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Thymic Shared Antigen-2: A Novel Cell Surface Marker Associated with T Cell Differentiation and Activation

Stuart P. Berzins,∗ Gayle M. Davey, Elise S. Randle-Barrett, Mark A. Malin, Brendan J. Classon, Stuart Fraser, and Richard L. Boyd

Thymic shared Ag-2 (TSA-2) is a 28-kDa, glycosphatidylinositol-linked cell surface molecule expressed on various T cell and thymic stromal cell subsets. It is expressed on most CD3\(^{+}\)CD4\(^{-}\)CD8\(^{-}\), CD4\(^{+}\)CD8\(^{+}\), and CD3\(^{hi}\)CD4\(^{+}\)CD8\(^{-}\) thymocytes but is down-regulated on ∼40% of CD3\(^{hi}\)CD4\(^{+}\)CD8\(^{-}\) thymocytes. Expression on peripheral TCR-αβ\(^{+}\) T cells is similar to that of CD3\(^{+}\) thymocytes, although a transient down-regulation occurs with cell activation. Consistent with the recent hypothesis that emigration from the thymus is an active process, recent thymic emigrants are primarily TSA-2\(^{-}\)low\(^{+}\) . TSA-2 expression reveals heterogeneity among subpopulations of CD3\(^{hi}\)CD4\(^{+}\)CD8\(^{-}\) thymocytes and TCR-γδ\(^{+}\) T cell previously regarded as homogenous. The functional importance of TSA-2 was illustrated by the severe block in T cell differentiation caused by adding purified anti-TSA-2 mAb to reconstituted fetal thymic organ culture. While each CD25/CD44-defined triple-negative subset was present, differentiation beyond the TN stage was essentially absent, and cell numbers of all subsets were significantly below those of control cultures. Cross-linking TSA-2 on thymocytes caused a significant Ca\(^{2+}\) influx but no increase in apoptosis, unless anti-TSA-2 was used in conjunction with suboptimal anti-CD3 mAb. Similar treatment of mature TSA-2\(^{+}\) T cells had no effect on cell survival or proliferation. This study reveals TSA-2 to be a functionally important molecule in T cell development and a novel indicator of heterogeneity among a variety of developing and mature T cell populations. The Journal of Immunology, 1999, 162: 5119–5126.

Although current models of thymopoiesis partition T cell development into discrete subsets, the process is more likely to be a continuum of tightly regulated functional and phenotypic changes. At present, each phase of thymocyte maturation is defined by the expression of a constellation of surface determinants rather than any single identifying Ag. However, as more determinants are reported, it is becoming increasingly apparent that many subpopulations thought to be homogeneous contain phenotypically and/or functionally distinct subsets (1). Because the complex thymocyte differentiation sequence is clearly dependent on the thymic stroma (2), the identification of new Ags, particularly those associated with the thymic microenvironment, should enable the pathways of thymopoiesis to become more fully defined.

We previously described a panel of mAbs raised against thymic stroma recognizing a variety of Ags expressed in distinct regions of the thymus (3, 4). Most stained either thymocytes or thymic stroma; however, some identified Ags coexpressed on both (4). One such mAb, MTS 35, recognized thymic shared Ag-1 (TSA-1),3 expressed on stromal tissue elements and immature thymocytes (5). The importance of TSA-1 during early thymopoiesis was demonstrated by the severe block in early thymopoiesis caused by treatment of fetal thymic organ cultures with the MTS 35 mAb (6).

Another mAb from that panel, MTS 32, also recognizes an Ag expressed on thymic stroma and developing thymocytes. This Ag, defined as thymic shared Ag-2 (TSA-2), is expressed on ∼90% of thymocytes and virtually all αβ\(^{+}\)CD4\(^{-}\)CD8\(^{-}\) cells but is negative or down-regulated on nearly one-half of αβ\(^{+}\)CD4\(^{+}\)CD8\(^{-}\) thymocytes and mature T cells. TSA-2\(^{-}\)low\(^{+}\) CD4\(^{+}\) thymocytes are reported to be functionally more mature than the TSA-2\(^{hi}\) cells, but this functional difference has not been described for peripheral T cells (7). By immunohistology, TSA-2 is strongly expressed in the thymic cortex, but medullary staining is surprisingly weak (2, 7). Interestingly, on thymocytes of the thymic leukemia-prone AKR mice, TSA-2 appears markedly down-regulated during the preleukemic phase and is virtually absent by the onset of disease, suggesting an association with the thymic dysfunction of AKR mice and their subsequent susceptibility to disease (8).

