Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide

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Despite accumulating evidence that regulatory T cells play a crucial role in preventing autoimmunity, the processes underlying their generation during immune repertoire formation are unknown. We show here that interactions with a single self-peptide can induce thymocytes that bear an autoreactive T cell receptor (TCR) to undergo selection to become CD4+CD25+ regulatory T cells. Selection of CD4+CD25+ thymocytes appears to require a TCR with high affinity for a self peptide because thymocytes that bear TCRs with low affinity do not undergo selection into this pathway. Our findings indicate that specificity for self-peptides directs the selection of CD4+CD25+ regulatory thymocytes by a process that is distinct from positive selection and deletion.

The ability of peripheral T cells to respond to foreign antigens while remaining unresponsive to the host's own tissues is determined largely by thymic selection processes. Positive selection rescues thymocytes from death by neglect based on the ability of their T cell receptors (TCRs) to interact with host major histocompatibility complex (MHC) molecules[1]. Conversely, negative selection contributes to T cell tolerance by eliminating autoreactive thymocytes in a process termed clonal deletion[1]. For αβ T cells, there is strong evidence that TCR specificity for self-peptide(s) presented by host MHC molecules can be crucial in determining whether a thymocyte is positively or negatively selected. In studies with TCR transgenic mice for which the nominal specificity of the mature T cell is known, peptides promote positive selection. These peptides include those that bear limited sequence similarity and reactivity compared to the TCR's cognate peptide[2–5] and others that antagonize the reactivity of mature T cells toward their cognate peptide[6,7]. In addition, very low doses of the cognate peptide itself can promote positive selection[8,9]. All these peptides share the ability to facilitate low avidity interactions between the TCR and host MHC molecules. In similar studies, peptides that contribute to high avidity interactions with host MHC molecules, including high concentrations of the cognate peptide or variant peptides that mature T cells recognize either as full or partial agonists, can induce thymocyte deletion[5,8–10].

The thymus also generates regulatory T cells that play a crucial role in maintaining self-tolerance[11]. For example, $CD4^+CD25^+$ T cells (which comprise ~5–10% of the peripheral $CD4^+$ T cells in nontransgenic mice) are generated intrathymically and can prevent a variety of $CD4^+$ T cell–mediated autoimmune diseases[11–13]. Because $CD4^+CD25^+$ T cells require TCR stimulation to mediate their suppressive activities *in vitro* and can regulate organ-specific autoimmune diseases *in vivo*, it is likely that these regulatory T cells are specific for peripheral self-peptides[13,14]. However, a lack of experimental systems in which the specificity of the regulatory T cell is known has precluded an understanding of the ligands and processes that direct their development.

We have described a transgenic mouse system in which self-peptide–specific $CD4^+$ T cells are regulated by $CD4^+CD25^+$ regulatory T cells[15]. When TCR transgenic (TS1) mice that bear T cells specific for the major $I\text{-}E^d$ determinant (S1) of influenza hemagglutinin (HA) were crossed to a lineage of HA transgenic (HA28) mice, the S1-specific T cells were not deleted. Instead, a large proportion of the S1-specific $CD4^+$ T cells were $CD25^+$ and functioned as regulatory T cells. The studies herein characterize the role of the S1 neoself-peptide in the thymic selection of these regulatory T cells and demonstrate that specificity for a self-peptide directs $CD4^+CD25^+$ T cell development.

Results

CD25+ regulatory T cells develop intrathymically

We first compared $CD4^+$ single positive (SP) thymocytes and peripheral lymph node (LN) $CD4^+$ T cells from TS1 and TS1×HA28 mice for CD25 expression by flow cytometry. Transgenic S1-specific T cells were identified with the clonotypic antibody 6.5[16]. As described, $6.5^{Hi}CD4^+$ T cells were abundant in the LNs of TS1×HA28 mice, although 6.5 expression was approximately 50% lower compared to their TS1 counterparts (**Fig. 1a**)[15]. In addition, approximately 50% of the $6.5^{Hi}CD4^+$ LN cells from TS1×HA28 mice were $CD25^+$, compared to 13% of their TS1 counterparts. $CD4^+$ SP thymocytes closely resembled the $CD4^+$ LN cells from TS1×HA28 mice: $6.5^{Hi}CD4^+$ SP thymocytes were abundant, 6.5 staining was approximately 50% lower compared to TS1 $CD4^+$ SP thymocytes and approximately 30% were $CD25^+$.

We next examined the frequencies of 6.5^{Hi} $CD4^+CD25^+$ and $CD25^-$ cells in TS1×HA28 mice at different stages of ontogeny. Although less abundant, $6.5^{Hi}CD4^+CD25^+$ cells arose with similar kinetics to their $CD25^-$ counterparts in 2- and 7-day-old mice (**Fig. 1b**). In 2-day-old TS1×HA28 mice, 12% of the $6.5^{Hi}CD4^+$ SP thymocytes were $CD25^+$,

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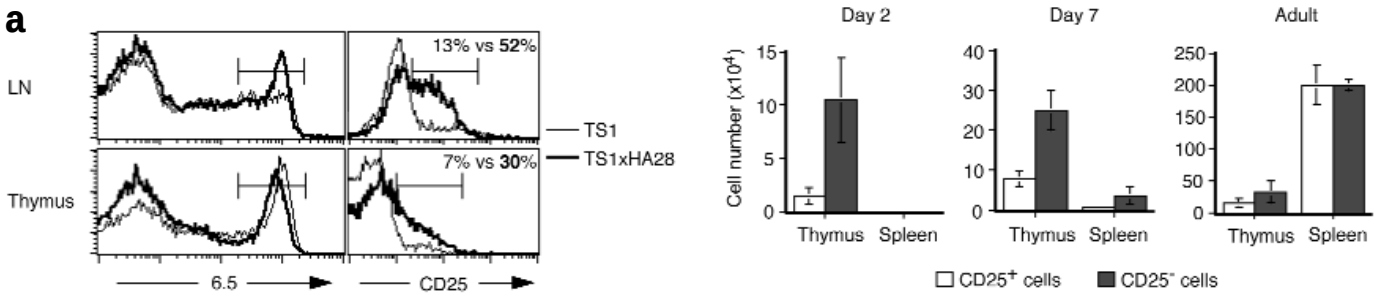

