Defective Development of Thymocytes Overexpressing the Costimulatory Molecule, Heat-stable Antigen

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Summary

Heat-stable antigen (HSA) is a small, glycosyl phosphatidylinositol–anchored protein that can act as a costimulatory molecule for antigen-dependent activation of helper T cells. In addition to being expressed on antigen-presenting B cells, HSA is also expressed during the initial stages of T cell development in the thymus. HSA levels are very high on immature $CD4^-$, $CD8^-$ double negative thymocytes, but are reduced on $CD4^+$, $CD8^+$ double positive cells undergoing selection in the thymus, and are entirely eliminated when these cells differentiate into immunologically competent $CD4^+$ or $CD8^+$ single positive T cells. To examine the potential roles of this molecule in T cell development and selection, we generated transgenic mice in which HSA was highly expressed on all classes of thymocytes. The consequence of deregulated HSA expression was a pronounced reduction in the numbers of double positive and single positive thymocytes, whereas the numbers of their double negative precursors were largely unaffected. These results demonstrate that downregulation of HSA expression at the double positive stage is a critical event in thymocyte development. The depletion of thymocytes resulting from HSA overexpression begins at the same time as the onset of negative selection, suggesting that HSA may provide signals that contribute to determining the efficiency of this process.

Heat-stable antigen (HSA)[1] has recently been identified as an important costimulatory molecule for the activation of murine Th cells (1, 2). The expression of HSA on APCs provides a second signal, subordinate to that provided by MHC-presented peptide, which induces the activation and proliferation of antigen-specific Th cells. In the absence of this second signal, which can also be provided by the B7 costimulatory molecule independently of or in cooperation with HSA (3, 4), the interacting Th cell is driven into an inactivated, nonproliferating state.

One type of APC that expresses HSA is the B cell. Levels of HSA expression vary both during B cell development and among functional classes of mature B cells. The earliest B cell precursors identifiable in bone marrow are HSA^- (5). Their immediate progeny acquire expression of moderate to high levels of HSA, with the highest levels being found on cycling pro-B cells (5). Pre-B cells with rearranged immunoglobulin genes and most mature primary B cells express moderate levels of HSA (5–9). However, the minor subset of virginal B cells that can differentiate into memory cells after antigen exposure has only very low levels of HSA, as do circulating memory B cells (8, 9). It is not yet known whether the regulated expression of HSA on B cells determines their functional capabilities as T cell–activating or –suppressing APCs.

HSA is also expressed during the initial stages of T cell development (10–14). HSA is found on the earliest T cell precursors that have been identified in the thymus (15, 16), and expression continues at high levels throughout the $CD4^-$ $CD8^-$ double negative (DN) stage (10, 12). A reduction in the level of HSA expression occurs with the differentiation of DN thymocytes into $CD4^+$ $CD8^+$ double positive (DP) thymocytes (10, 11). Subsequent maturation of the DP thymocytes into $CD4^+$ or $CD8^+$ single positive (SP) thymocytes is accompanied by loss of HSA expression, and HSA remains absent from these cells after they have migrated from the thymus as functional T cells (6, 11).

The loss of HSA expression before the emigration of matured T cells from the thymus is perhaps to be expected, because the presence of HSA on T cells might interfere with their recognition of or response to costimulating HSA presented by APCs. However, it is not obvious why HSA should

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; hGH, human growth hormone; HFN, HBSS/fetal bovine serum/sodium azide; HSA, heat-stable antigen.

be expressed at all on thymocytes before their maturation. As an initial step in determining the function of HSA in thymocyte development, we have generated transgenic mice in which thymocytes at all stages express high levels of HSA. The severe depletion of all thymocytes beyond the DN stage that occurs in these transgenic mice points to an important role for downregulation of HSA expression in the ability of thymocytes to survive selection and differentiate into mature T cells.

Materials and Methods

Construction of the HSA Transgene. The pLIT2 vector was derived from p1017 (17), with the lck proximal promoter downstream of the StuI site at about −1,000 bp replaced by the Ig μ enhancer and TCR β promoter of pJFEμVβMT (Jirik, F., University of British Columbia, unpublished results). A frame-shift was introduced into the human growth hormone (hGH) coding region at the BglII site to ensure that a functional hGH protein could not be expressed from the pLIT2 vector. The 270 bp HinFI fragment of pSL87c4 (18) containing the complete coding region of HSA was inserted between the promoter and the introns and polyadenylation site of the *hGH* gene (19) to generate the transgene construct pLIT2-HSA.

