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# On the Self-Referential Nature of Naive MHC Class II-Restricted T Cells[1]

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# On the Self-Referential Nature of Naive MHC Class II-Restricted T Cells[1]

**Christophe Viret, Xin He, and Charles A. Janeway, Jr.[2]**

**The use of mutant mice expressing a normal MHC class II molecule surface level but a severely restricted self-peptide diversity (H-2M$\alpha^{-/-}$) previously revealed that T cells carrying the E$\alpha_{52-68}$–I-A$^b$ complex-specific 1H3.1 TCR rely on self-peptide(s) recognition for both their peripheral persistence in irradiated hosts and their intrathymic positive selection. Here, we identify E$\alpha_{52-68}$ structurally related self-peptide(s) as a major contributor to in vivo positive selection of 1H3.1 TCR-transgenic thymocytes in I-A$^{b+}$/I-E$\alpha^-$ mice. This is demonstrated by the drastic and specific reduction of the TCR high thymocyte population in 1H3.1 TCR-transgenic (Tg) mice treated with the E$\alpha_{52-68}$–I-A$^b$ complex-specific Y-A*e* mAb. Self-peptide(s) recognition is also driving the maturation of T cells carrying a distinct MHC class II-restricted specificity (the E$\alpha_6$ $\alpha\beta$ TCR), since positive selection was also deficient in E$\alpha_6$ TCR Tg H-2M$\alpha^{-/-}$ thymi. Such a requirement for recognition of self-determinants was mirrored in the periphery; E$\alpha_6$ TCR Tg naive T cells showed an impaired persistence in both H-2M$\alpha^{-/-}$ and I-A$^b\beta^{-/-}$ irradiated hosts, whereas they persisted and slowly cycled in wild-type recipients. This moderate self-peptide(s)-dependent proliferation was associated with a surface phenotype intermediate between those of naive and activated/memory T cells; CD44 expression was up-regulated, but surface expression of other markers such as CD62L remained unaltered. Collectively, these observations indicate that maturation and maintenance of naive MHC class II-restricted T cells are self-oriented processes.** ***The Journal of Immunology*****, 2000, 165: 6183–6192.**

Apart from an early phase, the intrathymic development of conventional $\alpha\beta$ T cells relies on a TCR-MHC interaction that is allele specific and, depending on its nature, either rescues immature thymocytes from apoptosis and allows them to mature (positive selection) or causes their elimination by precipitating apoptosis (negative selection). This dual selection process concomitantly ensures the generation of a diverse mature TCR repertoire and the establishment of tolerance to most self-determinants expressed on bone marrow-derived cells (1–5). By definition, recognition of self-peptides is central to the process of deletion of autoreactive thymocytes. The question of whether this is also true for positive selection has been a matter of discussion (6–8). Pioneering works with natural mutations affecting MHC class I amino acid residues involved in peptide binding but not in the TCR-MHC interaction suggested that a direct contact between self-peptides and the TCR is required for positive selection of CD8$^+$ T cells (9, 10). This idea was reinforced by in vitro studies based on mouse fetal thymic organ culture; the use of thymic lobes from peptide transporter-deficient (TAP-1$^{-/-}$) and $\beta_2$-microglobulin-deficient ($\beta_2$m$^{-/-}$)[3] mice showed that the loading of MHC class I presentable peptides restores development of CD8$^+$CD4$^-$ thymocytes in a peptide sequence-dependent fashion (11, 12). Similar results were obtained using fetal thymic organ culture experiments conducted with MHC class I-restricted TCR-transgenic mice (13, 14). Naturally presented self-peptides able to drive positive selection of CD8$^+$ T cells were indeed recently identified (15, 16).

Experiments documenting a role for self-peptide recognition during positive selection of MHC class II-restricted T cells are more recent and were mainly conducted in vivo using manipulated mice with deficiency for components of the Ag processing and/or presentation pathways. For instance, mice lacking the $\alpha$ subunit of the peptide exchange factor H-2M (H-2M$\alpha^{-/-}$) (17–19) have a normal expression level of I-A$^b$ MHC class II molecules on their APCs but display a very restricted self-peptide complexity; they dominantly express the invariant chain-derived 81–104 peptide class II-associated invariant chain peptide (CLIP) bound to I-A$^b$ and a very low level of some other endogenous peptides (20). The minimal level of self-complexity was achieved by the generation of mice expressing only one peptide:MHC class II complex (I-Ab-Ep) (21, 22). These two systems revealed that when thymic stromal cells express very few or only one peptide in the context of MHC class II molecules, the maturation of CD4$^+$ T cells is severely impaired; from one-quarter to one-half of the normal CD4$^+$ T cell number are detected in the periphery. In addition, many of the selected CD4$^+$ T cells cannot be assimilated to those of a normal mouse because they strongly react against syngeneic APCs. It was also observed that six distinct CD4$^+$ T cell specificities that can efficiently develop within a normal thymic microenvironment fail to do so when they confront the restricted self-peptide complexity displayed by H-2M$\alpha^{-/-}$ thymic stromal cells (20, 23–25). Distinct experimental systems further demonstrated the self-peptide specificity of positive selection. 1) In D10 TCR $\beta$-chain Tg mice, the frequency of the D10 TCR $\alpha$-chain CDR3 loop, which is a peptide contacting point, is limited in the TCR$^{low}$ thymocyte population, but is dominant in the TCR$^{high}$ population, that is the thymocytes that have been positively selected (26).

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[3] Abbreviations used in this paper: $\beta_2$m, $\beta_2$-microglobulin; RAG, recombinase-activating gene; Tg, transgenic; LKLF, lung Kruppel-like transcription factor.



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2) Mutant mice lacking the cathepsin L proteinase display an incomplete degradation of invariant chain and therefore an altered self-peptide repertoire presented by MHC class II molecules on thymic epithelial cells. In these mice the number of CD4$^+$ T cells is reduced by 60–80% in the thymus and the periphery (27). 3) Mice with 95% of MHC class II molecules bound to a single peptide have a normal number of mature CD4$^+$ T cells, but further reduction of the peptide complexity impairs CD4$^+$ T cell maturation (28). Thus, a large corpus of observations validates the concept that positive selection of immature thymocytes relies on self-peptide–self-MHC complex recognition. Concomitant to these findings, it has been established that a survival signal must be delivered via repeated TCR-MHC interactions and is required for mature (CD3$^{high}$ CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$) peripheral $\alpha\beta$ T cells to persist. Such a requirement was documented using a graft of fetal thymus, transient restoration of thymic MHC class II molecule expression in situ, or adoptive transfer of mature naive T cells into MHC-deficient or MHC-mismatched hosts (25, 29–35) and is likely to involve dendritic cells (36, 37).

The necessity of a TCR-restricting MHC interaction for mature naive T lymphocytes to survive is highly reminiscent of the phenomenon of positive selection of immature $\alpha\beta$ thymocytes. This similarity prompted us to envision a higher order of symmetry; that is, to examine the possibility that T cell maintenance may rely on the recognition of self-peptide:self–MHC complexes (38), as is the case for intrathymic positive selection. We took advantage of the H-2M-deficient mice to address this issue. Besides the fact that the H-2M$\alpha^{-/-}$ thymic microenvironment did not support positive selection of 1H3.1 TCR $\alpha\beta$ Tg thymocytes, we found that persistence of mature naive 1H3.1 TCR Tg CD4$^+$ T cells is impaired in irradiated H-2M$\alpha^{-/-}$ recipients compared with irradiated normal hosts. We also observed that naive CD4$^+$ T cell persistence in irradiated wild-type recipients is associated with a peptide-specific low level of expansion that can be visualized using the cytoplasmic fluorescent dye CFSE (39). We concluded that the peripheral maintenance of mature naive CD4$^+$ T cells in irradiated recipients involves a low level of cell division induced by recognition of self-peptide–self-MHC complexes (25). This idea was further documented for CD4$^+$ T cells (40, 41) and extended to mature CD8$^+$ T cells (42).

In this study, we show that the self-peptide(s) involved in positive selection of the E$\alpha_{52-68}$:I-A$^b$-specific 1H3.1 TCR Tg thymocytes forms peptide–I-A$^b$ complex(es) structurally related to the E$\alpha_{52-68}$–I-A$^b$ complex. We then used a distinct MHC class II-restricted T cell specificity (the E$\alpha_6$ $\alpha\beta$ TCR) to assess the generality of the observations previously made with 1H3.1 TCR Tg mice; that is, the continuous requirement for self-peptide–self-MHC complex recognition for intrathymic positive selection and peripheral persistence in lymphopenic hosts.

## Materials and Methods

### *Animals*

Mice used were housed in the Yale immunobiology facility (New Haven, CT). C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The B6 I-A$^b\beta^{-/-}$ (MHC class II-deficient) (43) mice were purchased from Taconic Farms (Germantown, NY). The 1H3.1 (V$\alpha_1$-J$\alpha_{21}$/V$\beta_6$-D$\beta_{2.1}$-J$\beta_{2.6}$) and E$\alpha$6 (V$\alpha_{23.1}$-J$\alpha_{49}$/V$\beta_6$-D$\beta_{1.1}$-J$\beta_{2.1}$) TCR Tg mice were generated in this laboratory using pT $\alpha$ and $\beta$ cassette vectors (44) and were maintained on a B6 background. The H-2M$\alpha^{-/-}$ mice (B6–129 mixed background) were provided by L. Van Kaer (Howard Hughes Medical Institute, Nashville, TN) (17). The recombinase-activating gene-1 (RAG-1)[3]-deficient mice were a gift from D. Shatz (Howard Hughes Medical Institute, Yale University).