Figure 1. CD4⁺CD25⁺ S1–specific T cells are generated intrathymically. (**a**) CD25⁺ thymocyte development in TS1×HA28 mice. Flow cytometry was used to determine 6.5 expression on CD4⁺ LN cells and CD4⁺ SP thymocytes from TS1 and TS1×HA28 mice. The 6.5^{Hi} cells (indicated by horizontal lines) were then analyzed for CD25 expression. The percentages of 6.5^{Hi} CD4⁺CD25⁻ and CD25⁺ cells in TS1 *versus* TS1×HA28 mice are shown. Data are representative of at least six experiments. (**b**) CD25 expression during ontogeny. Thymi and spleens from 2-day-old, 7-day-old and adult TS1×HA28 mice were collected and cells stained with antibodies to CD4, CD8, CD25 and 6.5. The percentages of CD4⁺ SP, 6.5^{Hi}, CD25⁺ and CD25⁻ thymocytes and splenocytes from five mice per time-point were determined and absolute numbers calculated. Bars indicate mean±s.d..

whereas CD4⁺CD25⁺ splenocytes were undetectable (consistent with previous studies which indicated that 2-day-old mice contain few, if any, peripheral CD4⁺CD25⁺ T cells[17]). Thus, the development of the CD25⁺CD4⁺ SP thymocytes does not require peripheral interactions with a self-peptide and subsequent recirculation to the thymus.

As 6.5^{Hi}CD4⁺ splenocytes became detectable in 7-day-old mice, the relative frequencies of CD25⁺ and CD25⁻ T cells closely paralleled those of CD4⁺ SP thymocytes. In adult mice, approximately equal numbers of 6.5^{Hi}CD4⁺ splenocytes (but not 6.5^{Hi}CD4⁺ SP thymocytes) were CD25⁺ and CD25⁻, which suggested that homeostatic mechanisms might promote peripheral expansion of CD25⁺ T cells. Nevertheless, their close temporal relationship with 6.5^{Hi}CD25⁻CD4⁺ cells during ontogeny provides evidence that 6.5^{Hi}CD25⁺CD4⁺ T cells arise by thymic selection processes.

Radioresistant cells direct CD25⁺ T cell development

We examined the thymic elements responsible for inducing the development of CD4⁺CD25⁺ regulatory T cells by generating radiation bone marrow (BM) chimeras. The 6.5^{Hi}CD4⁺ LN cells from HA28 mice reconstituted with TS1 BM (TS1→HA28 chimeras) expressed decreased amounts of 6.5 and approximately half the 6.5^{Hi}CD4⁺ LN cells from these chimeras were CD25⁺ (**Fig. 2a**). Conversely, the cell surface phenotypes of TS1×HA28→BALB/c and TS1→BALB/c chimeras were indistinguishable. These findings show that radioresistant thymic elements are both necessary and sufficient for the selection of CD4⁺CD25⁺ T cells in TS1×HA28 mice.

Because radioresistant elements were responsible for the development of CD4⁺CD25⁺ T cells in TS1×HA28 mice, we repeated the TS1→HA28 BM chimera studies with the use of BM cells from TS1 mice that were