In the present article, we map the onset of TSA-2 expression to the CD44\(^{+}\)CD25\(^{+}\) stage of triple-negative (TN) cell differentiation, commensurate with TCR gene rearrangement and T lineage commitment (9), and report the failure of thymopoiesis beyond the TN stage within MTS 32-treated fetal thymic organ culture (FTOC). We further demonstrate a possible role for TSA-2 as a costimulatory molecule on developing thymocytes and as an important marker of differentiation among T cell subsets previously regarded as homogeneous. In particular, we describe the expression of TSA-2 on recent thymic emigrants as being significantly lower than that of either medullary thymocytes or mature peripheral T cells. TSA-2 expression may therefore enable the identification of thymocytes destined for immediate export and assist in

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3 Abbreviations used in this paper: TSA-1, thymic shared Ag-1; TSA-2, thymic shared Ag-2; TN, triple-negative; FTOC, fetal thymic organ culture; GPL, glycosphatidylinositol; PI-PLC, phosphatidylinositol-phosphatase C; HSA, heat shock
Materials and Methods

**Mice**

CBA/CaH (H-2\textsuperscript{d}) were obtained from Monash University and Walter and Eliza Hall Institute Central Animal Houses and housed under conventional conditions. Embryos were obtained from timed-mated females where the day of detection of a vaginal plug was designated Day 0.

**Flow cytometry**

Mice were killed by CO\textsubscript{2} asphyxiation, and freshly dissected thymus, spleen, or lymph nodes were gently pushed through a 200-μm pore size sieve in cold PBS-FCS (1%)-sodium azide (Az) (0.02%). Cell suspensions were washed by centrifugation (650 × \textit{g}_{\text{max}}, 5 min, 4°C), and the pellet was resuspended in PBS-FCS-Az. For spleen cell suspensions, RBCs were lysed by incubation in RBC lysis buffer (8.9 g/ml ammonium chloride) for 10 min at 4°C. Cell concentration and viability were determined in duplicate using a hemocytometer and ethidium bromide/acidine orange staining viewed under an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany). Cell suspensions were stained either in V-bottom tubes (1–3 × 10\textsuperscript{6} cells) or in 96-well round-bottom plates (<5 × 10\textsuperscript{5} cells). Cells were gently resuspended in 25 μl of appropriate mAb or secondary conjugate, incubated for 20–25 min at 4°C, washed by the addition of the 0.5–1 mM PBS-FCS-Az (0.2 ml for plates), and centrifuged (650 × \textit{g}_{\text{max}}, 5 min, 4°C).

Flow cytometry data were analyzed using Lysis II or Cell Quest software (Becton Dickinson).

**Fetal thymic organ culture**

For fetal liver cell-reconstituted FTOC, thymic lobes were removed from embryos at day 15 of gestation and cultured for 6 days in culture medium supplemented with 1.35 mM 2-deoxyguanosine. Briefly, four to six individual thymic lobes were placed on the surface of 0.45-m pore size filters (Gelman Sciences, Ann Arbor, MI) resting on Gelfoam gelatin sponges (Upjohn, Kalamazoo, MI) soaked in culture medium (RPMI 1640 adjusted to mouse osmolarity) and supplemented with 10% v/v FCS (Commonwealth Serum Laboratories, Melbourne, Australia), 2 mM glutamine, 10 mM HEPES (Flow Laboratories, Stanmore, Australia), 0.5 mg/ml folic acid, and 0.2 mg/ml glucose. After culture, filters with thymus lobes were washed twice by immersion in 50 ml of culture medium for at least 2 h at 37°C, 5% CO\textsubscript{2} in air. The lobes were removed from the filters and placed in Terasaki plates, one lobe/well, containing 5 × 10\textsuperscript{4} viable fetal liver cells in 30 μl of RPMI-FTOC medium. The Terasaki plates were gently inverted, forming a hanging drop, and incubated for 24 h at 37°C, 5% CO\textsubscript{2} in air.