Generation of Transgenic Mice. The 7,100-bp transgene portion of pLIT2-HSA was released by SstII digestion, purified by agarose gel electrophoresis, and injected into the pronuclei of (C57BL/6J × C3H)F_2 hybrid zygotes. Pups resulting from transplantation of injected zygotes into pseudopregnant females were analyzed for the presence of the transgene by Southern blot analysis of tail DNA hybridized with a 2.1-kb hGH probe or by PCR analysis using oligonucleotide primers flanking the HSA-hGH junction in the transgene.

Analysis of Transcripts. Total cellular RNA was purified from tissues as described (20), and separated by electrophoresis through agarose gels containing formaldehyde. After transfer to nylon membranes (ZetaProbe, BioRad, Richmond, CA) mRNAs were detected by hybridization as described (21), using DNA probes labeled with ^{32}P by random priming (22). The hGH probe included all hGH sequences within the pLIT2 vector, encompassing the entire hGH coding region and introns (19). The HSA probe was the 270-bp HinFI fragment of pSL87c4 (18).

Flow Cytometric Analysis of Protein Expression. Cells were released from tissues by disruption through a fine steel mesh and washed in HBSS containing 5% fetal bovine serum and 0.1% sodium azide (HFN). Staining with primary and secondary antibodies was done on ice in HFN. After washing in HFN, cells were analyzed on FACScan® or FACStar Plus® flow cytometers (Becton Dickinson & Co., Mountain View, CA). HSA was detected with purified and biotinylated mAb M1/69 (6). FITC-labeled CD3, CD4, and CD8 mAb were from Boehringer Mannheim (Indianapolis, IN), whereas the biotinylated CD8 mAb, PE-labelled CD4, and all anti-TCR mAb were from Pharmingen. The anti-IL-2 receptor α mAb was from the 7D4 hybridoma (American Type Culture Collection, Rockville, MD).

CD3 Stimulation and Proliferation Assay. $CD4^+$ or $CD8^+$ cells from thymus or axillary and inguinal lymph nodes were purified by sorting on the FACStar® flow cytometer. 5×10^4 cells were incubated in RPMI medium containing 50 μM 2-ME and 5% fetal bovine serum, in microtiter plate wells that had been coated with CD3 mAb (a generous gift from J. Bluestone, University of Chicago, Chicago, IL). After 66 h, 1 μCi of [^{3}H]thymidine was added to each well for a 6-h pulse-labeling period. The cells were then washed, and incorporated radioactivity was measured by liquid scintillation counting.

Results

Generation of Transgenic Mice Overexpressing HSA in Thymocytes. To achieve high levels of expression of HSA throughout all stages of thymocyte development, a cDNA encoding HSA was fused to a transcription-activating complex consisting of an immunoglobulin μ enhancer and the TCR β promoter (Fig. 1). The transgene also contained a region of the *lck* gene upstream of its proximal promoter (23), because this region was suspected to have an expression-enhancing function in thymocytes of transgenic mice.

Two transgenic mice were produced by injection of the HSA transgene into fertilized zygotes. One of the transgenic mice had about four integrated copies of the transgene, and was sterile (LIT2-20). The other transgenic mouse had an estimated 80 copies of the transgene and was fertile, serving as the founder of a breeding line (LIT2-37). Its offspring were crossbred to generate mice that were homozygous for the transgene locus (LIT2-37×37). These HSA transgenic mice developed normally and were healthy in a pathogen-free environment.

Expression of the HSA Transgene. The LIT2-37 transgenic line expressed the HSA transgene mRNA (specifically detected by hybridization with a hGH probe, Fig. 2 *A*) in thymic tissue at levels that were considerably higher than those of the endogenous HSA gene (detected along with the transgene transcript by an HSA probe, Fig. 2 *B*). Transgene mRNA levels were about twice as high in LIT2-37×37 homozygous transgenic mice versus LIT2-37 heterozygous transgenic mice. mRNAs from the transgene were found at moderate levels in lymph nodes, spleen, and bone marrow, and at very low levels in brain and heart (Fig. 2).