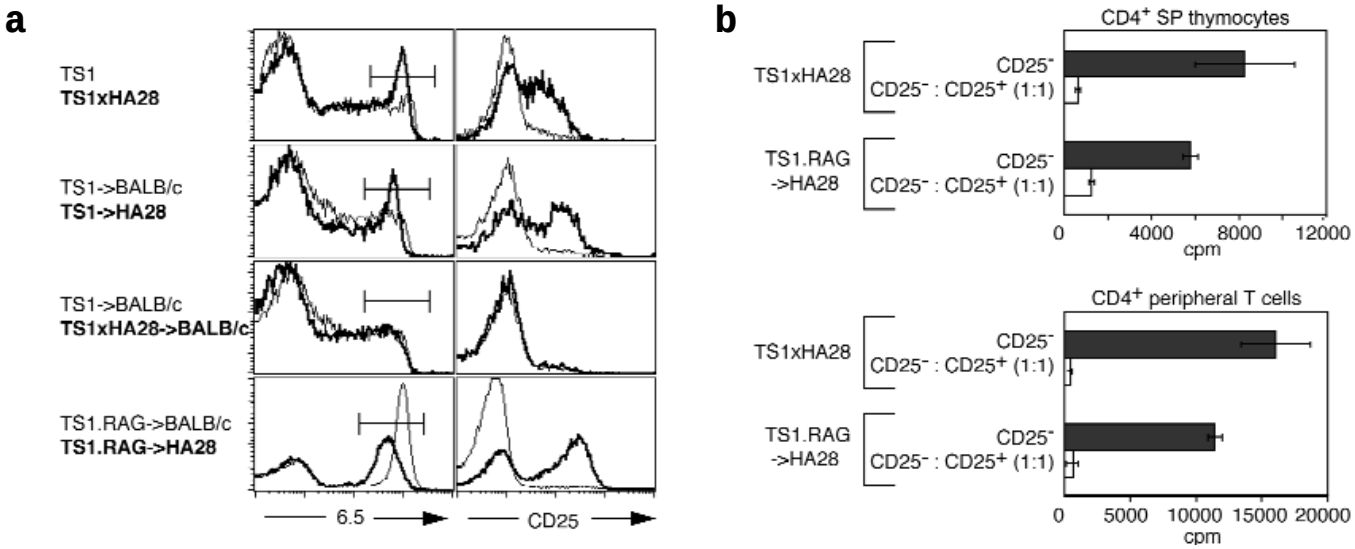

Figure 2. CD4⁺CD25⁺ regulatory T cells in TS1×HA28 mice are selected by radioresistant thymic elements and are not dependent upon allelic inclusion of endogenous TCR chains. (**a**) CD25⁺ T cell development in BM chimeras. LN cells from TS1, TS1×HA28 or irradiated BALB/c and HA28 hosts that had been reconstituted with TS1, TS1×HA28 or TS1.RAG$^{-/-}$ BM cells were stained with anti-CD4, anti-CD25 and 6.5. Expression of 6.5 on CD4⁺ LN cells is shown. (TS1, TS1→BALB/c and TS1.RAG$^{-/-}$→BALB/c profiles, thin lines; TS1×HA28, TS1→HA28, TS1×HA28→BALB/c and TS1.RAG$^{-/-}$→HA28 profiles, thick lines.) The 6.5^{Hi} cells (indicated by horizontal lines) were then analyzed for CD25 expression. Note that the CD4⁺6.5⁻ LN cells in TS1.RAG$^{-/-}$→BALB/c and TS1.RAG$^{-/-}$→HA28 chimeras do not express the transgenic V_β8.2 chain and, therefore, are of host origin (data not shown). For each treatment, at least four mice were analyzed. (**b**) CD4⁺ SP CD25⁺ cells from TS1×HA28 and TS1.RAG$^{-/-}$→HA28 mice suppress CD4⁺CD25⁻ T cell responses. CD4⁺ SP CD25⁻ thymocytes and CD4⁺CD25⁻ peripheral cells were purified by flow cytometry and stimulated with S1 peptide either alone or mixed 1:1 with CD4⁺ SP CD25⁺ thymocytes or peripheral cells derived from the same initial cell populations. Proliferation was measured by ^{3}[H]thymidine incorporation. Mean±s.d. of duplicate or triplicate wells are shown and are representative of two to four independent experiments.