Fetal liver cells were obtained from livers dissected from E14 embryos and were gently pushed through a 200-μm pore size wire mesh sieve. The cell suspension was centrifuged (400 × \textit{g}_{\text{max}}, 5 min, 4°C), and the pellet was resuspended in RPMI 1640, then gently layered over 5 ml of Ficoll-Paque and centrifuged (650 × \textit{g}_{\text{max}}, 45 min, 4°C). The precursor containing leukocytes at the RPMI/Ficoll-Paque interface were collected, washed by the addition of RPMI 1640, and centrifuged (650 × \textit{g}_{\text{max}}, 5 min, 4°C). Cells were adjusted to a final concentration of 1.7 × 10\textsuperscript{6} cells/ml in RPMI-FTOC medium. After 24 h of incubation to allow reconstitution, thymic lobes were removed from the filters and washed, and then labeled for acquisition on a FACScan (Becton Dickinson) with anti-rat Ig-FITC (Caltag, San Francisco, CA).

**Calcium flux**

Fresh thymocytes were labeled for 1 h at 37°C with 10 μM Indo-1 calcium dye, washed, and then incubated with anti-CD8, anti-CD3, or MTS 32 for 45 min at 4°C. The cells were again washed and then incubated with goat anti-rat (Caltag) at 37°C for 1, 3, and 10 min to establish cross-linking. The cells were collected on a FACS II using UV excitation at 351–364 nm and analyzed to determine the ratio of violet to blue (Ca\textsuperscript{2+}-bound Indo-1 free Indo-1) emission.

**Estimation of induced apoptosis**

Cell suspensions from the thymus of 6-wk-old mice were cultured over-night under standard conditions in 24-well plates previously coated with combinations of anti-CD3 (145-2C11), anti-TSA-2 (MTS 32) or control Ab (rat Ig). Cells were subsequently stained for expression of CD4 and CD8 with the proportion of CD4\textsuperscript{+}/CD8\textsuperscript{+} cells used to indicate relative apoptosis levels (10).

**FITC labeling of thymocytes**

Details of this technique are similar to those described elsewhere (11). Briefly, animals were anesthetized, and the chest opened to expose the thymic lobes. Each lobe was injected with ~10 μl of 350 μg/ml FITC (in PBS) which typically resulted in random labeling of 30–60% of the thymocyte population. The wound was closed with a surgical staple, and the mouse was warmed until fully restored from anesthesia. Mice were killed by CO\textsubscript{2} asphyxiation ~24 h postgraft, and lymphoid organs were removed for analysis. Instruments were washed between removal of each organ, and the FITC-injected thymus was always removed last to avoid cross-contamination of samples. The use of FITC required a PE-labeled secondary Ab to detect MTS 32 which is not otherwise used in the experiments described herein. The result is a reduced intensity of staining compared with other profiles, although TSA-2 high and low populations are still clearly distinguishable.

**Results**

**Biochemical analysis of TSA-2**

To determine the m.w. of TSA-2, detergent lysates of thymocyte membranes were separated by single-dimension SDS-PAGE, Western transferred, and blotted with mAb MTS 32. MTS 35, recognizing TSA-1, and MTS 1, recognizing an intracellular Ag, were used as specificity controls (Fig. 1).
A number of T cell molecules, including Thy-1, HSA, and members of the Ly6 family, are anchored to the cell surface by glycosphatidylcholinol (GPI) linkages (12). To determine whether TSA-2 was also linked by this means, thymocytes were treated with PI-PLC. Subsequent fluorescent labeling with MTS 32 and flow cytometric analysis revealed a decrease in TSA-2 expression compared with untreated control cells (Fig. 2). Similarly, MTS 33, detecting the Ly6 member Thb, also exhibited decreased reactivity, whereas the fluorescence intensity of CD4 (a transmembrane molecule) was unchanged. TSA-2 is therefore a single-chain molecule of ~26–28 kDa anchored to the cell membrane by a GPI linkage.