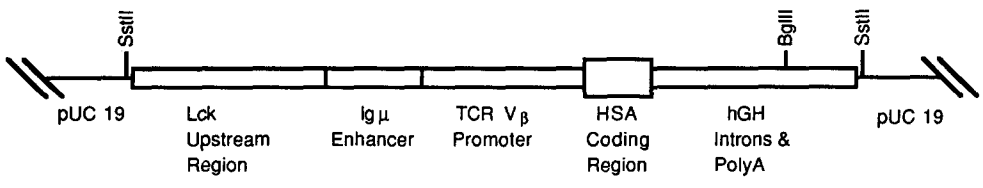

Figure 1. Structure of the HSA transgene. The pLIT2 vector contains the complete coding region of the HSA cDNA. Its transcription is driven by the promoter from the TCR β gene, coupled to the μ enhancer from the Ig H chain gene. A large fragment of the *lck* gene upstream of the proximal promoter is also present. Sequences from the *hGH* gene, including splice sites and a polyadenylation signal, are downstream of the HSA coding region and are included in the primary transcript.

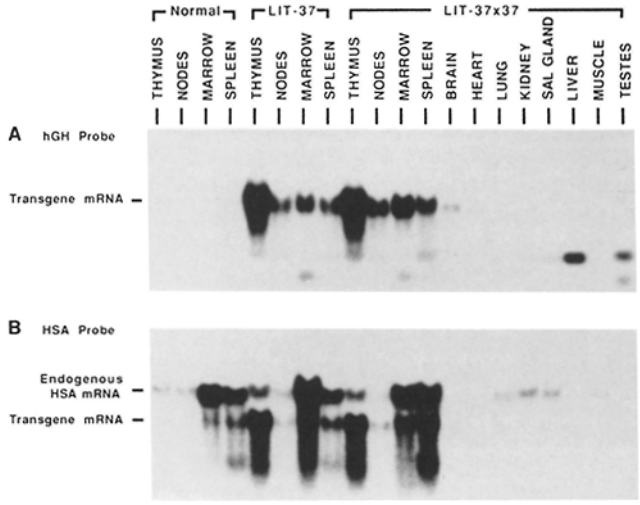

Figure 2. Analysis of transgenic and endogenous HSA transcripts. 10-μg samples of total cellular RNA from the indicated tissues of nontransgenic mice (*Normal*), heterozygous (*LIT-37*), or homozygous (*LIT-37×37*) transgenic mice were analyzed by Northern blot for the presence of transcripts derived from the HSA transgene (detected by the hGH as well as the HSA probes) and the endogenous HSA gene (detected by the HSA probe only). The small transcripts detected in some tissues by the hGH probe only are probably derived from the endogenous mouse growth hormone gene.

Flow cytometry was used to compare the expression of HSA protein on subpopulations of thymocytes in the LIT2-37×37 transgenic mice versus nontransgenic mice from the same litters (Fig. 3). HSA was expressed at high levels on most DN thymocytes of the nontransgenic mice, and these levels were only slightly elevated in the DN thymocytes from the transgenic mice. HSA expression was at intermediate levels in almost all DP thymocytes of nontransgenic mice, but in the transgenic mice a majority of DP thymocytes had high HSA levels, equivalent to those seen in DN thymocytes. Similarly, HSA levels were at high or intermediate levels in most SP thymocytes of transgenic mice, whereas in nontransgenic mice, SP thymocytes had only intermediate to nil HSA expression. Thus, in the transgenic mice, HSA expression was not significantly altered in DN thymocytes but was elevated above normal levels in DP thymocytes and extended to SP thymocytes that normally lack expression.

Depletion of Specific Thymocyte Subpopulations in HSA Transgenic Mice. All of the HSA transgenic mice had abnormally small thymuses. This was particularly obvious in the mice that were homozygous for the transgene locus. Histological staining showed that the thymic medullary regions of the transgenic mice were sparsely populated and the cortical regions were indistinct and very thin relative to those of normal mice. The thymuses of homozygous HSA transgenic mice contained about one tenth the number of total thymocytes as did those of nontransgenic littermates (Table 1). This was due to a 10–20-fold depletion of both the DP and SP populations, which normally constitute almost all of the cells in the thymus (Fig. 4, Table 1).