### *Adoptive transfer*

TCR Tg CD4$^+$ T cells from lymph nodes and spleen were prepared as single-cell suspensions and purified using magnetic beads (Bio Mag; Advanced Magnetic, Cambridge, MA) and the Y3JP (mouse IgG2a, anti-I-A$^b$), 14.8 (rat IgG2b, anti-CD45RA/B220), 53-6.72, and 2.43 (both rat IgG2b and anti-CD8) mAbs to remove APCs and CD8$^+$ T cells. Cells were washed three times and resuspended in normal saline. Injections were given i.v. into the retro-orbital plexus of the eye using 5–6 $\times$ 10$^6$ cells/200 $\mu$l/mouse unless otherwise indicated. The 6- to 10-wk-old recipient animals used were sublethally irradiated (600 rad, $^{137}$Cs source; Yale University Cancer Center) unless otherwise indicated. In some experiments purified mature T cells were dye-labeled before transfer using CFSE (Molecular Probes, Eugene, OR). Labeling was performed in normal saline at 10$^7$ cells/ml using 2 $\mu$l of a 5 mM CFSE stock solution for 10 min at 37°C. Cells were washed twice in saline and injected as indicated above. In all transfer experiments the donor and recipient were sex matched.

### *Immunostaining and flow cytometry*

Depending on experiments, spleen and lymph nodes (axillary, lateral axillary, superficial inguinal, and mesenteric) were removed, and cell suspensions were prepared. Splenic RBC were lysed using Tris-buffered ammonium chloride. Fluorescent-labeled mAbs were used for staining. Briefly, 0.2 $\times$ 10$^6$ cells were incubated in microtiter U-bottom plates with saturating concentrations of labeled mAb in 20 $\mu$l for 30 min on ice. Cells were washed twice and analyzed immediately without fixation. The mAbs used were anti-V$\beta$6-FITC (clone RR4-7), anti-C$\beta$-PE (H57-597), anti-CD90.2/Thy-1.2-PE (53-2.1), anti-CD44-PE (IM7), anti-CD62L-FITC (MEL14), anti-CD49d-biotin (R1-2), anti-CD45RB (16A), and anti-B220-PE (RA3-6B2) from PharMingen (San Diego, CA); anti-CD8$\alpha$-PE/FITC (53-6.7) from Life Technologies (Gaithersburg, MD); and anti-CD4-quantum red (H129.19) from Sigma (St. Louis, MO). The Y3JP (mouse IgG2a, anti-I-A$^b$) (45), Y17 (mouse IgG2b, anti-I-E) (46), 25.9.17 (mouse IgG2a, anti-I-A$^b$) (47), Y-A*e* (mouse IgG2b, anti-A$^b$+E$\alpha$) (48), 10.2.16 (mouse IgG2a, anti-I-A$^{k,r,f,s}$) (49), GK1.5 (rat IgG2b, anti-CD4), 53-6.72 and 2.43 (both rat IgG2b, anti-CD8), and 14.8 (rat IgG2b, anti-CD45RA/B220) mAbs were affinity purified in the laboratory using standard procedures. A FACScan flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA) were used to collect and analyze the data. Nonviable cells were excluded using forward and side scatter electronic gating. To estimate the ability of Y-A*e* to bind I-A$^b$ molecules loaded with variants of the E$\alpha_{52-68}$ peptide, C57BL/6 splenocytes were incubated 4 h at 37°C using 50 $\mu$g/ml of peptide in complete media. The cells were washed twice and costained for B220 and Y-A*e* epitope expression immediately. The mean fluorescence intensity of B220$^+$ cells was used for calculation.

### *Functional assays*

For T cell proliferation assay, T cell suspensions were prepared from lymph nodes and cultured in U-bottom 96-well plates (Becton Dickinson, Lincoln Park, NJ) for 3–4 days at 37°C in Click's Eagle-Hanks' amino acid medium (Irvine Scientific, Santa Ana, CA) supplemented with 5% heat-inactivated FCS (Intergen, Purchase, NY), 5 $\times$ 10$^{-5}$ M 2-ME (Bio-Rad, Richmond, CA), 2 mM L-glutamine, and 50 $\mu$g/ml gentamicin (Life Technologies). Depending on the experiment, T cells (30–50 $\times$ 10$^3$/well) were stimulated using irradiated B6 splenocytes as APCs (3 $\times$ 10$^5$ or less/well, 2000 rad) plus serial dilutions of synthetic E$\alpha_{52-68}$ peptide (ASFEAQGALANIAVDKA; single-letter amino acid code) or its variants in a total volume of 150 $\mu$l. The cells were incubated in duplicate wells, and 1 $\mu$Ci of [$^3$H]thymidine/well was added to the culture during the last 12 h. The plates were then harvested, and counts per minute were determined using liquid scintillation counting. For inhibition experiments, purified mAbs (3–5 $\mu$g/ml) were sterile-filtered and added to microcultures. The peptides were synthesized and analyzed by mass spectroscopy at the W. M. Keck Biotechnology Resource Center (Yale University).

## Results

### *Specific interference with positive selection in vivo identifies E$\alpha_{52-68}$ structurally related self-peptide(s) as a major contributor(s) to the maturation of the E$\alpha_{52-68}$:I-A$^b$-specific 1H3.1 TCR Tg thymocytes*

The 1H3.1 $\alpha\beta$ TCR is specific for the 52–68 portion of the I-E$\alpha$ chain complexed to I-A$^b$ MHC class II molecules (50, 51). Such a complex is also specifically recognized by the Y-A*e* mAb (48, 50–52). Thus, APCs from I-A$^{b+}$/I-E$\alpha^+$ mice (e.g., B10.A (5R))

stain positively for Y-A*e* and activate 1H3.1 TCR Tg T cells in a Y-A*e*-inhibitable manner. Conversely, APCs from I-$A^{b+}$/I-E$\alpha^-$ mice (e.g., C57BL/6) (53) stain negatively for Y-A*e* and are unable to activate 1H3.1 TCR Tg T cells (50, 51, 54). Consequently, a severe intrathymic negative selection is observed in 1H3.1 TCR Tg mice when I-E$\alpha$ expression is due to an endogenous functional I-E$\alpha$ gene (1H3.1 TCR Tg B10.A (5R)) or is driven by a transgene (1H3.1 TCR/I-E$\alpha$ double Tg) (54) (our unpublished observations). We previously observed that neonatal injection of Y-A*e* mAb was able to specifically interfere with the process of intrathymic deletion in 1H3.1 TCR/I-E$\alpha$ double-Tg mice (54); a low, but significant, fraction of V$\beta$6 high $CD4^+CD8^-$ thymocytes was rescued in the presence of Y-A*e*, and E$\alpha_{52-68}$-responsive T cells were detectable in the periphery. It was also striking to note that the Y-A*e* treatment greatly increased thymic cellularity. These observations could be most simply explained by the concomitant blockade of both positive and negative selection of 1H3.1 TCR Tg thymocytes by Y-A*e*. Thus, in the presence of Y-A*e*, a limited number of transgenic thymocytes could be positively selected, and some of these could then escape negative selection. This hypothesis is well in line with the observation that normal C57BL/6 (I-$A^{b+}$/I-E$\alpha^-$) mice repeatedly treated with Y-A*e* have an impaired capacity to respond to E$\alpha_{52-68}$ upon immunization (A. Y. Rudensky, C. E. Grubin, and C. A. Janeway, Jr., unpublished observations) (55). This result suggested that positive selection of $CD4^+$ T cells able to respond to E$\alpha_{52-68}$ involves self-peptides that are sufficiently structurally related to E$\alpha_{52-68}$ to be recognized by Y-A*e* in the context of I-$A^b$. To directly test this possibility, we injected newborn C57BL/6 1H3.1 TCR Tg mice with Y-A*e* and analyzed the phenotype of thymic cell suspension 12–15 days later by immunostaining and flow cytometry. The I-E-specific Y17 mAb was used as a control because its isotype matches the Y-A*e* isotype (IgG2b). Fig. 1*A* shows that after 2 wk of treatment, the fraction of V$\beta6^{high}$ thymocytes, that is, the population of cells that are beyond the stage of positive selection, is drastically reduced by the administration of Y-A*e*. V$\beta$6 distribution was virtually unchanged by Y-17 mAb treatment, indicating that the effect seen in the presence of Y-A*e* is specific (Fig. 1*A*, *bottom panels*). In addition, the administration of isotype-matched 10.2.16 mAb, which reacts to many I-A molecules but not to I-$A^b$, had no effect (data not shown). Y-A*e* mAb treatment also resulted in increased thymic cellularity. Consequently, the number of $CD4^+CD8^+$ thymocytes was augmented; in the experiment depicted in Fig. 1*A*, numbers of $CD4^+CD8^+$ cells were: saline, $32.4 \times 10^6$; Y-A*e*, $81.84 \times 10^6$; and Y17, $34.08 \times 10^6$. These observations are consistent with an accumulation of immature thymocytes at the $TCR^{low}$ $CD4^+CD8^+$ stage and therefore with specific interference with positive selection of 1H3.1 TCR Tg thymocytes. The mAb treatment of transgene negative littermates revealed that in the presence of Y-A*e* the fraction as well as the absolute number of thymocyte subpopulations were virtually unchanged compared with those in vehicle- or Y17-treated mice (Fig. 1*B*). The fact that a normal number of mature $CD4^+$ thymocytes is present in Y-A*e*-treated non-Tg mice indicates that the inhibitory effect on positive selection of 1H3.1 TCR Tg thymocytes in vivo cannot be explain by depletion of thymic stromal cells. It also indicates that Y-A*e* is selectively reacting to only a fraction of MHC class II molecules expressed in C57BL/6 thymic stromal cells. Since 1H3.1 TCR Tg thymocytes do not mature in the thymus of 1H3.1 TCR Tg I-$A^b\beta^{-/-}$ mice (Fig. 2), their development in C57BL/6 mice cannot be supported by either classical or nonclassical MHC class I molecules, but only by I-$A^b$ molecules. Therefore, the inhibition of positive selection in Y-A*e*-treated 1H3.1 TCR Tg mice indicates that Y-A*e* effectively reacts to a C57BL/6 self-peptide(s) presented in the context of I-$A^b$. Thus, H-2M -dependent (25) self-peptide–I-$A^b$ complexes able to be recognized by Y-A*e* represent major contributors to positive selection of the E$\alpha_{52-68}$–I-$A^b$ complex-specific 1H3.1 TCR Tg thymocytes in C57BL/6 (I-$A^{b+}$/I-E$\alpha^-$) mice. Presumably such complexes contain self-peptides with structural characteristics related to those of the E$\alpha_{52-68}$ peptide.

