activity
Nurse cells complexes were isolated essentially by following the procedure described in Ref. 23. Briefly, thymuses were collected, cut in small pieces, and subjected to protease digestion and PBS gradient. Caspase activity was determined with the “Salforhodamine Multicaspase Activity Kit Apoptosis Detection” from Biomol following the manufacturer’s recommendations. Thymic organ cultures from normal mice were performed as described in Ref. 8 to assay for caspase activity in ex vivo-treated thymocytes. Eighteen hours after incubation with or without 1 μg of TS, single cell suspension was prepared in RPMI 1640. Thymocytes were seeded (1 × 10⁶/well) in 24-well plates (Falcon; Becton Dickinson Labware). The same kit as before was used and fluorescence was quantified in a luminescence spectrometer (Thermo Spectronic-Amino¢ Bowman Series 2). In extracts from the same cells, caspase 1, 3, and 8 activities were recorded in 30 μg of total protein using the “caspase colorimetric substrate/inhibitor QuantiPak” from Biomol. Caspase 8 activity was assayed in the presence of 1 μM caspase 3 inhibitor. Blank control for each caspase was performed in the presence of the corresponding inhibitor, except for caspase 8 that was assayed in the presence of caspase 3 and 8 inhibitors (all at 1 μM).

Results
Sex dependence of TS-induced thymocyte apoptosis
The administration of TS, an enzyme able to mobilize sialyl residues on the cell surface, induces in vivo thymocyte apoptosis that usually is evidenced as clusters of TUNEL-reactive cells (8). However, as shown in Fig. 1A, female mice were unexpectedly resistant to TS-induced thymocyte depletion even after a three-dose schedule. These observations lead us to assay gonadectomized male mice that were insensitive to the same enzyme administration schedule (Fig. 1A). No changes in thymus weight (data not shown),
DP thymocyte percentage (75 ± 7% vs 64 ± 7%), or thymocyte count were found; contrasting with the strong cell depletion observed in TS-treated control male mice (Fig. 1A; see also Ref. 8). To test for the involvement of male sex hormones, male mice were implanted with flutamide (a nonsteroidal antiandrogen)-releasing pellet, and 1 wk later the three TS dose schedule was administered. Flutamide significantly ameliorated the thymocyte depletion induced by TS (Fig. 1B), clearly supporting the involvement of the AR/testosterone interaction. To test female-derived thymocytes susceptibility to TS-induced apoptosis, they were embedded in a male context through a bone marrow chimeric mouse approach. The TS administration to these chimeras was able to deplete the female-derived thymocytes when placed inside the male recipient, meanwhile no effect at all was observed in the reciprocal experiment (Fig. 1C).

In another set of assays, the apoptosis induction was tested by H&E and TUNEL staining in organs taken 18 h after a single TS dose (either of 1 or 10 μg). Female mice displayed 0–1 apoptotic separate cells by either technique (×400 field), even after 10-μg TS injection, contrasting with male animals that showed ~5 clusters of apoptotic bodies/field when treated with 1 μg of TS (n = 3, for each assay, see Fig. 2, A–C). Hormone reconstitution assays were conducted with gonadectomized male mice that were implanted with testosterone-releasing pellets and, due to the known effect of androgens on thymus reduction, 20 days were allowed to reach a hormonal steady state before TS administration (1 μg). TS-induced apoptosis was readily detectable in these animals (0–1 separate cells vs 4 TUNEL reactive clustered cells/field ×400, in untreated vs TS-treated mice, n = 3; see also Fig. 2, D–F vs G–I). Because the testosterone level was exogenously fixed, and no other major supply of hormone was available, we conclude that TS-induced apoptosis requires the presence of the androgen in physiologic amounts. Furthermore, TS injection was able to induce thymocyte apoptosis in female mice implanted with testosterone pellets 20 days before (0–1 separate cells vs 3–4 clusters of TUNEL reactive cells/field ×400, in untreated vs TS-treated animals, n = 3; see also Fig. 2, J–L vs M–O). Collectively, these results are clearly stressing the central role played by androgens in TS-induced thymocyte apoptosis.