In striking contrast to the loss of most DP and SP thymocytes, the numbers of DN thymocytes were not significantly altered in the transgenic mice (Table 1). As a result, DN cells

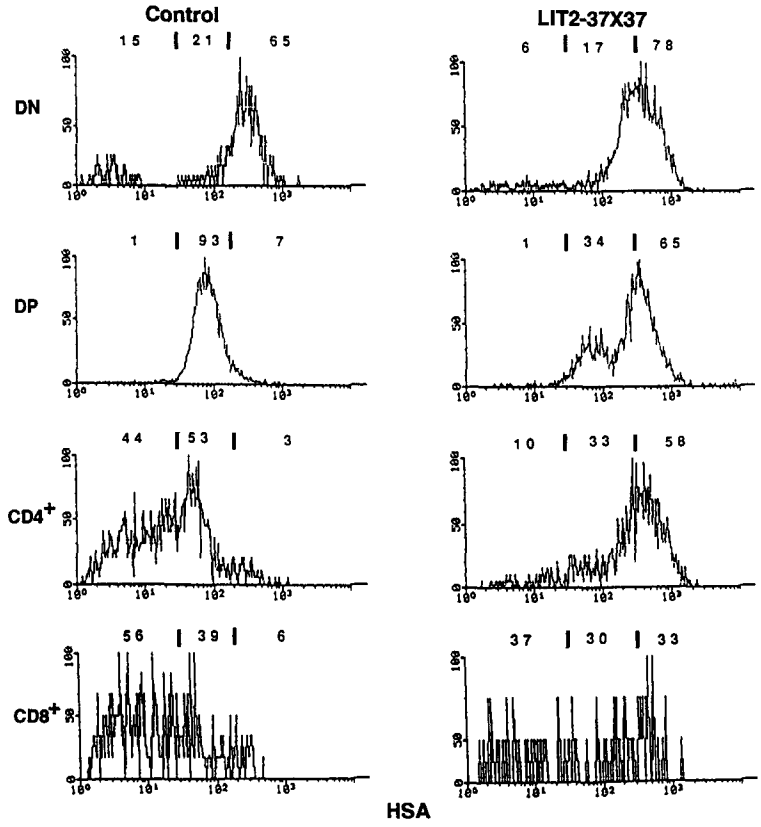

Figure 3. Expression of HSA on thymocyte subpopulations in normal and HSA transgenic mice. Total thymocytes from a 4-wk-old LIT2-37×37 homozygous transgenic mouse and its normal, nontransgenic littermate (*Control*) were stained with FITC-conjugated CD4, PE-conjugated CD8, biotinylated M1/69 (anti-HSA) mAb, and Cy5-streptavidin, and analyzed by flow cytometry. The numbers above the histograms indicate the percentages of cells expressing nil, low, or high levels of HSA.

Table 1. *Thymocyte Subpopulations in Normal and HSA Transgenic Mice*

Thymocyte subpopulation	Cell number × 10^6		Cell number ratio
	Normal	Transgenic	Transgenic/Normal
Total thymocytes	240	22	0.09
$CD4^-CD8^-$	9.6	7.0	0.70
$CD4^+CD8^+$	180	11	0.06
$CD4^+CD8^-$	31	2.6	0.08
$CD4^-CD8^+$	14	0.7	0.05
IL-$2R^+$	2.4	2.9	1.20

Thymocytes from a 9-wk-old LIT2-37×37 homozygous transgenic mouse and its normal, nontransgenic littermate were stained with FITC- or PE-conjugated mAb directed against the indicated antigens and analyzed by flow cytometry. Cell numbers were derived from direct counts of all cells freed from the thymus by disruption through a fine steel mesh. The transgenic/normal cell number ratio indicates the degree of depletion of each population in the transgenic mice.