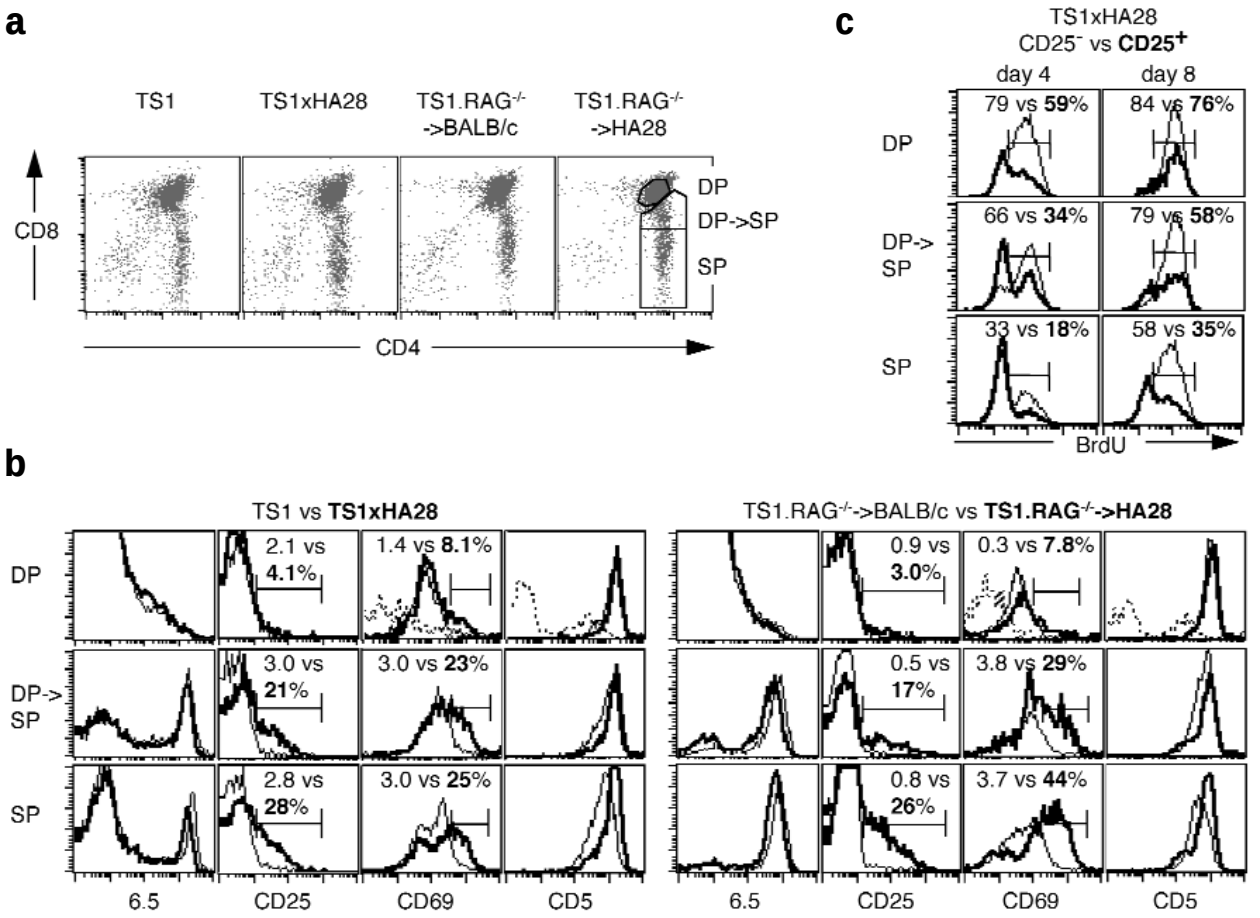

Figure 3. Phenotypic changes accompanying CD4⁺CD25⁺ regulatory thymocyte development. (**a**) CD4 *versus* CD8 profiles show the distribution of thymocytes in TS1, TS1×HA28, TS1.RAG$^{-/-}$→BALB/c and TS1.RAG$^{-/-}$→HA28 mice. Mice had an average thymic cellularity of 90×10^6, 68×10^6, 23×10^6 and 29×10^6, respectively. The regions that contain thymocytes at different stages of maturation are indicated. (**b**) Thymocytes acquire a cell surface phenotype characteristic of CD4⁺CD25⁺ regulatory T cells at the CD4⁺ SP stage. Thymocytes were stained with antibodies to CD4, CD8, 6.5 and either CD25, CD69 or CD5. Histograms show the amounts of 6.5 (on all thymocytes) or of CD25, CD69 and CD5 (on 6.5^{Hi} thymocytes) present in each region. (Left: TS1, thin lines; TS1×HA28 thick lines. Right: TS1.RAG$^{-/-}$→BALB/c, thin lines; TS1.RAG$^{-/-}$→HA28, thick lines. Both: CD69 and CD5 expression on DN cells from TS1 mice, dashed lines.) The percentages of 6.5^{Hi}CD4⁺CD69^{Hi} or CD25⁺ cells in TS1 *versus* TS1×HA28 mice are shown. (**c**) BrdU labeling of CD25⁺ thymocytes at the DP, DP→SP and SP stages of development. TS1 and TS1×HA28 mice were injected with BrdU for 4 or 8 days. Thymocytes, LN cells and splenocytes were collected and stained with anti-CD4, anti-CD8, anti-CD25, anti-BrdU and 6.5. BrdU incorporation in 6.5^{Hi}CD25⁻ or CD25⁺ populations is shown for DP, DP→SP and CD4⁺ SP thymocytes gated as in **a**. The percentages of BrdU⁺ cells in CD25⁻ *versus* CD25⁺ populations are shown. Data are representative of three to seven mice in at least three independent experiments.

rendered incapable of allelic inclusion by mating onto a recombination activating gene 2 (RAG-2)–deficient background (TS1.RAG$^{-/-}$ mice). We carried out these studies because allelic inclusion allows a large fraction of 6.5⁺ T cells to express more than one TCRα chain[16,18] and the coexpression of endogenous α chains could potentially be involved in the selection of CD4⁺CD25⁺ thymocytes in TS1×HA28 mice. In comparison to their TS1.RAG$^{-/-}$→BALB/c counterparts, CD4⁺ T cells from TS1.RAG$^{-/-}$→HA28 chimeras expressed lower 6.5 and approximately half of the 6.5^{Hi}CD4⁺ T cells were CD25⁺ (**Fig. 2a**).

To confirm that the CD25⁺ cells that arose in the TS1.RAG$^{-/-}$→HA28 chimeras were regulatory, we used cell sorting to fractionate 6.5^{Hi}CD4⁺ SP thymocytes and 6.5^{Hi}CD4⁺ peripheral cells from either TS1×HA28 or TS1.RAG$^{-/-}$→HA28 mice into CD25⁻ and CD25⁺ subsets. The ability of purified CD25⁻ cells to proliferate in response to S1 peptide in the presence or absence of equal numbers of purified CD25⁺ cells was then examined. In all cases, addition of CD25⁺ cells substantially inhibited proliferation of the purified CD25⁻ cells (**Fig. 2b**). Together, these data indicate that thymocytes that can only express the 6.5 TCR undergo selection to become CD4⁺CD25⁺ regulatory T cells.

Phenotypic changes with CD25⁺ thymocyte development

We next examined the selection processes that give rise to the development of CD4⁺CD25⁺ thymocytes. With the use of flow cytometric analysis, thymocytes were fractionated into regions corresponding to distinct stages of thymocyte development: the CD4⁺CD8⁺ double positive (DP) stage, the DP→ CD4⁺ SP transition and the CD4⁺ SP stage (**Fig. 3a**). We initially compared the expression of 6.5 and CD25 between these different populations in thymocytes from TS1 and TS1×HA28 mice and in thymocytes from TS1.RAG$^{-/-}$→BALB/c and TS1.RAG$^{-/-}$→HA28 BM chimeras. We found that 6.5 and CD3 (data not shown) were expressed in equivalent amounts on 6.5^{Hi} cells at the DP stage but were expressed in lower amounts on the CD4⁺ SP cells from TS1×HA28 mice and TS1.RAG$^{-/-}$→HA28 BM chimeras (**Fig. 3b**). For CD25, a slightly higher percentage of 6.5^{Hi} cells from TS1×HA28 mice and