### *Plasticity in Y-A*e *reactivity*

To estimate the capacity of Y-A*e* to recognize a range of diverse peptide–I-$A^b$ complexes, we analyzed the recognition of several length variants as well as mutants of E$\alpha_{52-68}$ by Y-A*e* and the 1H3.1 TCR using immunostaining and proliferation assay (Fig. 3). Several irrelevant peptides able to bind I-$A^b$ molecules (56) were used as a negative control: $\beta_2m_{48-58}$, $CD22_{25-39}$, and LDL receptor 486–501. As expected, these control peptides did not generate Y-A*e* signal or proliferative T cell response. Y-A*e* was able to accommodate virtually all truncations tested at both the $NH_2$ and COOH termini as well as elongation at the $NH_2$ terminus (Fig. 3*A*). We then tested E$\alpha$ peptide variants carrying point mutations. Since 1H3.1 T cells react well to the 56–64 truncated form of the peptide, the I-$A^b$ binding motif is likely to involve the alanine residues 56, 59, 61, and 64 (A. K. Barlow, R. Medzhitov, and C. A. Janeway, Jr., unpublished observations). We therefore analyzed mutants in which these positions were unmodified (Fig. 3*B*). Although the panel of mutants tested was limited, we observed that recognition by Y-A*e* can accommodate more changes than recognition by 1H3.1 TCR. For instance, point mutation at positions 58, 60, and 62 were relatively well tolerated by Y-A*e*, while such changes completely extinguished the 1H3.1 T cell proliferative response (Fig. 3*B*). In addition, changes at positions 54 and 55 did not reduce the Y-A*e* reactivity and in some cases increased it (F-54-V and E-55-N). In contrast, position 63 was critical, since both I-63-D and I-63-A mutants abrogated Y-A*e* binding while they are able to reduce the response of 1H3.1 T cells to B10.A (5R) and therefore still able to bind to I-$A^b$ (data not shown). Thus, it appears that Y-A*e* can accommodate multiple amino acid changes as well as several length variations, suggesting that several C57BL/6 self-peptides as well as their putative truncation variants can potentially confer Y-A*e* recognition to I-$A^b$ molecules.

### *Deficient positive selection of E*$\alpha$*6 TCR Tg thymocytes confronted with a restricted self-peptide complexity*

To further asses the importance of self-peptide recognition during intrathymic positive selection, we again used H-2M-deficient mice to analyze the maturation of a distinct MHC class II-restricted T cell specificity. The E$\alpha$6 $\alpha\beta$ TCR ($V\alpha_{23.1}$-$V\beta_6$) specifically recognizes the E$\alpha_{52-66}$–I-$A^b$ complex, but not the E$\alpha_{52-68}$–I-$A^b$ complex recognized by the 1H3.1 $\alpha\beta$ TCR ($V\alpha_1$-$V\beta_6$) (57). E$\alpha$6 TCR $\alpha\beta$ Tg mice (C. Viret and C. A. Janeway, Jr., unpublished observations) were bred to H-2M$\alpha^{-/-}$ mice to generate E$\alpha$6 TCR Tg H-2M-deficient mice. Results from immunostaining and flow cytometric analysis of lymphoid organs (Fig. 4*A*) show that the positive selection of immature E$\alpha$6 TCR Tg thymocytes is clearly altered in the absence of H-2M heterodimers; compared with TCR Tg H-2M$\alpha^{+/+}$ thymus (*middle panels*), a higher number of $CD4^+CD8^+$ (double-positive) thymocytes appears in the TCR Tg H-2M$\alpha^{-/-}$ thymus (*bottom panels*; TCR Tg, $41.4 \times 10^6$; TCR Tg H-2M$\alpha^{-/-}$, $67.4 \times 10^6$), and fewer mature $CD4^+CD8^-$ thymocytes are detected (TCR Tg, $32.8 \times 10^6$; TCR Tg H-2M$\alpha^{-/-}$, $10.2 \times 10^6$). In accordance, the V$\beta6^{high}$ thymocyte fraction is severely reduced in the TCR Tg H-2M$\alpha^{-/-}$ thymus (Fig. 4*A*, *central* histograms). In the periphery, some V$\beta6^+$ T cells accumulated in the TCR Tg H-2M$\alpha^{-/-}$ spleen and lymph nodes (Fig. 4*B*, *mid-*

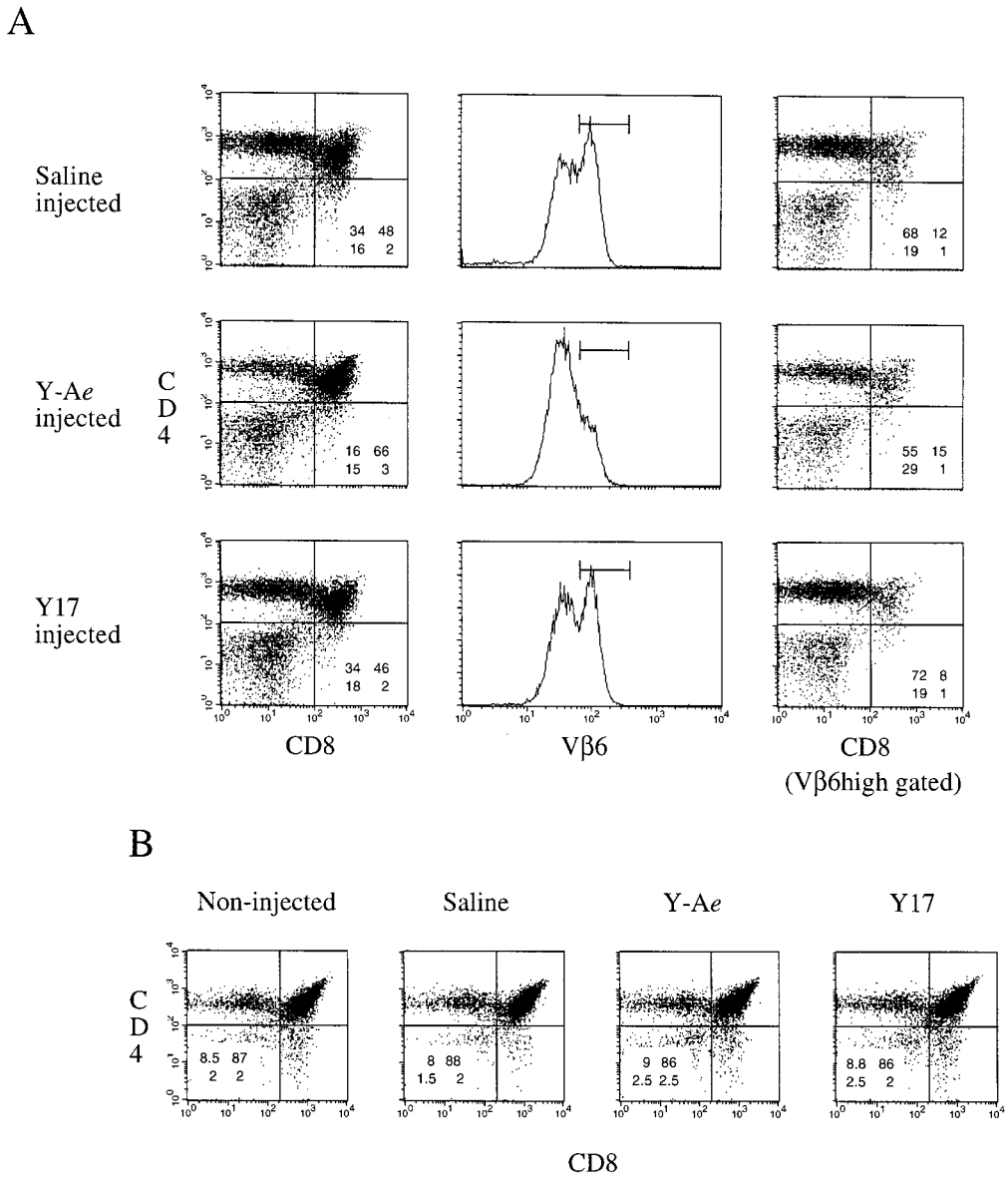


**FIGURE 1.** Y-A*e*-recognizable self-peptide–self-MHC complexes are major contributors to positive selection of 1H3.1 TCR Tg thymocytes in vivo. *A*, Immunostaining and flow cytometric analysis of thymic cell suspensions from mAb-treated mice. 1H3.1 TCR Tg newborn mice were injected i.p. every 2 days with 50 $\mu$g of the Y-A*e* mAb specific for the E$\alpha_{52-68}$–I-A$^b$ complex, the isotype-matched I-E-specific Y17 mAb, or vehicle (saline). Mice were sacrificed after 2 wk of treatment, and thymic cell suspensions were stained and analyzed by flow cytometry immediately. The *central panels* represent the V$\beta$6 histograms; the *left* and *right panels* show the CD4/CD8 distribution, respectively, with and without electronic gating on the V$\beta$6 high thymic population. Quadrant statistics are indicated. The profiles are representative of three experiments. In this particular experiment the cellularity was: saline injected, $67.5 \times 10^6$; Y-A*e* injected, $124 \times 10^6$; and Y17 injected, $74.1 \times 10^6$. *B*, mAb treatment of non-Tg littermates. In this experiments the cell numbers were: noninjected, $137.1 \times 10^6$; vehicle, $144.8 \times 10^6$; Y-A*e*, $142.1 \times 10^6$; and Y17, $136.3 \times 10^6$.