Expression of TSA-2 on thymocyte subsets

Immunofluorescence staining of thymic sections shows TSA-2 is expressed at high levels on thymocytes and stromal elements of the cortex. Although the high density of thymocytes in the cortex makes it difficult to determine the precise stromal reactivity, flow cytometry shows ~20% of freshly isolated thymic stromal cells to be TSA-2+ (13) along with the majority of thymocytes.

The most immature TN cells (CD44highCD25–) (9) are primarily TSA-2– with pre-T cells first expressing TSA-2 as uncommitted CD3–CD4–CD8– (TN) prothymocytes during the transition from CD25+CD44+ to CD25–CD44–, commensurate with TCR-β gene rearrangement and commitment to the T cell lineage (Fig. 3). TSA-2 is therefore one of the very earliest markers of the T cell lineage. High levels are maintained through the double-negative (DN) stage, but down-regulation may then occur, particularly on CD3+CD4+CD8– cells where ~40% eventually become TSA-2low (Fig. 4). Down-regulation of TSA-2 is also evident on some CD4+CD8+ cells but to a lesser extent than on CD4+CD8– thymocytes.

FIGURE 2. TSA-2 is linked to the cell surface via a GPI anchor. Fluorescent labeling of thymocytes treated with PI-PLC causes a decrease in TSA-2 expression. The fluorescence intensity of transmembrane molecule CD4 was unchanged.

An interesting relationship was found to exist between the levels of TSA-2 and CD4 on the CD4+CD8– subset of thymocytes. Separation of the CD4+CD8– quadrant into high, intermediate, and low levels of CD4 expression revealed the expression of TSA-2 staining to be inversely proportional to that of CD4. Specifically, CD4+CD8– cells with relatively low CD4 levels expressed high levels of TSA-2, but those with the highest CD4 expression had vastly reduced TSA-2 levels. Both CD4(low)TSA-2(low) and CD4(high)TSA-2(high) thymocytes showed similar Thb and Thy-1 expression and were CD3(high), αβTCR(high), and HSA(low), suggesting similar levels of maturity. There were, however, subtle differences in the levels of CD3 and αβ TCR that supported the findings of Vicari et al. (7) that TSA-2low cells were more mature than their TSA-2high counterparts (Fig. 5). The relationship between CD4 and TSA-2 expression on CD4+CD8– thymocytes is not seen among DP cells or peripheral T cells, nor did the reduced TSA-2 expression on some CD4+CD8+ thymocytes relate to CD8 levels.

TSA-2 expression was also investigated on TCR-αβCD4+CD8– and TCR-γδ+ T cells, both of which showed considerable heterogeneity. TCR-γδ+ T cells expressing CD8+ had generally high TSA-2 expression, whereas CD8– TCR-γδ+ T cells had no (or low) TSA-2 (Fig. 6). Although the phenotype of γδ TCR+ subpopulations varies with age (14, 15), TSA-2 expression on peripheral γδ+ cells was similar to that seen in the thymus at all ages tested. TCR-αβCD4+CD8– cells in both the thymus and periphery expressed low to intermediate but not high levels of TSA-2.

Ontogeny

In light of earlier findings that TSA-2–CD4+CD8– thymocytes were functionally and phenotypically more mature than their TSA-2+ counterparts (7), the expression of TSA-2 during early thymus development was analyzed. Thymocytes from individual CBA mice from birth (day 0) through to day 7 were compared with those from adult mice (6–14 wk) for levels of CD3, HSA, and TSA-2 in conjunction with CD4 and CD8 expression (Fig. 7).