constitute about 30% of thymocytes in the HSA transgenic mice, as opposed to 4% in the nontransgenic mice (Fig. 4). The DN population of thymocytes is heterogeneous, being composed of both immature and mature T cell types. DN thymocytes that express IL-2 receptor and that do not have detectable TCR on their surfaces are at an early stage in development and serve as precursors for DP cells (24). DN cells with this immature phenotype were not depleted at all in the transgenic mice (Table 1). The DN population also includes minor subsets of thymocytes that already express either αβ-TCR or γδ-TCR. DN cells expressing αβ-TCR are functionally mature and appear to have developed from TCR^- DN precursors via an intermediate stage that may have expressed at least CD8, and possibly CD4 as well (25, 26). These αβ-TCR^+ DN cells were highly depleted in the transgenic mice, to an extent equivalent to the depletion of the DP and SP cells (Fig. 5). In contrast, the DN thymocytes expressing γδ-TCR were at normal levels in the HSA transgenic mice. This is compatible with the onset of the depleting effects of HSA overexpression occurring at the DN to DP transition, because the γδ-TCR and αβ-TCR lineages diverge before this point (27), and the γδ-TCR expressing thymocytes do not appear to pass through a DP stage before their maturation (28).

There was a small but significant decrease in the average levels of expression of αβ-TCR in the DP cells of the transgenic versus normal mice (Fig. 6). The decline in CD3 expression in this population was even more pronounced (Fig. 6), indicating that there are many DP cells in the transgenic mice that express low levels of αβ-TCR but virtually no CD3. Most SP cells from the transgenic mice expressed both αβ-TCR and CD3 at levels that were only slightly reduced from those on SP cells of normal mice (Fig. 6). However, the subpopulations of SP cells lacking TCR and CD3 expression were considerably more abundant in the transgenic mice, particularly among the $CD4^+$ cells.

The degree of depletion of DP and SP thymocytes in the transgenic mice was correlated with the level of HSA expression attained, as mice heterozygous for the transgene had a less severe phenotype than did their homozygous littermates, with only a threefold reduction in the numbers of DP and SP thymocytes (Fig. 4). Conversely, the sterile founder transgenic mouse, which had very high levels of HSA expression

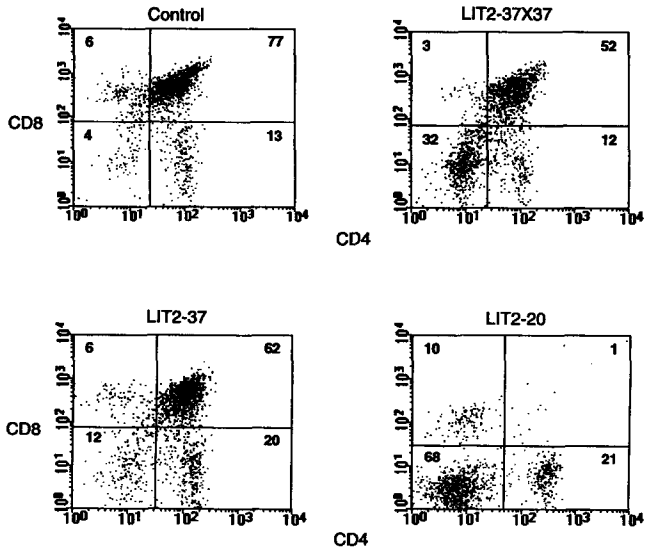

Figure 4. Thymocyte subpopulations in normal and HSA transgenic mice. Total thymocytes from a 9-wk-old LIT2-37×37 homozygous transgenic mouse and its nontransgenic littermate (*Control*), a 12-wk-old LIT2-37 heterozygous transgenic mouse, and the 25-wk-old LIT2-20 founder transgenic mouse were stained with FITC-conjugated CD4 mAb and PE-conjugated CD8 mAb, and analyzed by flow cytometry. Numbers in quadrants indicate the percentage of the total population.