*dle* and *bottom panels*), but the majority were expressing the CD8 coreceptor. This CD8 skewing is also visible in the thymic compartment (Fig. 4*A*, *lower right panel*). These observations indicated that immature thymocytes carrying E$\alpha$6 $\alpha\beta$ TCR specificity do not efficiently develop in the H-2M$\alpha^{-/-}$ thymic microenvironment, and therefore they rely on recognition of self-peptide–self-MHC complexes for their positive selection in wild-type C57BL/6 mice.

### *Self-peptide(s)-specific low expansion of mature naive E$\alpha$6 TCR $\alpha\beta$ Tg CD4$^+$ T cells in irradiated syngeneic recipients*

We previously observed that persistence of adoptively transferred 1H3.1 TCR $\alpha\beta$ Tg CD4$^+$ mature naive T cells in irradiated syngeneic recipients is associated with a self-peptide(s)-specific low level of cell division (25). Ernst et al. (41) reported a similar result using adoptive transfer of CD4$^+$ DO11 TCR Tg T cells, but noticed that another MHC class II-restricted T cell specificity seems to behave differently; OT-II TCR Tg CD4$^+$ T cells revealed minimal expansion after transfer into irradiated hosts. It was therefore of interest to examine additional CD4$^+$ TCR Tg T cells in such experimental conditions. We adoptively transferred purified naive E$\alpha$6 TCR Tg CD4$^+$ RAG-1$^{-/-}$ T cells into irradiated normal C57BL/6 vs I-A$^b\beta^{-/-}$ and H-2M$\alpha^{-/-}$ mice and analyzed their persistence in spleen and lymph nodes at different time points after transfer. The results obtained (Fig. 5) were entirely consistent with those obtained using 1H3.1 TCR Tg mice; both I-A$^b\alpha\beta$ and H-2M $\alpha\beta$ heterodimers (i.e., I-A$^b$ molecules presenting a normal array of self-peptides) are required for persistence of mature naive E$\alpha$6

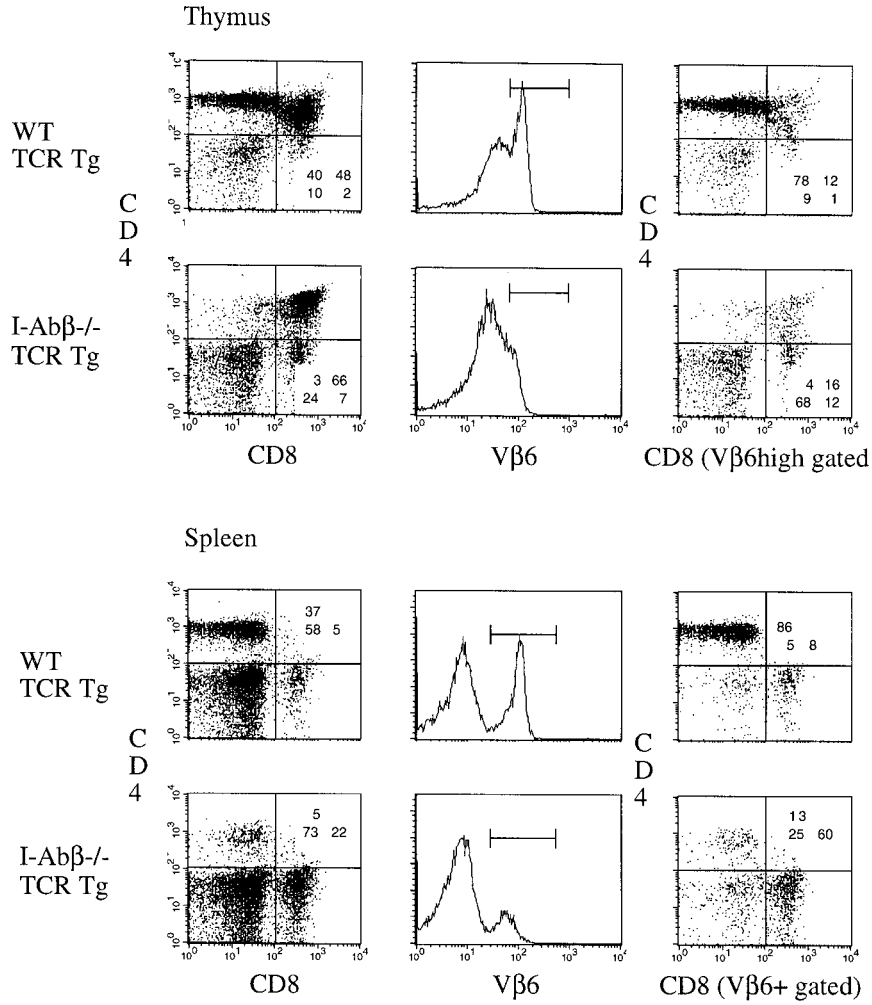


**FIGURE 2.** Deficient intrathymic positive selection of 1H3.1 TCR Tg thymocytes in the absence of MHC class II molecules. Immunostaining and flow cytometric analysis of thymic (*top*) and splenic (*bottom*) cell suspensions from 5-wk-old 1H3.1 TCR Tg MHC class II-deficient (I-A$\beta^{b-/-}$) mice. A TCR Tg I-A$\beta^{b+/+}$ littermate is included as a control. The profiles are organized as described in Fig. 1*A*. Note that in 1H3.1 TCR Tg I-A$\beta^{b-/-}$ mice, V$\beta$6$^{high}$ thymocytes are lacking, and the few V$\beta$6$^+$ cells detected in the spleen are mostly CD8$^+$ and express a low level of TCR. In this particular experiment the thymic cellularity was: TCR Tg, $83.1 \times 10^6$; and TCR Tg I-A$\beta^{b-/-}$, $49.4 \times 10^6$.

TCR $\alpha\beta$ Tg CD4$^+$ T cells in secondary lymphoid organs of irradiated C57BL/6 (B6) recipients (Table I). Using CFSE-labeled naive E$\alpha$6 TCR Tg CD4$^+$ T cells, we also observed that such a peripheral persistence correlates with a certain level of cell division in secondary lymphoid organs (see C57BL/6 recipients in Fig. 5). This expansion results from the recognition of self-peptide–I-A$^b$ complexes because it is not observed in recipient mice with a restricted self-peptide repertoire (see H-2M $\alpha^{-/-}$ recipients). Thus, it appears that under these conditions, expansion of mature naive TCR Tg MHC class II-restricted T cells occurs for three of four specificities tested (Refs. 25 and 41 and this report).

### *Phenotypic changes associated with self-peptide recognition-driven low expansion of naive CD4$^+$ T cells*

We tried to characterize the activation status of MHC class II-restricted T cells that slowly cycle upon recognition of self-epitopes in lymphopenic hosts. Before transfer, 1H3.1 TCR Tg CD4$^+$ T cells were 94% CD44$^{low}$, 93–95% CD45RB$^{high}$/CD62L$^{high}$, and negative for both CD25 and CD69 surface expression in accordance with their naive status (data not shown). After transfer, we repeatedly observed that the cycling activity correlates with an increase in the surface expression of CD44; cells that achieved the highest number of cycles had the highest expression level of CD44 (Fig. 6*A*). This was observed for cells recovered from both spleen and lymph nodes and is fully consistent with several studies showing that TCR transgenic and polyclonal naive CD4$^+$ and CD8$^+$ T cells up-regulate CD44 surface expression upon transfer into lymphopenic recipients (41, 42, 58–60). The surface expression of other markers was not changed upon homeostatic proliferation; the CD62L (Fig. 6*A*) expression level was virtually unmodified, and CD25 and CD69 expressions remained negative (data not shown). Only CD49d expression appeared slightly increased during expansion (Fig. 6*A*). Although the Ag receptor expression is frequently down-modulated on activated T cells, we observed that the surface expression of the TCR as well as of the CD4 coreceptor were unaffected upon expansion of 1H3.1 TCR Tg CD4$^+$ T cells in irradiated hosts (Fig. 6*B*). These observations indicated that the moderate expansion of naive CD4$^+$ T cells induced by recognition of self-peptide–self-MHC complexes is associated with a surface phenotype clearly distinct from those of both naive and activated/memory T cells.