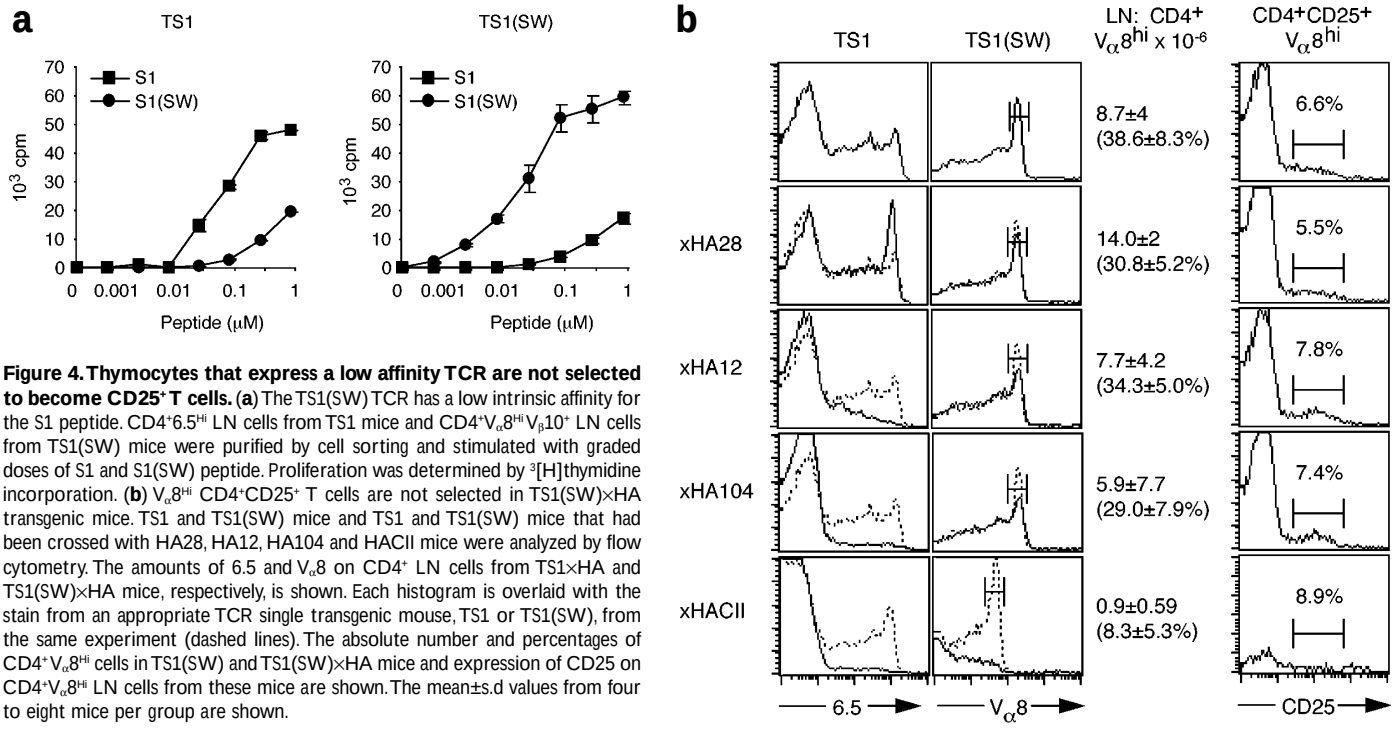

Figure 4. Thymocytes that express a low affinity TCR are not selected to become CD25⁺ T cells. (**a**) The TS1(SW) TCR has a low intrinsic affinity for the S1 peptide. CD4⁺6.5^{Hi} LN cells from TS1 mice and CD4⁺V$_\alpha$8^{Hi}V$_\beta$10⁺ LN cells from TS1(SW) mice were purified by cell sorting and stimulated with graded doses of S1 and S1(SW) peptide. Proliferation was determined by ^{3}[H]thymidine incorporation. (**b**) V$_\alpha$8^{Hi} CD4⁺CD25⁺ T cells are not selected in TS1(SW)×HA transgenic mice. TS1 and TS1(SW) mice and TS1 and TS1(SW) mice that had been crossed with HA28, HA12, HA104 and HACII mice were analyzed by flow cytometry. The amounts of 6.5 and V$_\alpha$8 on CD4⁺ LN cells from TS1×HA and TS1(SW)×HA mice, respectively, is shown. Each histogram is overlaid with the stain from an appropriate TCR single transgenic mouse, TS1 or TS1(SW), from the same experiment (dashed lines). The absolute number and percentages of CD4⁺V$_\alpha$8^{Hi} cells in TS1(SW) and TS1(SW)×HA mice and expression of CD25 on CD4⁺V$_\alpha$8^{Hi} LN cells from these mice are shown. The mean±s.d values from four to eight mice per group are shown.

TS1.RAG$^{-/-}$→HA28 BM chimeras were CD25⁺ at the DP stage compared to TS1 and TS1.RAG$^{-/-}$ counterparts. However, as cells matured to the SP stage, the frequency of 6.5^{Hi} CD25⁺ cells increased approximately sevenfold. Thus, cell surface changes that are characteristic of 6.5^{Hi}CD4⁺ T cells in TS1×HA28 mice and TS1.RAG$^{-/-}$→HA28 BM chimeras are acquired at relatively late stages of thymocyte development and are fully manifested at the mature CD4⁺ SP stage.

We also examined cell surface molecules that are typically associated with T cell activation and thymic selection events. Up-regulation of CD69 and CD5 at the DP stage has been associated with positive selection of developing thymocytes[19,20]. Some studies have found that these molecules are up-regulated in thymocytes undergoing deletion, with higher expression correlating with higher affinity or avidity interactions with self-MHC complexes[21,22]. By comparing the relative expression of CD69 and CD5 in TS1 or TS1.RAG$^{-/-}$→BALB/c thymocytes (where the 6.5 TCR is positively selected) with TS1×HA28 or TS1.RAG$^{-/-}$→HA28 thymocytes, we aimed to identify phenotypic changes accompanying the selection of CD25⁺ thymocytes.