**Requirement of the AR expression in the TEC**

The involvement of androgen signaling in the TS-induced thymocyte apoptosis was assayed with the AR−/− mice. TS administration to these male animals did not induce apoptosis as revealed by thymus histology (Fig. 3, A and B). Because the TS-induced apoptosis occurs inside the nurse cell complex, where both TEC and thymocytes are present, a bone marrow chimeric mouse approach was designed to define the cell type involved in the androgen interaction. Bone marrow from AR−/− mice was transferred to lethally irradiated wild-type male recipients. TS administration to these chimeras induced thymocyte apoptosis (5–6 clusters of TUNEL reactive cells/field ×400; see also Fig. 3, C and D) resembling those observed after treatment of normal male animals. In contrast,

Thymocyte apoptosis induction after sialyl residue mobilization requires a functional AR in the TEC. A–B, AR−/− mice. C–D, Chimeric mice constructed by transfer of AR−/− bone marrow to irradiated normal male recipients. E–F, Chimeric mice constructed by transfer of normal male mouse bone marrow to irradiated AR−/− recipients. All animals received a single TS dose (1 μg) 18 h before thymus collection. A, C, and E, H&E ×200. B, D, and F, H&E ×1000, arrowheads denote apoptotic bodies.

Thymocyte molecules and pathways involved

The CD43 is a highly sialylated mucin that counts for ~25–30% of the total cell surface sialyl residue being also a suggested binder for the TS (25, 26). CD43 is implicated in several biologic functions as an adhesion and costimulatory molecule and is reported as an essential molecule in the TS-induced thymocyte apoptosis. In support, TNF-α production was enhanced after TS injection as observed through fluorescence either by microscopy (Fig. 4, I and J) or flow cytometry (13.4% ± 1.4 from control vs 18.8% ± 3 from TS treatment, p < 0.05).

Because TNF-α pathway is known to induce apoptosis via caspasas, a fluorescent pan caspase-inhibitor was tested. This inhibitor reacts with activated caspasas and then will accumulate inside the apoptotic cells. As shown in Fig. 4K, apoptotic thymocytes were highlighted inside nurse cells obtained after in vivo TS treatment. When thymus organ cultures were treated overnight with TS (1 μg/ml), they also displayed increased caspase activity (Fig. 4L). To extend this analysis, the activity of caspases 1, 3, and 8 was then tested. Caspase 3-like activity was readily measurable (Fig. 4M), meanwhile caspase 1 and 8 activities were not detected.

Discussion

The exogenous alterations of the surface sialylation catalyzed by the TS leads thymocytes to apoptosis in vivo where the interaction with the TEC is required because no apoptosis is induced in isolated cells (8). In this study, we report the strict requirement of androgens in the sialyl residue mobilization-induced apoptosis from several different approaches (see Figs. 1 and 2). Females and flutamide-treated male animals were resistant to apoptosis induction by TS and similar findings were achieved with the AR−/− male mice; meanwhile testosterone-replenished gonadectomized males were rendered sensitive. Although thymus from TS-treated female mice does not show damage or cell depletion, thymocytes derived from female bone marrow donors were sensitive when embedded in a male context. Accordingly, the androgen treatment of female mice abrogated their thymocyte resistance to TS-induced apoptosis.