## Discussion

The deficient maturation of 1H3.1 TCR Tg CD4$^+$ thymocytes in mice expressing a normal level of I-A$^b$ molecules but a severely restricted self-peptide complexity (the H-2M$\alpha^{-/-}$ mice) revealed a central role for self-peptide(s) recognition for positive selection of immature 1H3.1 T cells. The fact that the Y-A*e* mAb, which, like an $\alpha\beta$ TCR, interacts with both the peptide and polymorphic residues of I-A$^b$ (such as I-A$\alpha_{57}$ and I-A$\beta_{65-67}$) (48) interferes with positive selection of 1H3.1 TCR Tg thymocytes in vivo, validates this conclusion and indicates that the self-peptide(s) involved is structurally related to E$\alpha_{52-68}$. This result is consistent

A

| | | Y-A*e* | 1H3.1 |
|---|---|---|---|
| **E$\alpha$52-68** | **A S F E A Q G A L A N I A V D K A** | 100 | 100 |
| 52-67 | A S F E A Q G A L A N I A V D K | 113 | 138 |
| 52-66 | A S F E A Q G A L A N I A V D | 104 | 115 |
| 54-66 | F E A Q G A L A N I A V D | 93 | 119 |
| 53-66 | S F E A Q G A L A N I A V D | 103 | 178 |
| 54-67 | F E A Q G A L A N I A V D K | 98 | 132 |
| 52-65 | A S F E A Q G A L A N I A V | 101 | 139 |
| 51-68 | F A S F E A Q G A L A N I A V D K A | 99 | 121 |
| 50-68 | K F A S F E A Q G A L A N I A V D K A | 109 | 98 |
| $\beta$2-m 48-58 | T Q F H P P H I E I Q | 1 | 0 |
| CD22 25-39 | D W T V D H P Q T L F A W E G | 0 | 0 |
| LDLr.486-501 | R N I Y W T D S V P G S V S V A | 0 | 0 |

B

| | | Y-A*e* | 1H3.1 |
|---|---|---|---|
| **E$\alpha$52-68** | **A S F E A Q G A L A N I A V D K A** | 100 | 100 |
| | **A A** A Q G A L A N I A **A A** | 56 | 176 |
| F-54-V | A S **V** E A Q G A L A N I A V D | 123 | 125 |
| E-55-S | A S F **S** A Q G A L A N I A V D | 112 | 149 |
| E-55-N | A S F **N** A Q G A L A N I A V D | 121 | 148 |
| G-58-V | A S F E A Q **V** A L A N I A V D | 76 | 8 |
| L-60-A | A S F E A Q G A **A** A N I A V D | 96 | 0 |
| N-62-V | A S F E A Q G A L A **V** I A V D | 78 | 3 |
| I-63-D | A S F E A Q G A L A N **D** A V D | 1 | 4 |
| I-63-A | A S F E A Q G A L A N **A** A V D | 1 | 0 |

**FIGURE 3.** Plasticity in E$\alpha_{52-68}$:I-A$^b$ recognition by Y-A*e*. Reactivity of the Y-A*e* and 1H3.1 synonymous immune receptors to distinct length variants (*A*) and mutants (*B*) of the E$\alpha$ peptide. The $\beta_2$-microglobulin ($\beta_2$-m)$_{48-58}$, CD22$_{25-39}$, and LDL receptor 486–501 I-A$^b$-binding peptides were used as controls. Peptides are listed using single-letter amino acid code. Mutations are indicated in bold and are described on the *left*. Y-A*e* binding was estimated by immunostaining and flow cytometry after in vitro peptide loading of C57BL/6 splenocytes. Only B220$^+$ cells were analyzed. The fluorescence intensity of nonpulsed cells was subtracted from peptide-pulsed samples. The values represent the percentage of the value obtained for WT E$\alpha_{52-68}$ loading. The ability to stimulate 1H3.1 T cells was assessed by a proliferation assay using purified naive 1H3.1 TCR Tg CD4$^+$ T cells as effectors and irradiated C57BL/6 splenocytes as APCs. Similar data were obtained by concomitantly measuring IL-2 secretion (data not shown). The values represent the percentage of the response to the WT E$\alpha_{52-68}$ peptide. All the values were calculated with peptide doses corresponding to the exponential phase of the response curve to the WT E$\alpha_{52-68}$ peptide (typically 3 $\mu$g/ml). Data are representative of three staining experiments and three functional assays.

with the observation that Y-A*e*-treated C57BL/6 mice have an impaired ability to mount a specific response upon immunization with E$\alpha_{52-68}$ (55). However, under the experimental conditions used by Rudensky (55), it remained possible that a residual amount of Y-A*e* could interfere with the in situ priming of naive T cells. Our observation that Y-A*e* interferes with positive selection of 1H3.1 TCR Tg thymocytes in vivo underscores such a possibility and supports the idea that the intrathymic recognition of Y-A*e*-recognizable self-peptide–I-A$^b$ complexes contributes to positive selection of E$\alpha_{52-68}$–I-A$^b$ complex-specific T cells.

The data imply that in C57BL/6 mice, thymic epithelial cells can assemble self-peptide–I-A$^b$ complexes involving peptides structurally close enough to E$\alpha_{52-68}$ to confer recognition by Y-A*e*. We tried to visualize in situ the presentation of these complexes. We analyzed C57BL/6 thymic sections using immunohistochemistry and immunohistofluorescence. Like others (55, 61), we failed to detect visualizable Y-A*e* binding (data not shown). We then tried to analyze cortical (cTEC1–2) and medullary (mTEC1C6) thymic epithelial cell lines derived from C57BL/6 mice (62) by immunofluorescence and flow cytometry and did not detect significant Y-A*e* staining even after treatment with IFN-$\gamma$, which increases MHC class II molecule expression (result not shown). We conclude that the expression of Y-A*e*-recognizable self-peptide–I-A$^b$ complexes on C57BL/6 thymic stromal cells can be detected through interference with thymocyte development, but is not visualizable by staining using standard developing techniques. This could reflect a particularly low affinity binding of Y-A*e*, the fact that such complexes may be rare, or a combination of both phenomena. These complexes may well be rare, because we were able to repeatedly detect a significant Y-A*e* signal when analyzing fully mature, but not immature, dendritic cells derived from bone marrow progenitors in vitro (54), that is, only on dendritic cells that have MHC class II molecules with a prolonged half-life and virtually all localized on the plasma membrane (63, 64). The fact that all mature dendritic cells stain positively also indicates that a Y-A*e*-recognizable epitope(s) is expressed on all and not a population of C57BL/6 bone marrow-derived dendritic cells. Although we cannot exclude that an unknown mechanism restores a low transcription level of the deficient I-E$\alpha$ gene, the Y-A*e*-recognizable self-peptide–I-A$^b$ complex(es) expressed in unmanipulated C57BL/6 mice is unlikely to involve the E$\alpha_{52-68}$ peptide itself for two reasons. First, using a walking RT-PCR we failed to detect any part of the transcript region encoding the E$\alpha_{52-68}$ sequence in C57BL/6 APCs (54). Second, while mature C57BL/6 dendritic cells are clearly Y-A$e^+$, they do not activate 1H3.1 T cells. This is in sharp contrast with the fact that the loading of C57BL/6 splenocytes with doses of the E$\alpha_{52-68}$ peptide that do not generate a visualizable Y-A*e* signal can efficiently activate 1H3.1 T cells (54).

In C57BL/6 mice the presentation of an E$\alpha_{52-68}$-independent, Y-A*e*-recognizable epitope(s) appears able to cause both positive (this report and Ref. 55) and negative (54) intrathymic selection of CD4$^+$ T cells. Therefore, such epitopes are most likely constitutively expressed on both thymic epithelial cells and bone marrow-derived cells. Since in these two cell types, the lysosomal degradation of proteins involves distinct members of the cystein proteinase family (cathepsins L and S, respectively) (65), we conclude that the generation of a peptide fragment(s) able to confer Y-A*e* binding to I-A$^b$ molecules does not require a dedicated lysosomal proteinase. However, the question of whether the sequence of such peptides is related in the two cell types remains open. These self-peptides could be relatively diverse, because it appears that, at least in the case of the recognition of the E$\alpha_{52-68}$–I-A$^b$ complex, Y-A*e* recognition can accommodate multiple sequence and length variations. A similar conclusion was reached by Baldwin et al. (66), who observed that the MCC88–103–I-E$^k$ complex-specific G35 mAb is able to specifically inhibit in vitro and in vivo positive selection of transgenic thymocytes carrying the same specificity (the 5C.C7 $\alpha\beta$ TCR). In this case G35 was able to detectably react in situ with both thymic epithelial cells and dendritic cells.