CD69 and CD5 were more highly expressed on 6.5^{Hi} DP cells compared to DN cells in all mice examined, which reflects early events associated with positive selection of the 6.5 TCR (**Fig. 3b**). As was observed for CD25, a slightly higher percentage of 6.5^{Hi} cells from TS1×HA28 mice and TS1.RAG$^{-/-}$→HA28 BM chimeras were CD69^{Hi} at the DP stage and, as cells matured to the SP stage, the frequency of 6.5^{Hi}CD69^{Hi} cells increased (**Fig. 3b**). CD5 was also expressed at higher amounts on TS1×HA28 and TS1.RAG$^{-/-}$→HA28 thymocytes, although the differences did not become fully evident until the CD4⁺ SP stage (**Fig. 3b**). Thus, selection of 6.5^{Hi}CD25⁺ thymocytes in TS1×HA28 mice is accompanied by relatively late increases in the expression of cell surface markers associated with thymic selection in the presence of a high affinity ligand. In addition, the expression of these markers at different stages of development is similar among thymocytes that express the 6.5 TCR in the presence or absence of endogenous TCR rearrangements.

To further characterize the selection events associated with the generation of CD25⁺CD4⁺ SP thymocytes, we injected TS1×HA28 mice with 5-bromodeoxyuridine (BrdU) every 12 h for either 4 or 8 days and examined its incorporation by 6.5^{Hi}CD25⁺ and 6.5^{Hi}CD25⁻ thymocytes (**Fig. 3c**). The CD25⁺ thymocytes incorporated BrdU more slowly than their CD25⁻ counterparts. This was most apparent for the DP→SP and SP thymocytes after 4 days: approximately half as many CD25⁺ as CD25⁻ thymocytes were BrdU⁺. The percentage of BrdU⁺CD25⁺ cells increased after 8 days of labeling, although the percentage of BrdU⁺CD25⁻ cells also increased such that the CD25⁻ cells still contained a higher percentage of BrdU⁺ cells. Because CD25⁺ cells at the DP→SP transition (which are CD4⁺CD8^{Lo} and do not have a peripheral T cell counterpart) became BrdU⁺ before much labeling was detected among peripheral CD4⁺ T cells (<5% BrdU⁺CD4⁺CD25⁺ cells on day 8; data not shown), these data further indicate that the CD4⁺CD25⁺ thymocytes are not mature recirculating CD4⁺ T cells that encountered antigen in the periphery. In addition, as suggested by their appearance during ontogeny (**Fig. 1b**), these labeling studies provide evidence that CD25⁺CD4⁺ thymocytes arise by a similar pathway but are generated more slowly than their CD25⁻ counterparts.

Low affinity precludes CD25⁺ T cell development

The reactivity of the 6.5 TCR with a variety of analog peptides containing amino acid substitutions in the S1 determinant has been characterized[23]. One such analog peptide occurs naturally in the influenza virus strain A/SW/33 (SW) and contains two amino acid substitutions relative to the native S1 determinant. CD4⁺ LN cells from TS1 mice are 20-fold more sensitive to the S1 determinant, their index peptide, than to S1 (SW) (**Fig. 4a**). We generated a second TCR transgenic mouse—TS1(SW)—whose T cells are specific for S1(SW) and use the V$_\alpha$8.3-V$_\beta$10 variable region genes from a T cell hybridoma derived from a SW-immunized mouse[24]. CD4⁺ LN cells from TS1 and TS1(SW) mice displayed similar sensitivities to the S1 and S1(SW) determinants, respectively, differing

threefold in the amount of peptide required for half-maximal stimulation. However, the TS1(SW) T cells were 100-fold more sensitive to the S1(SW) determinant than to S1, which indicated that the intrinsic affinity of the 6.5 TCR for the S1 determinant is of the order of ~100-fold higher than that of the TS1(SW) TCR.

To examine whether T cells with a low intrinsic affinity for the S1 determinant develop into $CD25^+$ cells in response to the S1 peptide in HA28 mice, we compared the frequencies of $CD25^+$ expression by LN cells from TS1(SW) and TS1(SW)×HA28 mice (**Fig. 4b**). TS1(SW)-specific T cells were detected based on $V_\alpha8$ expression because $V_\beta10$ is found on >99% of TS1(SW) T cells (data not shown). Both the absolute number and the percentage of $CD25^+$ cells among $V_\alpha8^{Hi}$ $CD4^+$ LN cells in TS1(SW)×HA28 mice were similar to those found in TS1(SW) mice (**Fig. 4b**). This contrasts with TS1×HA28 mice in which approximately half the $6.5^{Hi}CD4^+$ T cells were $CD25^+$ (**Fig. 1a**). Thus, thymocytes bearing a TCR with a low intrinsic affinity for the S1 peptide did not develop into $CD4^+CD25^+$ thymocytes in response to HA in TS1(SW)×HA28 mice.