Although the overall proportion of TSA-2– thymocytes was slightly decreased at days 0–1 relative to the adult thymus, TSA-2 expression within the CD4+CD8– subset from E18 (the earliest time point at which functional single-positive thymocytes can be detected (16, 17)) to day 1 was uniformly high. The down-regulation of TSA-2 on the CD4+CD8– subset to levels similar to that of adult thymocytes, first occurred around day 2, with normal adult
proportions present from day 3 onward. Before day 3, TSA-2^low^ cells were found primarily in the CD4^-^CD8^-^ subset.

**TSA-2 expression on activated T cells**

Since TSA-2 down-regulation occurred only on relatively mature T cells, we investigated whether this was a consequence of activation. TSA-2 expression was generally lower on activated (CD69^+^ or CD25^+^) than nonactivated T cells in the thymus and periphery of adult mice (Fig. 8). On peripheral T cells, stimulation with anti-CD3 mAb, Con A, or PMA/ionomycin also resulted in significant down-regulation of TSA-2, particularly within the CD4^-^CD8^-^ subset. The typically high TSA-2 expression levels on mature T cells and the identical expression patterns for naive (CD4^lo^) and memory (CD4^hi^) phenotype T cells (data not shown) indicated that this down-regulation was transient.

As well as an indicator of activation, CD69 is also transiently expressed on apoptosing CD4^-^CD8^-^ thymocytes (18, 19). It was therefore possible that TSA-2 down-regulation on CD69^-^ thymocytes reflected imminent apoptosis rather than activation. However, TSA-2 expression on the CD4^-^CD8^-^ cells was universally high (data not shown), indicating the correlation was limited to activated, viable cells.

**Recent thymic emigrants (RTE)**

Given the consistent down-regulation of TSA-2 on subsets of mature thymocytes, we investigated the possibility that down-regulation was also an indicator of migration to the peripheral pool. RTE were identified in the spleen, lymph nodes, and blood 24 h after intrathymic injection of FITC. TSA-2 down-regulation was

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**FIGURE 5.** TSA-2 is inversely expressed on CD4^-^CD8^-^ thymocytes relative to the level of CD4 expression. On CD4^-^CD8^-^ thymocytes that express the lowest (lo) levels of CD4, TSA-2 is expressed at uniformly high (hi) levels. However, CD4^-^CD8^-^ thymocytes expressing the highest levels of CD4 express only low to intermediate levels of TSA-2. The CD4^-^lo^, CD4^-^int^, and CD4^-^hi^ subsets of the CD4^-^CD8^-^ population show similar expression of CD3, αβ TCR, and HSA; however, the level of CD3 expression is highest on CD4^-^hi^TSA-2^-^ cells. No variation was observed on CD8^-^ cells or on any other T cell subset examined. CD4^-^CD8^-^ peripheral T cells showed a similar but less distinct correlation between TSA-2 expression and CD4 levels.
observed on up to 80% of CD4+ and 40% of CD8+ RTE. Remarkably, the extent of down-regulation was found to be significantly greater for RTE than for either mature thymocytes or mature T cells (Fig. 9). In addition to providing further evidence that RTE are distinct from T cells resident in the periphery, these data support the hypothesis that some form of activation precedes the export of T cells from the thymus (20, 21).

Role of TSA-2 in thymocyte development

The up-regulation of TSA-2 as CD3+CD4-CD8- thymocytes differentiate from the CD44+CD25+ to the CD44-CD25+ subset suggested a functional role in early thymopoiesis. To address this question, purified MTS 32 (anti-TSA-2) mAb was added to dGuo FTOCs reconstituted with E14 fetal liver cells. At the end of the 18-day culture period, the thymic lobes were analyzed by flow cytometry to determine whether T cell differentiation had been affected.