The analysis of E$\alpha$6 TCR Tg H-2M$\alpha^{-/-}$ mice revealed that CLIP as well as the few other peptides presented by I-A$^b$ molecules in H-2M-deficient mice (20) are unable to support an efficient positive selection of immature E$\alpha$6 TCR Tg thymocytes. Along with other studies (20, 23–25), this phenotype indicates that seven of seven CD4$^+$ T cell specificities tested fail to undergo positive selection when a normal amount of MHC class II molecules presents a very narrow set of self-peptides. The requirement for self-peptide recognition during positive selection of E$\alpha$6 TCR Tg thymocytes was mirrored in the periphery for the survival of mature E$\alpha$6 TCR Tg T cells; the persistence of naive E$\alpha$6 TCR Tg CD4$^+$ T cells was impaired in irradiated H-2M-deficient recipients. Thus, self-peptide–I-A$^b$ complexes unable to promote positive selection of E$\alpha$6 TCR Tg T cells are unable to support the peripheral maintenance of naive E$\alpha$6 TCR Tg T cells. Interestingly, in a distinct

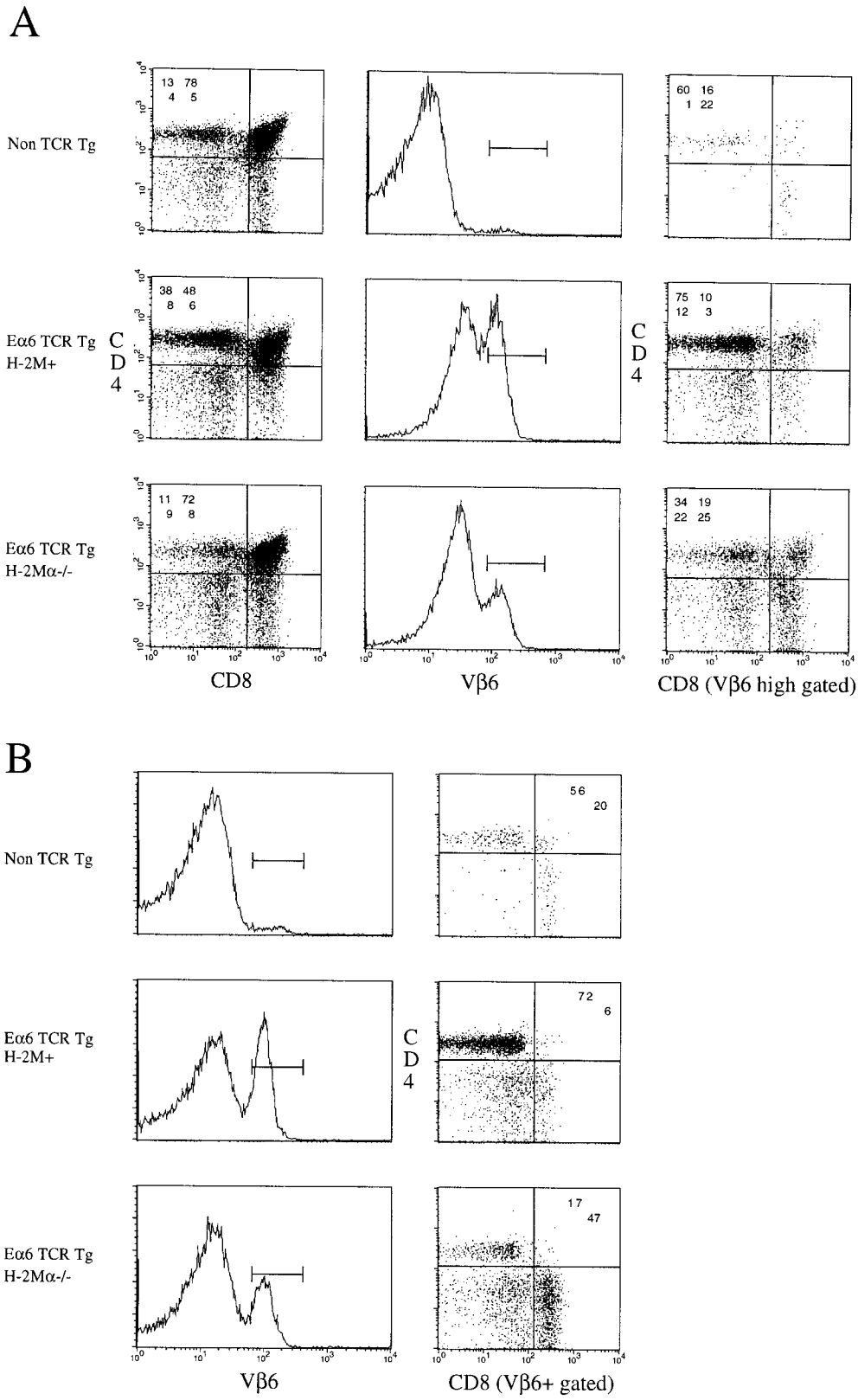


**FIGURE 4.** Deficient positive selection of the E$\alpha$6 $\alpha\beta$ TCR Tg immature thymocytes in the H-2M$\alpha^{-/-}$ thymic microenvironment. *A*, FACS analysis of thymic cell suspensions prepared from 4.5-wk-old E$\alpha$6 TCR Tg H-2M$\alpha^{+/+}$ and H-2M$\alpha^{-/-}$ littermate mice. A TCR Tg-negative littermate is included. The *central panels* show the V$\beta$6 histogram. The *left* and *right panels* show the CD4/CD8 distribution, respectively, without and with electronic gating on the V$\beta$6$^{high}$ thymocyte population. Quadrant statistics are indicated. In the experiment depicted the cellularity was: TCR Tg H-2M$\alpha^{+/+}$, 86.4 $\times$ 10$^6$; and TCR Tg H-2M$\alpha^{-/-}$, 93.6 $\times$ 10$^6$. *B*, FACS analysis of spleen cell suspensions. The *right panels* indicate the CD4/CD8 distribution after electronic gating on the V$\beta$6$^+$ cells. Lymph node cell suspension showed a similar profile (data not shown). Thymic and lymph nodes profiles are representative of three sets of mice analyzed.

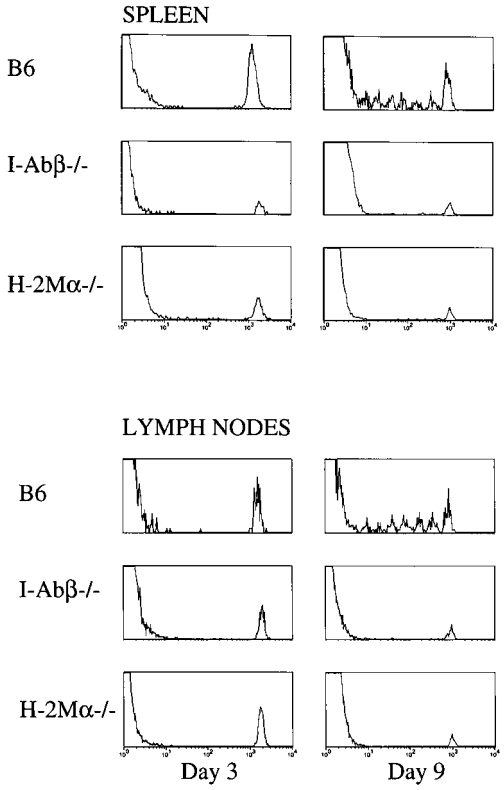


**FIGURE 5.** Both the restricting MHC element (I-A$^b$) and a normal array of self-peptides are required for the low expansion-associated persistence of adoptively transferred E$\alpha$6 TCR $\alpha\beta$ Tg CD4$^+$ T cells in irradiated recipient mice. CFSE-labeled naive E$\alpha$6 TCR Tg CD4$^+$ RAG-1$^{-/-}$ T cells ($6 \times 10^6$) were transferred into normal C57BL/6 mice (B6), MHC class II-deficient mice (I-A$^b\beta^{-/-}$), and H-2M-deficient mice (H-2M$\alpha^{-/-}$). Cell suspensions from spleen (*top panels*) and lymph nodes (*bottom panels*) were prepared on days 3 and 9 after transfer and were analyzed by cytofluorometry (*x*-axis, log fluorescence; *y*-axis, relative cell number).

system, the poor positive selection of CD4$^+$CD8$^-$ TCR Tg thymocytes was correlated with a failure to accumulate mature CD4$^+$ TCR Tg T cells in the periphery (67).

The self-peptide(s)-dependent persistence of E$\alpha$6 TCR Tg T cells in irradiated wild-type recipients is associated with a low level of cell division. This phenomenon has been observed for 1H3.1 CD4$^+$ T cells (25) and D0.11 CD4$^+$ T cells, but not for OT-II CD4$^+$ T cells (41). The reason why OT-II T cells do not measurably divide in irradiated syngeneic recipients is unknown. It is possible that a longer time is required to visualize their expansion. This could be explained, for instance, by the rare expression of self-peptide:MHC class II complex(es) able to provide a survival signal to OT-II TCR Tg T cells. The peripheral low expansion of mature $\alpha\beta$ CD4$^+$ T cells observable in such experimental systems is certainly physiologically relevant, because the continuous administration of 5-bromo-2-deoxyuridine revealed that in thymectomized adult mice both CD4$^+$ and CD8$^+$ T cells that display a naive T cell phenotype show a moderate, but clearly detectable, level of expansion in vivo (68). These expanding cells include cells that retain as well as cells that regain naive T cell markers. This may suggest that the up-regulation of CD44 surface expression that we and others (41, 42, 58–60) observe on expanding cells is potentially transient. Apart from a slight increase in CD49d expression, we did not detect a significant change in surface expression of other markers, such as CD62L, CD69, or CD25. It has been frequently observed that the TCR expression level can

Table I. *Representative numbers of CD4$^+$V$\beta$6$^+$ T cells recovered from the spleen of irradiated normal C57BL/6, I-A$^b\beta^{-/-}$, and H-2M$\alpha^{-/-}$ mice after adoptive transfer of E$\alpha$6 TCR Tg CD4$^+$ RAG-1$^{-/-}$T cells*[a]

| Day | C57BL/6 | I-A$^b\beta^{-/-}$ | H-2M$\alpha^{-/-}$ |
|---|---|---|---|
| 3 | $0.34 \times 10^6$ | $0.24 \times 10^6$ | $0.26 \times 10^6$ |
| 8 | $2.05 \times 10^6$ | $0.04 \times 10^6$ | $0.1 \times 10^6$ |

[a] A total of $5–6 \times 10^6$ E$\alpha$6 TCR Tg CD4$^+$ RAG-1$^{-/-}$ T cells was injected i.v. into irradiated wild-type or mutant recipient mice. At days 3 and 8, spleen cell suspensions were ammonium chloride-treated, enumerated, and stained for CD4 and V$\beta$6 before analysis by flow cytometry. The absolute numbers were calculated using the formula: (cellularity $\times$ percentage CD4$^+$V$\beta$6$^+$ cells)/100.