We next examined whether TS1(SW) T cells might develop into $CD4^+CD25^+$ T cells in mice in which S1 mediates deletion of the 6.5 TCR. We have described two lineages of HA transgenic mice (HA12 and HA104) in which 6.5^+ thymocytes are deleted to graded degrees[18]. TS1(SW) mice were crossed with HA12 and HA104 mice and also with transgenic mice (HACII) that express the PR8 HA under the control of a MHC class II (I-Eα) promoter[25] (**Fig. 4b**). Whereas 6.5^+CD4^+ T cells were substantially deleted in TS1×HA12 mice, there was little or no reduction in the frequency of $V_\alpha8^{Hi}$ $CD4^+$ T cells in TS1(SW)× HA12 mice. There was a modest decrease in the number of $V_\alpha8^{Hi}$ $CD4^+$ T cells in TS1(SW)×HA104 mice that correlated with the increased deletion of 6.5^+ T cells in TS1×HA104 mice, and both TS1×HACII and TS1(SW)×HACII mice exhibited profound deletion of the respective clonotype-bearing T cells. In addition, similar percentages of $V_\alpha8^{Hi}$ $CD4^+CD25^+$ T cells were present in the LNs of TS1(SW) and TS1(SW)×HA transgenic mice. Thus, even when they develop in the presence of the S1 self-peptide under conditions that can to varying degrees induce deletion of the 6.5 TCR, thymocytes that bear the low affinity TS1(SW) TCR do not undergo selection into $CD25^+$ T cells. Under some circumstances they can be deleted but their low affinity for S1 appears to preclude their development into $CD4^+CD25^+$ T cells in response to S1 peptide.

Discussion

In TS1 mice, thymocytes that bear the 6.5 TCR are positively selected through interactions with MHC class II molecules expressing either a particular self-peptide or a collection of self-peptides[26]. In TS1×HA28 mice, an additional self-peptide (S1) substantially altered the development of thymocytes that bear the 6.5 TCR, which leads to the development of S1-specific $CD4^+CD25^+$ T cells. It is worth noting that the S1 peptide is an agonist ligand for the 6.5 TCR[27] and that thymocytes and T cells in TS1×HA28 mice that express low amounts of the 6.5 TCR along with high amounts of endogenous α chains (and which therefore have lower avidities for the S1 peptide) are not $CD25^+$[15] (and data not shown). In addition, thymocytes that bear the TS1(SW) TCR—whose intrinsic affinity for the S1 peptide is 1% that of the 6.5 TCR—did not develop into $CD25^+$ T cells, even in mice in which the S1 peptide induced deletion of the 6.5 TCR. Although it is possible that the failure of thymocytes bearing the TS1(SW) TCR to undergo selection to become $CD25^+$ is in some way related to the timing of expression of this transgenic TCR, these data suggest that selection of $6.5^+CD4^+CD25^+$ thymocytes in TS1×HA28 mice depends on the high affinity of the 6.5 TCR for S1 peptide.

Inasmuch as interactions with a defined ligand (the S1 peptide) lead to the export of thymocytes that exhibit unique phenotypic and functional characteristics, the generation of $CD25^+$ T cells in TS1×HA28 mice can be viewed as a thymic selection process that has characteristics of, and yet is distinct from, positive selection. Like positive selection, CD69 and CD5 were up-regulated on 6.5^{Hi} cells from TS1 and TS1×HA28 mice, and expression of these molecules on 6.5^{Hi} cells in TS1×HA28 mice was elevated compared to expression in TS1 mice. This is consistent with selection *via* a high affinity or avidity self-peptide[21,22]. However, unlike positive selection or other previously described thymic selection events, generation of regulatory cells was associated with CD25 expression.

It is possible that S1 peptide is expressed by cortical epithelial cells in an amount that provides an appropriate combination of high specificity and low overall avidity that alters positive selection and leads to the generation of $CD25^+$ thymocytes. Indeed, low doses of an agonist peptide were previously found to promote selection of $TCR^{Hi}CD8^+$ thymocytes that were unable to proliferate in response to their agonist ligand, resembling the anergic phenotype of $CD4^+CD25^+$ T cells[28,29]. Expression in cortical epithelial cells has also been shown to promote positive selection of self-reactive T cells in the absence of much deletion, as occurs in TS1×HA28 mice[30]. However, the data here are also consistent with thymocytes that express the 6.5 TCR undergoing equivalent positive selection events in BALB/c and HA28 mice while continuing interactions with the S1 peptide presented by "tolerizing" antigen-presenting cells in HA28 mice induce the development of $CD25^+$ regulatory thymocytes. There is evidence in other systems that medullary antigen-presenting cells can induce anergy in developing thymocytes[31].

It is also not yet clear why 6.5^{Hi} thymocytes are exported as a mixture of $CD25^+$ and $CD25^-$ cells in TS1×HA28 mice. The fact that $TS1.RAG^{-/-}\rightarrow$HA28 mice still present a 50:50 mixture of $6.5^{Hi}CD25^-$ and $CD25^+$ cells indicates that $6.5^{Hi}CD25^-$ cells are not excluded from $CD25^+$ T cell development because of endogenous α chain usage. Perhaps $CD25^+$ cells themselves block the development of additional $CD25^+$ thymocytes by interfering with signals that are requisite for their development, in a manner that is similar to their ability to suppress the proliferation of coexisting T cells. Alternatively, the use of a TCR transgenic mouse may have overwhelmed niches that mediate selection into the CD25 pathway, although studies in nontransgenic systems have provided evidence to support the hypothesis that the thymus naturally exports mixtures of antigen-reactive and suppressor T cell populations with shared specificities[32,33].

Our findings show that, as a means of regulating thymocytes bearing a TCR with a high intrinsic affinity for a self-peptide, the development into $CD4^+CD25^+$ regulatory T cells is an alternative to deletion. Because high avidity interactions with self-peptides have been found, in several systems, to be associated with thymocyte deletion, it has previously been suggested that $CD4^+CD25^+$ T cells might be selected based on low affinity (or low avidity) interactions with self-peptides[11,34]. However, selecting regulatory T cells based on their low affinity for a self-peptide(s) could lead to peripheral T cells with limited specificity for the selecting peptide(s). Indeed, positive selection of thymocytes is promoted by interactions with low affinity ligands and provides an effective repertoire for responses to foreign antigens by allowing thymocytes with diverse specificities to be exported to the periphery. Because selection of $CD4^+CD25^+$ thymocytes appears to depend on a high TCR affinity for the selecting peptide, this process instead promotes the export of regulatory T cells whose TCRs have the potential to be highly reactive to and specific for self-peptides that

might be encountered in the periphery. It will be important in future studies to understand how these selection processes are perturbed, or can be manipulated, in autoimmunity.