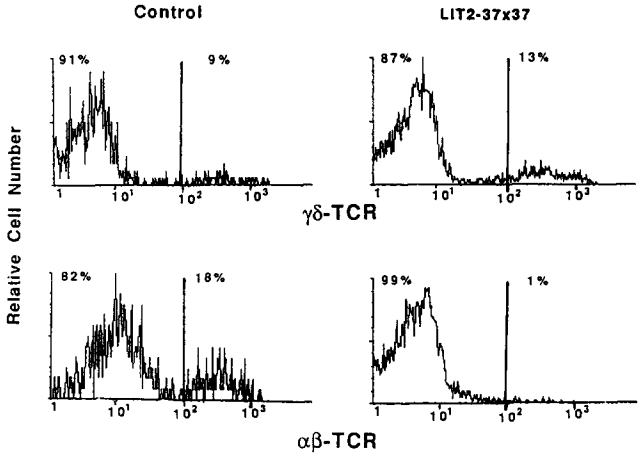

Figure 5. γδ- and αβ-TCR expressing DN thymocytes in normal and HSA transgenic mice. Total thymocytes from an 8-wk-old LIT2-37×37 homozygous transgenic mouse and its nontransgenic littermate (*Control*) were stained with FITC-conjugated CD4 and CD8 mAb, and PE-conjugated anti-γδ-TCR or anti-α/β-TCR mAb and analyzed by flow cytometry. DP and SP cells were excluded from the analysis by gating on the basis of CD4 and/or CD8 expression.

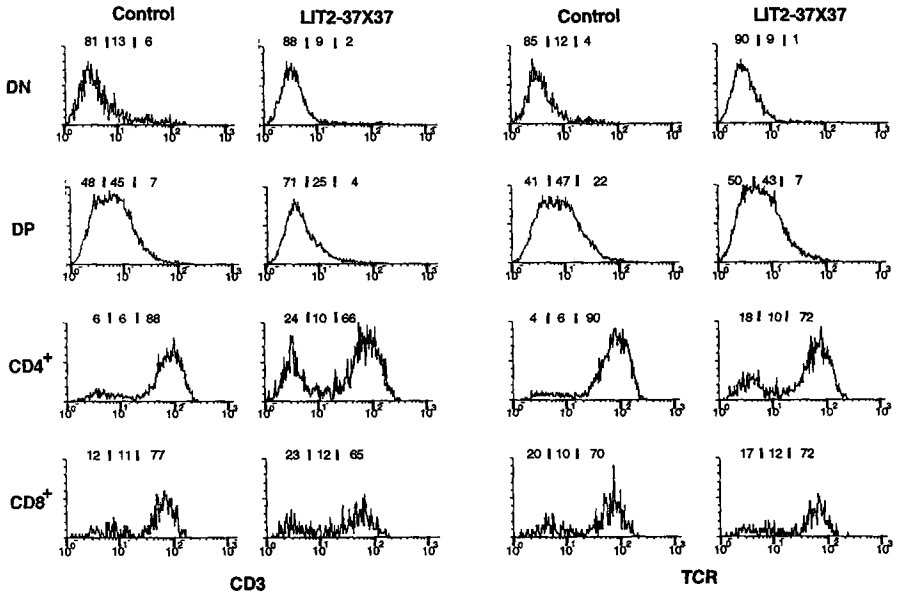

Figure 6. Expression of CD3 and $\alpha\beta$-TCR on thymocytes of normal and HSA transgenic mice. Total thymocytes from a 9-wk-old LIT2-37×37 homozygous transgenic mouse and its nontransgenic littermate (*Control*) were stained with PE-conjugated CD4, FITC-conjugated CD3, or anti-$\alpha\beta$-TCR mAb, and biotinylated CD8 mAb followed by allophycocyanin-conjugated streptavidin, and then analyzed by flow cytometry. The numbers above the histograms indicate the percentages of cells expressing nil, low, or high levels of CD3 or α/β-TCR.

in thymocytes, had an almost complete ablation of the DP thymocyte population (LIT2-20, Fig. 4).

Mature T Cell Populations in HSA Transgenic Mice. Peripheral CD8+ T cells in lymph nodes and spleen were reduced about 10-fold in the HSA transgenic versus nontransgenic mice (Fig. 7). This may simply reflect their reduced output from the thymus, as it is in proportion to the depletion of CD8+ SP thymocytes in the transgenic mice. However, the reduction in numbers of CD4+ peripheral T cells was not as dramatic and was proportionately less than the reduction in the numbers of their CD4+ SP thymocyte precursors (Fig. 7). This implies that there was a selective expansion or survival of CD4+ versus CD8+ T cells after their exit from the thymus.