At least 4–5-fold fewer cells were recovered from cultures treated with 200 μg/ml anti-TSA-2 mAbs than from untreated cultures or those treated with 200 μg/ml control mAbs such as rat IgM 33 (anti-ThB), or MTS 1 (isotype control). Lower concentrations (25–100 μg/ml) of MTS 32 also inhibited thymopoiesis, but to a lesser extent; therefore, 200 μg/ml was adopted as the standard treatment.
Flow cytometric analysis of thymocytes from anti-TSA-2-treated cultures (200 μg/ml) revealed a profound depletion of CD4\(^+\)CD8\(^-\), CD4\(^-\)CD8\(^+\), and CD4\(^-\)CD8\(^-\) cells, and although CD4\(^-\)CD8\(^-\) cell numbers were proportionally increased, the total number of CD4\(^-\)CD8\(^-\) cells was usually <30% of cultures treated with control mAbs (Fig. 10). All CD25- and CD44-defined TN subsets were present in approximately normal proportions (data not shown).

In comparison with control cultures, the DP compartment was proportionally the most affected, often reduced 10-fold and in some cases virtually absent. Although the generation of DP cells from the TN subset is dependent on TCR\(\beta\)-chain and RAG-1 expression by CD44 int/low CD25\(^+\) cells, expression of RAG-1 (as determined by in situ hybridization) was normal in treated cultures. Anti-TSA-2 mAb synergizes with anti-CD3 to induce apoptosis in thymocytes

One possible explanation for the sharp decrease in the number of cells recovered from anti-TSA-2-treated FTOCs was that rather than blocking thymocyte differentiation, the mAb was inducing apoptosis, particularly at the susceptible CD4\(^+\)CD8\(^-\) stage of development. To investigate this possibility, thymocytes were cultured overnight in flat-bottom plates coated with anti-TSA-2, and the resulting down-regulation of CD4\(^+\)CD8\(^-\) to CD4\(^\text{int}\)CD8\(^\text{int}\) (indicative of thymocyte apoptosis (10, 22)) was compared with that of control cultures. Forward scatter analysis and subsequent annexin staining was used to confirm thymocyte apoptosis in the CD4\(^\text{int}\)CD8\(^\text{int}\) subset (data not shown).

Thymocytes cultured overnight in plates coated with anti-CD3 showed increased levels of apoptosis (data not shown), whereas levels in anti-TSA-2-coated plates were similar to that of control cultures. However, increasing concentrations of anti-TSA-2 added in combination with suboptimal levels of anti-CD3 caused dose-dependent increases in the level of apoptosis (Fig. 11). Anti-TSA-2 alone induced neither proliferation nor apoptosis in peripheral T lymphocyte cultures, and the combination of anti-TSA-2 and anti-CD3 caused proliferation levels no greater that caused by anti-CD3 alone (data not shown).

Calcium influx

Thymocytes loaded with the calcium dye Indo-1 were labeled with the anti-TSA-2 mAb MTS 32, which was then cross-linked using rabbit anti-mouse Ig. A shift in the log ratio of violet/blue fluorescence toward the violet spectrum was observed after 3 min, which increased at the 10-min interval and was still present 25 min after thymocyte triggering (Fig. 12). This signal was more rapid than that of the anti-CD3-positive control, and there was no effect using anti-CD8 as a negative control. Therefore, consistent with its proposed role as a costimulatory molecule, cross-linking of TSA-2 results in a prolonged calcium release indicative of signal transduction associated with ligand binding.
The transition from CD44- to CD44+ thymocytes begins rearrangement of the TCR-β complex, indicating that thymocytes can result from signaling through TSA-2; however, the specific mechanisms leading to this transition are not fully understood. The effect of the mAb) before the addition of the MTS 32 that blocks proliferation of multiple thymocyte subsets. From which others could develop, or caused the widespread deletion of existing cell populations. Apoptosis in thymocytes is likely to reflect the increased signaling caused by cross-linking TSA-2 and CD3 with intact mAb, rather than the engagement of the natural ligand(s) within the thymus. Cross-linking TSA-2 on peripheral T cells had no effect on the rate of either apoptosis or cell proliferation, with or without CD3 costimulation.