It is reported that the androgen-derived deadly signal is originated by the TEC in vivo (24). These results are in agreement with our findings where the AR−/− thymocytes were susceptible to the apoptosis induced after sialyl residue mobilization in the chimeric mouse approach (see Fig. 3). Androgens might induce a lectin-like molecule in the TEC able to recognize the modified sialylation pattern on the CD43 mucin that, after clustering this marker, induces the corresponding intracellular pathway that leads to TNF-α-derived death. In support, the increment of TNF-α synthesis is reported after CD43 cross-linking (32). TNF-α has been involved in the apoptosis of isolated thymocytes after in vitro treatment with testosterone (31). Because the apoptosis induced by the sialyl residue mobilization required androgens, the participation of the TNF-α deathly pathway was assayed in this study. In vivo, the TNF-α is constitutively expressed in the thymus, and believed not to have a role in thymocyte selection or organ development (33, 34). However, a role of the TNF-α apoptotic pathway in the deletion of the sialylation-altered thymocytes was found, because the thymi from the TS-treated TNFR1/TNFR2 KO mouse do not display a significant apoptosis increase. From the results obtained with the TNFR single KO mice, the TNFR1 death pathway was found responsible for the apoptosis observed. However, the number of apoptotic cells recorded in the TNFR2 KO mouse was lower than that found in the TS-treated normal mice, supporting a collaborative effect among both receptors (35, 36). By another way, the enhancement of TNF-α production by thymocytes after in vivo
CD43, the main sialylated surface molecule of lymphocytes, has been involved in the interaction with TS. It is suggested that CD43-TS interaction participates in activation-induced cell death rescue of peripheral T cells as evaluated in in vitro assays (26). However, another research group disregarded its involvement (40). In fact, the reported rescue does not involve sialyl residue mobilization, suggesting that only the lectin-like activity is required. These findings strongly contrast with the strict dependence on enzymatic activity to induce thymocyte apoptosis by the TS treatment (8, 9). The role of the CD43 in thymocyte development remains uncertain. It is known that the 130-kDa form of this molecule became down-regulated during thymocyte development (41). Some CD43 glycoforms, whose main variations reside in the sialylation pattern, are involved in the maturation of isolated thymocytes (42). However, the lack of CD43 seems not to disturb the normal in vivo T cell development as shown with the KO mouse model (43). The TS-driven sialyl residue mobilization allowed us to postulate a role for CD43 in the thymocyte development in vivo. The relevant point in fact seems to be the modification of the CD43 sialylation topology rather than the CD43 presence itself. To induce cell death, the modified CD43 mucin sialylation might be misrecognized by the TEC that responds delivering an apoptotic signal. In support, hyposialylation of this marker induces apoptosis in T cells after cross-linking with the appropriate mAbs (29). Other authors working with the ST3-Gal-I sialyltransferase KO mice that contains CD43 undersialylated glycoforms have also postulated that the altered CD43 might interact in peripheral compartments with multivalent lectin-like molecules to induce CD8 T cell apoptosis (44). In this study, we postulate a sialylation-dependent checkpoint in the thymic compartment working on the DP thymocyte population in males. Despite its known participation in thymocyte and T cell apoptosis (2, 45), the presence of terminal α(2, 3) sialyl residues (the only substrate for the TS) does not interfere with the galectin-1 binding and therefore it seems not to be involved. However, the possibility to alter the accessibility of the CD43 glycoconjugates to the galectin-1 by sialylation/desialylation of its terminal galactoses remains.

From the results reported in this study, we can conclude that three players (CD43, androgens, and the TNFR1 pathway) are concomitantly required to induce apoptosis in those thymocytes displaying an altered sialylation pattern. The TS-modified cell surface might be mimicking putative wrong endogenous glycosylation patterns, and then a modified sialylation system surveillance mechanism can be postulated. This control system does not works in females and therefore those thymocytes that bear the modified glycoform/s mimicked by the TS administration will be allowed to continue with their maturation process. The challenging hypothesis arises that these modified cells might be associated, at least in part, with the observed differential autoimmune, tumoral, and infectious diseases susceptibility among sexes (reviewed in Refs. 12 and 13),

cultures. p < 0.002, Student's t test. L, Fluorescent pan caspase inhibitor accumulation on thymocytes. M, Relative rates of caspase 3-like activity. All in vivo TS administrations consisted of a single 1-μg dose applied i.v. 18 h before collecting thymuses. A–B, ×200; C–D, ×1000; E–J, ×400; K, ×600.
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Disclosures

The authors have no financial conflict of interest.

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