CD4+ and CD8+ SP T cells from lymph nodes of HSA transgenic mice had normal proliferative responses to CD3 stimulation (Table 2), as did SP thymocytes. This result indi-

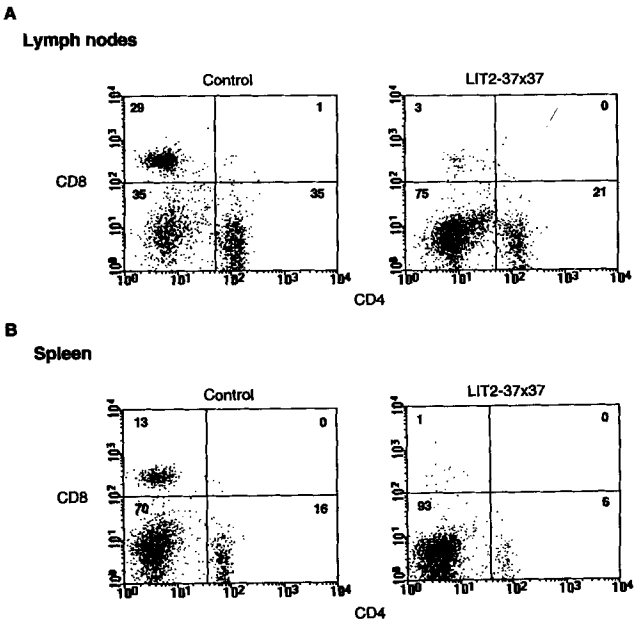

Figure 7. T cell subpopulations in lymph nodes and spleens of normal and HSA transgenic mice. Total axillary and inguinal lymph node cells or spleen cells from a 9-wk-old LIT2-37×37 homozygous transgenic mouse and its nontransgenic littermate (*Control*) were stained with FITC-conjugated CD4 mAb and PE-conjugated CD8 mAb, and analyzed by flow cytometry. Numbers in quadrants indicate the percentage of the total population.

Table 2. *Proliferative Responses of SP Thymocytes and T Cells to CD3 Stimulation*

		Thymidine incorporation	
		Normal	Transgenic
		cpm	
Thymocytes	CD4+	57,121 ± 4,467	29,563 ± 4,033
	CD8+	80,465 ± 1,396	85,533 ± 8,574
Lymph node T cells	CD4+	14,450 ± 2,997	28,121 ± 3,526
	CD8+	99,573 ± 6,910	53,245 ± 4,595

CD4+ and CD8+ SP cells were purified from thymus or axillary and inguinal lymph nodes of LIT2-37 × 37 HSA transgenic mice and normal, nontransgenic littermates by cell sorting, incubated in microtiter plate wells coated with CD3 mAb for 3 d and then pulsed with [^{3}H]thymidine for 6 h. The data are from a single experiment, with standard deviations derived from four separate incubations of each cell sample. Similar proliferative responses were seen in all eight cell populations in three other independent experiments. However, the differences in the amounts of thymidine incorporation between different populations were not consistently seen in separate experiments, and therefore are not significant. In the absence of stimulating CD3 mAb, thymidine incorporation was <200 cpm in all populations.

cates that the limited number of mature T cells developing in the thymuses of the HSA transgenic mice were functionally mature and did not have downstream defects in their abilities to transduce signals received by the TCR–CD3 complex.

Discussion

The introduction of a transgene directing high levels of HSA expression resulted in a severe defect in the production of T cells of the $\alpha\beta$-TCR lineage. Depletion of thymocytes in the HSA transgenic mice was first evident in early DP cells, as all thymocytes before this stage were unaffected and all thymocytes and T cells beyond this stage were depleted. It is possible that all of the depleting effects of the HSA transgene occur at the DP stage, with the reduced numbers of SP thymocytes and peripheral T cells reflecting the previous elimination of most of their DP precursors.