Some effects of anti-TSA-2 mAb in FTOC were probably indirectly mediated through surrounding TSA-2+ thymocytes or stromal cells. The most immature TN cells (CD44+CD25+) are primarily TSA-2+ but were substantially reduced in number in treated FTOC, suggesting an indirect effect. Because fetal liver was also primarily TSA-2+ (data not shown, also Ref. 4), the influence of TSA-2+ stroma should not be underestimated, particularly because it is now clear that thymopoiesis cannot occur in the absence of thymic stroma (2). We are therefore investigating the poorly defined function of stromal cells, with particular emphasis on the relationship between TSA-2+ stroma and the earliest developing thymocytes, including the regulation of stem cell entry into the thymus.

While a functional role in early thymopoiesis is implied by the rapid up-regulation of TSA-2 during TN differentiation and the block in development caused by anti-TSA-2 mAb, TSA-2 also identifies heterogeneity among more mature thymocyte populations previously regarded as homogeneous. A number of molecules are used to define the maturity of thymocytes, including HSA (25, 26), Ly6C (27), and Qa2 (28); however, TSA-2 demonstrates phenotypic heterogeneity among CD3+CD4+CD8- cells of apparent similar maturity.

Minor differences in the level of CD4 on CD3+CD4+CD8- thymocytes are usually regarded as inconsequential, with no obvious differences in the expression of maturity markers such as HSA and CD4+CD8- TCR among CD4+ and CD4- thymocytes (excluding CD4low TN cells). However, CD3+CD4+CD8- cells expressing proportionally low CD4 levels have markedly different levels of TSA-2 than CD4high and even CD4low cells. High levels of TSA-2 are found on CD4low cells, but only low to intermediate TSA-2 levels on cells with the highest CD4 expression. The functional significance of this difference is currently under investigation, but several lines of evidence suggest TSA-2low cells are destined for imminent export from the thymus.

Previous reports describe TSA-2low CD4+CD8- thymocytes as functionally more mature than TSA-2high counterparts, although the significance of TSA-2 down-regulation was not clear (7). Although no difference in maturity is reported between CD3highCD4high and CD3highCD4low thymocytes, close examination of CD3 expression reveals a marginally higher expression on TSA-2low CD4high than on TSA-2highCD4low cells. In light of our findings that TSA-2 down-regulation can result from the activation of mature T cells, down-regulation of TSA-2 on medullary thymocytes appears to be restricted to the most mature cells of the CD3+CD4+CD8- subpopulation.

Discussion

We have previously described the mAb MTS 32 as recognizing an Ag expressed on developing and mature T cells (4). The Ag, defined as TSA-2, is further characterized here as a 26–28-kDa GPI-linked protein with remarkable patterns of expression on T cell subsets and an important role in early T cell development. TSA-2 is first expressed on CD3+CD4+CD8- (TN) cells during the transition from CD44+CD25+ to CD44-CD25 as developing thymocytes begin rearrangement of the TCR-β gene (23). With fetal liver reconstituted FTOC as a model of early thymopoiesis (24), treatment with purified MTS 32 caused a marked reduction of all major thymocyte populations compared with control cultures, suggesting a functional role for the TSA-2 Ag in normal T cell development. The near complete absence of CD4+CD8- (DP) and single-positive thymocytes in treated cultures meant that TN cells were proportionally least affected, with normal proportions of all CD44/CD25-defined subsets, albeit with cell numbers still significantly below normal. This suggested anti-TSA-2 was acting in one of three ways. The mAb either blocked the collective proliferation of TN cells, thereby preventing development of DP cells, reduced the number of TN progenitor cells from which others could develop, or caused the widespread deletion of multiple thymocyte subsets.