be down modulated upon encounter of antigenic peptide–MHC complexes. We therefore analyzed the TCR expression level on expanding T cells and found that in contrast with a study focusing on CD8$^+$ T cells (59), cycling mature 1H3.1 and E$\alpha$6 TCR Tg CD4$^+$ T cells do not down-regulate the surface expression level of their TCR in irradiated recipients. The CD4 coreceptor expression was also unchanged. Thus, the surface phenotype of mature T cells that slowly cycle in response to recognition of self-peptide–MHC class II complexes is clearly distinct from those of both naive and activated/memory T cells. The signaling events underlying such an intermediate state are virtually unknown. It is possible that the survival signal delivered to naive T cells involves the Ras mitogen-activated protein kinase signaling pathway. For instance, Ras-mitogen-activated protein kinase-activated kinases suppress in vivo the activity of the proapoptotic protein BAD and activate the cAMP response element-binding protein (CREB) transcription factor that promotes cell survival (69). Indeed, the same pathway

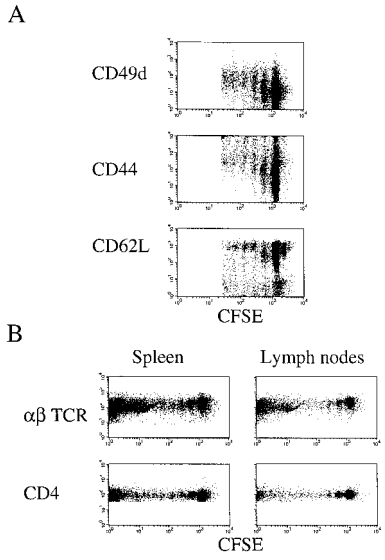


**FIGURE 6.** Surface phenotype associated with the expansion of 1H3.1 TCR Tg CD4$^+$ T cells induced by self-peptide–self-MHC complex recognition in an irradiated syngeneic host. CFSE-labeled 1H3.1 TCR Tg CD4$^+$ T cells ($6 \times 10^6$) were i.v. injected into irradiated normal C57BL/6 recipients, and secondary lymphoid organs were analyzed at different time points after transfer using immunostaining and flow cytometry. *A*, CD49d, CD44, and CD62L surface expressions on day 12 after transfer. *B*, TCR and CD4 coreceptor surface expressions 10 days after transfer. Expression of the $\alpha\beta$ TCR was detected using the C$\beta$-specific H57 mAb. For clarity, the results in *A* were plotted after gating out the background FL1 fluorescence; they represent analysis of ammonium chloride-treated splenocytes. Similar results were obtained using lymph node cell suspensions. Experiments conducted using E$\alpha$6 TCR Tg CD4$^+$ T cells showed consistent results (data not shown).

appears to be important for positive selection of thymocytes, since p44 mitogen-activated protein kinase-deficient mice display impaired thymocyte maturation beyond the $CD4^+CD8^+$ stage; the $TCR^{high}$ thymocyte population is reduced by >50%, and both $CD4^+CD8^-$ and $CD4^-CD8^+$ subsets are affected (70). It is also known that the Bcl-2 protein is highly expressed in mature peripheral T cells and is required for survival of mature lymphocytes, since a dramatic loss of T and B cells by apoptosis is observed in young mice lacking Bcl-2 (for review, see Ref. 71). Bcl-2 expression seems to be coupled to the TCR-mediated signal, because it is up-regulated during positive selection and persists in mature T cells in the periphery. However, multiple additional proteins are likely to be involved. For instance, mice lacking the lung Kruppel-like transcription factor (LKLF) in lymphocytes ($LKLF^{-/-}$/$RAG\text{-}2^{-/-}$ chimeric mice) have a normal mature B cell compartment, but show severely compromised survival of mature splenic and lymph node T cells; the number is reduced by 90% (72). Mature naive T cells are also absent. LKLF is expressed in mature $CD4^+$ and $CD8^+$ T cells including medullary thymocytes, is undetectable in immature thymocytes, and is down-regulated after T cell activation. It is therefore possible that LKLF gene expression is turned on upon intrathymic positive selection, persists upon repeated TCR/self-peptide–MHC low-affinity interactions, and is turned off when a high-affinity TCR/foreign peptide-MHC interaction occurs.

In conclusion, we report here that $E\alpha_{52-68}$ structurally related, endogenous, self-peptide(s) contributes to positive selection of the $E\alpha_{52-68}$:$I\text{-}A^b$ specific 1H3.1 TCR Tg thymocytes. Also shown is that the restricted self-peptide complexity present in H-2M-deficient mice can support neither efficient intrathymic positive selection nor peripheral maintenance of adoptively transferred E$\alpha$6 TCR Tg T cells into the irradiated host. A similar parallel has been observed previously for 1H3.1 TCR Tg T cells. Thus, for multiple CD4 T cell specificities, alteration of the self-peptide repertoire impairs both thymocyte maturation and peripheral persistence of mature naive T cells. Such a phenomenon indicates that the selection and maintenance of the TCR repertoire of mature $CD4^+$ T cells are part of a continuum centered on the recognition of self-peptide–self-MHC class II complexes.

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## References

1. von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell 76:219.*
2. Nossal, G. J. 1994. Negative selection of lymphocytes. *Cell 76:229.*
3. Robey, E., and B. J. Fowlkes. 1994. Selective events in T cell development. *Annu. Rev. Immunol. 12:675.*
4. Janeway, C. A., Jr. 1994. Thymic selection: two pathways to life and two to death. *Immunity 1:3.*
5. Kisielow, P., and von Boehmer, H. 1995. Development and selection of T cells: facts and puzzles. *Adv. Immunol. 58:87.*
6. Allen, P. M. 1994. Peptides in positive and negative selection: a delicate balance. *Cell 76:593.*
7. Schumacher, T. N., and H. Ploegh. 1994. Are MHC-bound peptides a nuisance for positive selection? *Immunity 9:721.*
8. Jameson, S. C., K. A. Hogquist, and M. J. Bevan. 1995. Positive selection of thymocytes. *Annu. Rev. Immunol. 13:93.*
9. Nikolic-Zugic, J., and M. J. Bevan. 1990. Role of self-peptides in positively selecting the T cell receptor repertoire. *Nature 344:65.*
10. Sha, W. C., C. A. Nelson, R. D. Newberry, J. K. Pullen, L. R. Pease, J. H. Russell, and D. Y. Loh. 1990. Positive selection of transgenic receptor-bearing thymocytes by $K^b$ antigen is altered by $K^b$ mutations that involve peptide binding. *Proc. Natl. Acad. Sci. USA 87:6186.*
11. Hogquist, K. A., M. A. Gavin, and M. J. Bevan. 1993. Positive selection of $CD8^+$ T cells induced by major histocompatibility complex binding peptides in fetal thymic organ culture. *J. Exp. Med. 177:1469.*
12. Ashton-Rickardt, P. G., L. Van Kaer, T. N. M. Schumacher, H. L. Ploegh, and S. Tonegawa. 1993. Peptide contributes to the specificity of positive selection of $CD8^+$ T cells in the thymus. *Cell 73:1041.*
13. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17.*
14. Ashton-Rickardt, P. G., A. Bandeira, J. R. Delaney, L. Van Kaer, H. P. Pircher, R. M. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T cell selection in the thymus. *Cell 76:651.*
15. Hogquist, K. A., A. J. Tomlinson, W. C. Kieper, M. A. McGargill, M. C. Hart, S. Naylor, and S. C. Jameson. 1997. Identification of a naturally occurring ligand for thymic positive selection. *Immunity 6:389.*
16. Hu, Q., C. R. B. Walker, C. Girao, J. T. Opferman, J. Sun, J. Shabanowitz, D. F. Hunt, and P. G. Ashton-Rickardt. 1997. Specific recognition of thymic self-peptides induces the positive selection of cytotoxic T lymphocytes. *Immunity 7:221.*
17. Martin, W. D., G. G. Hicks, S. K. Mendiratta, H. I. Leva, E. H. Ruley, and L. Van Kaer. 1996. 72 −2 77 mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell 84: 543.*
18. Miyazaki, T., P. Wolf, S. Tourne, C. Waltzinger, A. Dierich, N. Barois, H. Ploegh, C. Benoist, and D. Mathis. 1996. Mice lacking H2-M complexes, enigmatic elements of the MHC class Ii peptide loading pathway. *Cell 84:531.*
19. Fung-Leung, W. P., C. D. Surh, M. Liljedahl, J. Pang, D. Leturcq, P. A. Peterson, S. R. Webb, and L. Karlsson. 1996. Antigen presentation and T cell development in H2-M deficient mice. *Science 271:1278.*
20. Grubin, C. E., S. Kovats, P. deRoos, and A. Y. Rudensky. 1997. Deficient positive selection of $CD4^+$ T cells in mice displaying altered repertoire of MHC class II bound self-peptides. *Immunity 7:197.*
21. Ignatowicz, L., J. Kappler, and P. Marrack. 1996. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell 84:521.*
22. Fukui, Y., T. Ishimoto, M. Utsuyama, T. Gyotoku, T. Koga, K. Nakao, K. Hirokawa, M. Katsuki, and T. Sasazuki. 1997. Positive and negative $CD4^+$ thymocyte selection by a single MHC class II/peptide ligand affected by its expression level in the thymus. *Immunity 6:401.*
23. Tourne, S., T. Miyazaki, A. Oxenius, L. Klein, T. Fehr, B. Kyewski, C. Benoist, and D. Mathis. 1997. Selection of a broad repertoire of $CD4^+$ T cells in mice 72 −2 77 αo/o mice. *Immunity 7:187.*
24. Surh, C. D., D. S. Lee, W. P. Fung-Leung, L. Karlsson, and J. Sprent. 1997. Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of $CD4^+$ T cells. *Immunity 7:209.*
25. Viret, C., F. S. Wong, and C. A. Janeway, Jr. 1999. Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. *Immunity 10:559.*
26. Sant'Angelo, D. B., G. Waterbury, B. E. Cohen, W. D. Martin, L. Van Kaer, A. C. Hayday, and C. A. Janeway, Jr. 1997. The imprint of intrathymic self-peptides on the mature T cell receptor repertoire. *Immunity 7:517.*
27. Nakagawa, T., W. Roth, P. Wong, A. Nelson, A. Farr, J. Deussing, J. A. Villadangos, H. Ploegh, C. Peters, and, A. Y. Rudensky.1998. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science 280:450.*
28. Barton, G. M., and A. Y. Rudensky. 1999. Requirement for diverse, low-abundance peptides in positive selection of T cells. *Science 283:67.*
29. Takeda, S., H. R. Rodewald, H. Arakawa, H. Bluethmann, and T. Shimizu. 1996. MHC Class II molecules are not required for survival of newly generated $CD4^+$ T cells, but affect their long-term life span. *Immunity 5:217.*
30. Tanchot, C., F. A. Lemonier, B. Perarnau, A. A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science 276:2057.*
31. Rooke, R., C. Waltzinger, C. Benoist, and D. Mathis. 1997. Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenovirus. *Immunity 7:123.*
32. Kirberg, J., A. Berns, and H. von Boehmer. 1997. Peripheral T cell survival requires continual ligation of the T cell receptor to MHC-encoded molecules. *J. Exp. Med. 186:1269.*
33. Nesic, D., and S. Vukmanovic. 1998. MHC class I is required for peripheral accumulation of $CD8^+$ thymic emigrants. *J. Immunol. 160:3705.*
34. Boursalian, T. E., and K. Bottomly. 1999. Survival of naive CD4 T cells: roles of restricting versus selecting MHC class II and cytokine milieu. *J. Immunol. 162: 3795.*
35. Witherden, D., N. van Oers, C. Waltzinger, A. Weiss, C. Benoist and D. Mathis. 2000. Tetracycline-controllable selection of $CD4^+$ T cells: half-life and survival signals in the absence of major histocompatibility complex class II molecules. *J. Exp. Med. 191:355.*
36. Brocker, T. 1997. Survival of mature CD4 T lymphocytes is dependent on MHC class II-expressing dendritic cells. *J. Exp. Med. 186:1223.*
37. Delon, J., N. Bercovici, G. Raposo, R. Liblau, and A. Trautmann. 1998. Antigen-dependent and -independent $Ca^{2+}$ responses triggered in T cells by dendritic cells compared with B cells. *J. Exp. Med. 188:1473.*
38. Janeway, C. A., Jr., A. Kupfer, C. Viret, T. Boursalian, J. Goverman., K. Bottomly, and D. B. Sant'Angelo. 1998. T cell development, survival and signalling: a new concept of the role of self peptide:self MHC complexes. *Immunologist 6:5.*
39. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods 171:131.*