The onset of thymocyte depletion occurs just when the presence of the transgene is starting to override the progressive decline in HSA expression that normally accompanies the maturation of thymocytes. This implies that HSA acts quantitatively in restricting the ability of thymocytes to pass through the DP stage of their development. The human homolog of HSA, CD24 (29), can initiate the transduction of complex intracellular signaling, involving both calcium mobilization and tyrosine phosphorylation via the activation of a CD24-associated tyrosine kinase (30, 31). Similar mechanisms of signal transduction can presumably be initiated by HSA in thymocytes, and the escalation of such signaling by HSA overexpression is a probable cause of the depletion of thymocytes in the transgenic mice.

The defect in thymocyte development beyond the DN stage could be due to an inhibitory effect of abnormally high levels of HSA on proliferation. While SP cells and more mature DP cells are not dividing, there is proliferation of at least some immature DP cells (32, 33), and a suppression of their proliferative potential could result in the observed depletion in the numbers of these cells and their progeny in the HSA transgenic mice. However, such an effect would require that DP cells be uniquely sensitive to inhibition by HSA, as most DN cells are dividing despite their high levels of HSA expression (33).

The efficiency of negative selection of thymocytes expressing $\alpha\beta$-TCR specific for self-antigens is a major determinant of the numerical output of thymocytes (34–36), and therefore the perturbation of this process could also explain the effects of HSA overexpression on thymocyte development. Mice expressing TCR transgenes specific for self-antigens have normal numbers of DN cells but greatly reduced numbers of DP and SP cells (34, 37, 38), as a result of elimination of DP cells expressing the transgenic TCR. The timing and scale of thymocyte depletion resulting from negative selection of self-antigen–specific TCR in these transgenic mice are very similar to those seen in the HSA transgenic mice. Overexpression of HSA in DP thymocytes could result in an increased probability of these cells undergoing negative selection, e.g., by escalating a secondary signal that lowers the threshold for apoptosis-inducing signals generated by the TCR–CD3 complex. There was a bias towards lower than normal TCR and CD3 expression levels among the limited numbers of DP and SP thymocytes that were found in the thymuses of the HSA transgenic mice, which would be expected if the threshold for TCR-derived signals mediating negative selection had been generally lowered. An amplifying effect on apoptosis-inducing signals from TCR has recently been described for Thy-1 (39), another glycosyl phosphadtidylinositol-linked protein expressed on thymocytes.

One discrepancy in the correlation between the cell types sensitive to HSA overexpression versus negative selection is that $\alpha\beta$-TCR$^+$ DN thymocytes (or their precursors) appear to be nonresponsive to negative selection in both normal and TCR transgenic mice (40–43), but these thymocytes were depleted in the HSA transgenic mice. $\alpha\beta$-TCR$^+$ DN cells are normally distinguished from most thymocytes by their lack of HSA expression (44, 45), as well as by their apparent ability to evade negative selection. An intriguing possibility is that depletion of these cells in the HSA transgenic mice was due to their acquisition of susceptibility to negative selection as a consequence of overexpression of HSA.

Transgenic mice in which positive selection of thymocytes is eliminated by the expression of a TCR transgene specific for an inappropriate MHC have a phenotype that is quite distinct from that of the HSA transgenic mice, with normal numbers of DP thymocytes but depleted numbers of SP thymocytes expressing the transgenic TCR (34, 37). This difference indicates that overexpression of HSA is not deleting thymocytes by inhibiting positive selection of MHC-specific TCR, which appears to become a significant determinant of thymocyte numbers only in the late DP and SP stages.

There is considerable evidence that HSA may be involved in regulating the antigen-dependent activation and differentiation of B cells. The subpopulation of primary B cells that express high levels of HSA are nonresponsive to proliferative signals provided by direct stimulation of their IgM antigen receptors (46), and are unable to form long-lasting memory B cells after exposure to antigen (8, 9). These HSA$^+$ B cells are capable of activation and differentiation into antibody-secreting cells with appropriate T cell help (46), which may be partially mediated by the presumptive HSA ligand/receptor expressed on Th cells. The responses of thymocytes to stimulation of their TCR antigen receptors may be similarly dependent on or modulated by HSA-derived signals, with consequent effects on their expansion versus deletion at the DP stage. It will be of great interest to determine exactly what processes in thymocyte development are affected by HSA, and to identify the source of the external signals that stimulate HSA on thymocytes.

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