The absence of overt apoptosis in anti-TSA-2-treated FTOC and the limited effect of anti-TSA-2 mAb on normal FTOC (i.e., without prior thymocyte depletion and reconstitution) suggested the reduced cellularity of treated FTOC was due to a block of differentiation and/or proliferation, rather than deletion of existing cells. Specifically, whereas MTS 32-treated fetal liver reconstituted FTOC failed to progress beyond the TN stage and had significantly reduced cell numbers, cultures initiated as normal FTOC (i.e., no depletion of preexisting thymocytes) did develop CD4+CD8- cells and suffered minimal cell loss. The implication is that many preexisting thymocytes in normal FTOC have passed through the TN stage (or have received T cell differentiation signals that negate the effect of the mAb) before the addition of the MTS 32 that enables normal development of these cells. Anti-TSA-2 treatment of reconstituted FTOC, however, ensures that the entire maturation pathway of every thymocyte is subject to anti-TSA-2 treatment. Effects on early thymopoiesis would therefore impact on all cell subsets.

There is some evidence that a more widespread deletion of thymocytes can result from signaling through TSA-2; however, the extent of signaling required is probably unlikely to occur in vivo. Using thymocyte cell suspensions, cross-linking TSA-2 led to a Ca2+ influx which, in conjunction with signaling through the CD3 complex (through anti-CD3 mAb), caused significantly greater levels of apoptosis than anti-CD3 mAb did alone. Cross-linking TSA-2 alone caused no increase in apoptosis levels. Therefore, MTS 32 mAb binding TSA-2 in FTOC may elevate the signaling occurring during early T cell differentiation, leading to either a block in development or cell death. In our opinion, the lack of overt apoptosis within treated FTOC is again consistent with anti-TSA-2 causing a differentiation block during early thymopoiesis rather than deletion of existing cell populations. Apoptosis in thymocytes is likely to reflect the increased signaling caused by cross-linking TSA-2 and CD3 with intact mAb, rather than the engagement of the natural ligand(s) within the thymus. Cross-linking TSA-2 on peripheral T cells had no effect on the rate of either apoptosis or cell proliferation, with or without CD3 costimulation.
Remarkably, while the proportions of TSA-2− and TSA-2+ cells among single-positive medullary thymocytes and mature peripheral T cells were virtually identical, a vastly increased proportion of TSA-2low cells was seen among the RTE population. The transient loss of TSA-2 during emigration suggests recent activation and is consistent with export from the thymus being an active rather than passive process (17, 18). Furthermore, it provides strong support that RTE are distinct from both medullary thymocytes and cells of the peripheral T cell pool (29, 30).

Recent reports have suggested that RTE may divide immediately before export in response to an unknown stimulus that triggers the export of T cells from the thymus but does not induce up-regulation of traditional activation markers such as CD25 or CD69 (20, 31). TSA-2 down-regulation by RTE may provide the means by which medullary thymocytes destined for immediate export can be identified, thereby enabling the poorly understood process of thymic emigration to be more easily studied.

The prevalence of TSA-2 down-regulation among CD3+CD4+CD8− cells implicates MHCI+ APCs in the process. Although TCR transgenic mice are being used to test this proposition, it is interesting to note that before neonatal day 2 CD3+CD4+CD8− thymocytes are universally TSA-2high, with a clear TSA-2low subset emerging, coincident with the availability of blood-borne MHCI+ dendritic cells. Most circulating cells are excluded from the thymus, but dendritic cells readily enter during the neonatal period (32), thereby increasing presentation to CD3+CD4+CD8− cells. TSA-2 heterogeneity is not, however, limited to mainstream T cells. Universally high TSA-2 expression is seen on γδ TCR+ cells expressing CD8, but only intermediate or low levels occur on CD8− γδ TCR+ cells. TSA-2 is also down-regulated on ~50% of αδ+CD4+CD8− cells, consistent with the reported functional heterogeneity of these cells (33).

TSA-2 is thus defined as a novel 26–28-kDa GPI-anchored molecule, expressed on T lineage cells in a manner linked to both the maturity and activation status of the cell. Its distribution suggests that TSA-2 is an important marker of heterogeneity and stimulation among thymocytes, particularly the CD3+CD4+CD8− cells of the thymic medulla and minor T cell subsets such as recent thymic emigrants, γδ TCR−, and αδ+CD4−CD8+ T cells. Investigation of the functional differentiation of mature peripheral T cells defined by TSA-2 expression is currently being undertaken.

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References