40. Bender, J., T. Mitchell, J. Kappler, and P. Marrack. 1999. CD4$^+$ T cell division in irradiated mice requires peptides distinct from those responsible for thymic selection. *J. Exp. Med. 190:367.*
41. Ernst, B., D. S. Lee, J. M. Chang, J. Sprent, and C. D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity11:173.*
42. Goldrath, A. W., and M. J. Bevan. 1999. Low-affinity ligands for the TCR drive proliferation of mature CD8$^+$ T cells in lymphopenic hosts. *Immunity 11:183.*
43. Grusby, M., R. S. Johnson, V. E. Papaioannou, and L. H. Glimcher. 1991. Depletion of CD4$^+$ T cells in major histocompatibility complex class II deficient mice. *Science 253:1417.*
44. Kouskoff, V., K. Signorelli, C. Benoist, and D. Mathis. 1995. Cassette vectors directing expression of T cell receptor genes in transgenic mice. *J. Immunol. Methods 180:273.*
45. Janeway, C. A., Jr., P. J. Conrad, E. A. Lerner, J. Babich, P. Wettstein, and D. B. Murphy. 1984. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cell bound Ia antigens as targets of immunoregulatory T cells. *J. Immunol. 132:662.*
46. Lerner, E. A., L. A. Matis, C. A. Janeway, Jr., P. P. Jones, R. H. Schwartz, and D. B. Murphy. 1980. Monoclonal antibody against an Ir gene product? *J. Exp. Med. 152:1085.*
47. Ozato, K., N. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol. 124:533.*
48. Murphy, D. B., S. Rath, E. Pizzo, A. Y. Rudensky, A. George, J. K. Larson, and C. A. Janeway, Jr. 1992. Monoclonal antibody detection of a major self peptide.MHC class II complex. *J. Immunol. 148:3483.*
49. Oi, V. T., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol. 81:115.*
50. Rudensky, A. Y., P. Preston-Hurlburt, S. C. Hong, A. Barlow, and C. A. Janeway, Jr. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature 353:622.*
51. Rudensky, A. Y., S. Rath, P. Preston-Hurlburt, D. B. Murphy, and C. A. Janeway, Jr. 1991. On the complexity of self. *Nature 353:660.*
52. Murphy, D. B., D. Lo, S. Rath, R. L. Brinster, R. A. Flavell, A. Slanetz, and C. A. Janeway, Jr. 1989. A novel MHC class II epitope expressed in thymic medulla but not cortex. *Nature 338:765.*
53. Mathis, D., C. Benoist, V. E. Williams, M. Kanter, and H. O. McDevitt. 1983. Several mechanisms can account for defective E$\alpha$ gene expression in different mouse haplotypes. *Proc. Natl. Acad. Sci. USA 80:273.*
54. Viret, C., and C. A. Janeway, Jr. 2000. Functional and phenotypic evidence for presentation of E$\alpha$52–68 structurally related self-peptide(s) in I-E$\alpha$ deficient mice. *J. Immunol. 164:4627.*
55. Rudensky, A. Y. 1995. Endogenous peptides associated with MHC class II and selection of CD4 T cells. *Semin. Immunol. 7:399.*
56. Chervonsky, A. V., R. M. Medzhitov, L. K. Denzin, A. K. Barlow, A. Y. Rudensky, and C. A. Janeway, Jr. 1998. Subtle conformational changes induced in major histocompatibility complex class II molecules by binding peptides. *Proc. Natl. Acad. Sci. USA 95:10094.*
57. Barlow, A. K., X. He, and C. A. Janeway, Jr. 1998. Exogenously provided peptides of a self-antigen can be processed into forms that are recognized by self-T cells. *J. Exp. Med. 187:1403.*
58. Muranski, P., B. Chmielowski, and L. Ignatowicz. 2000. Mature CD4$^+$ T cells perceive a positively selecting class II MHC/Peptide complex in the periphery. *J. Immunol. 164:3087.*
59. Oehen, S., and K. Brduscha-Riem. 1999. Naive cytotoxic T lymphocytes spontaneously acquire effector function in lymphocytopenic recipients: a pitfall for T cell memory studies? *Eur. J. Immunol. 29:608.*
60. Kieper, W. C., and Jameson, S. C. 1999. Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. *Proc. Natl. Acad. Sci. USA 96:13306.*
61. Surh, C. D., E. K. Gao, H. Kosaka, D. Lo, C. Ahn, D. B. Murphy, L. Karlsson, P. Peterson, and J. Sprent. 1992. Two subsets of epithelial cells in the thymic medulla. *J. Exp. Med. 176:495.*
62. Mizuochi, T., M. Kasai, T. Kokuho, T. Kakiuchi, and K. Hirokawa. 1992. Medullary but not cortical thymic epithelial cells present soluble antigens to helper T cells. *J. Exp. Med. 175:160.*
63. Pierre, P., S. J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R. M. Steinman, and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature 388:787.*
64. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
65. Nakagawa, T. Y., and Rudensky, A. Y. 1999. The role of lysosomal proteinases in MHC class II-mediated antigen processing and presentation. *Immunol. Rev. 172:121.*
66. Baldwin, K. K., P. A. Reay, L. Wu, A. Farr, and M. M. Davis. 1999. A T cell receptor-specific blockade of positive selection. *J. Exp. Med. 189:13.*
67. Barthlott, T., R. J. Wright, and B. Stockinger. 1998. Normal thymic selection of TCR transgenic CD4 T cells, but impaired survival in the periphery despite the presence of selecting MHC molecules. *J. Immunol. 161:3992.*
68. Tough, D. F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med. 179:1127.*
69. Bonni, A., A. Brunet, A. E. West, S. R. Datta, M. A. Takasu, and M. E. Greenberg. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science 286: 1358.*
70. Pages, G., S. Guerin, D. Grall, F. Bonino, A. Smith, F. Anjuere, P. Auberger, and J. Pouyssegur. 1999. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science 286:1374.*
71. Chao, D. C., and S. J. Korsmeyer. 1998. BCL-2 family: regulators of cell death. *Annu. Rev. Immunol. 16:395.*
72. Kuo, C. T., M. L. Veselits, and J. M. Leiden. 1997. LKLF: a transcriptional regulator of single-positive T cell quiescence and survival. *Science 277:1986.*