Methods

Mice. HA12 and HA28 mice (which contain DNA encoding the NH_2-terminal 237 amino acids of the PR8 HA linked to the SV40 early region promoter-enhancer sequences) and HA104 mice (which express full-length HA under the same promoter) have been described[35–37]. HACII mice express full-length HA under the control of the I-Eα promoter and express high amounts of HA on MHC class II$^+$ cells[25] (A. J. Reed *et al*., unpublished data). TS1 mice express a TCR that is specific for the PR8 HA I-E^d–restricted determinant site 1[16]. TS1(SW) mice were generated by introducing the rearranged V_α8.3-V_β10 genes from the S1(SW)-specific hybridoma—S106-1—into the TCR expression vectors pTαcass and pTβcass[24,36,38]. HACII and TS1(SW) transgenic mice were generated by J. Richa at the University of Pennsylvania Transgenic Mouse Facility by injecting C57BL/6×BALB/c zygotes and founder mice were backcrossed for at least three generations to BALB/c mice before use. HA12, HA28, HA104 and TS1 mice were backcrossed to BALB/c mice for at least ten generations before use in this study. All mice were maintained in sterile microisolators at the Wistar Institute Animal Facility and were between 6 and 16 weeks of age when analyzed.

Flow cytometry. Thymocytes and LN cells were analyzed by three-, four- or five-color flow cytometry on a FACScan, FACSCalibur or FACStar^{Plus}, respectively (Becton Dickinson, San Jose, CA). Data analysis was carried out with the computer program CELLQuest (Becton Dickinson) and the number of events collected per sample was between 50,000 and 500,000. Purified subpopulations were sorted by an EPICS Elite flow cytometer (Coulter Corporation, Miami, FL) and were typically of ≥95% purity. The following antibodies were used for analysis: fluorescein isothiocyanate (FITC)–anti-CD4 (Gibco-BRL, Gaithersburg, MD), phycoerythrin (PE)–anti-CD4 (Gibco-BRL), allophycocyanin–anti-CD4 (PharMingen, San Diego, CA), PE-Texas Red–anti-CD4 (Caltag, Burlingame, CA), PE–anti-CD5 (PharMingen), PE–anti-CD8 (Gibco-BRL), anti-CD8-allophycocyanin-cy7 (Caltag), FITC–anti-CD25 (PC61, gift of S. Carding), PE–anti-CD25 (3C7, PharMingen), PE–anti-CD69 (PharMingen), FITC–anti-V_β8 (Caltag) and biotin-6.5[16]. Streptavidin-Red670 (Gibco-BRL) or allophycocyanin-streptavidin (PharMingen) was used to detect biotinylated reagents.

Bone marrow chimeras. HA28 and BALB/c mice, 5–7 weeks of age, were irradiated with 900 rad of γ-irradiation. Donor BM was stained with biotinylated antibodies to CD4 and CD8 followed by streptavidin conjugated to magnetic beads and subjected to MACS depletion according to manufacturer's protocol (Miltenyi Biotec, Auburn, CA). T cell–depleted BM was used to reconstitute hosts 24 h after irradiation. Neomycin (2 mg/ml) and polymixin B (100 U/ml, Schein, Florham Park, NJ) were given to mice on the day of irradiation and for 2 weeks after irradiation. Mice were analyzed 2–4 months after BM reconstitution.

BrdU labeling studies. Mice were injected intraperitoneally with 200 μl of BrdU (3 mg/ml, Sigma, St. Louis, MO) every 12 h for 4 or 8 days. BrdU incorporation was detected by intracellular staining. Cells were surface stained with anti-CD4, anti-CD8, anti-CD25 and 6.5 and then fixed for 20 min in cytofix (PharMingen). Fixed cells were incubated in DNaseI solution (100 U/ml of DNaseI, 0.15 M NaCl, 4.2 mM $MgCl_2$, 100 μM HCl) for 30 min at room temperature. Cells were then stained with FITC–anti-BrdU (Becton Dickinson, San Jose, CA) in cytoperm solution (PharMingen).

Proliferation analysis. CD4$^+$6.5^{Hi} and CD4$^+$$V_\beta$10$^+$ V_α8^{Hi} LN cells (3.5×10^4) from TS1 and TS1(SW) mice, respectively, were purified by FACS and cultured with irradiated BALB/c splenocytes (5×10^5) and graded doses of S1 (SFERFEIFPKE) or S1(SW) (SFEKFEIFPKT) peptides (synthesized and HPLC purified by the Wistar Institute peptide synthesis facility). Cells were cultured in supplemented IMDM that contained 10% fetal bovine serum in 96-well flat-bottomed plates. Cultures were pulsed with 0.5 μCi per well of ^{3}[H]thymidine 72 h after activation and collected 16 h later. Similarly, CD4$^+$ SP CD25$^-$ and CD4$^+$ SP CD25$^+$ thymocytes (3.5×10^4) and CD4$^+$CD25$^+$ LN and spleen cells ($3.0–4.5\times10^4$) from TS1×HA28 and TS1.RAG$^{-/-}$ →HA28 mice were isolated by cell sorting and stimulated with 3 μM (thymocytes) or 0.3 μM (LN and spleen) S1 peptide and cultured as above.

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