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On the Self-Referential Nature of Naive MHC Class II-Restricted T Cells

Christophe Viret, Xin He, and Charles A. Janeway, Jr.

The use of mutant mice expressing a normal MHC class II molecule surface level but a severely restricted self-peptide diversity (H-2Mα−/−) previously revealed that T cells carrying the Eα52–68-I-Aβ 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 Eα52–68 structurally related self-peptide(s) as a major contributor to in vivo positive selection of 1H3.1 TCR-transgenic thymocytes in I-Aβ+/I-Eα− 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 Eα52–68-I-Aβ complex-specific Y-Ae mAb. Self-peptide(s) recognition is also driving the maturation of T cells carrying a distinct MHC class II-restricted specificity (the Eααβ TCR), since positive selection was also deficient in Eα TCR Tg H-2Mα−/− thymi. Such a requirement for recognition of self-determinants was mirrored in the periphery; Eα TCR Tg naive T cells showed an impaired persistence in both H-2Mα−/− and I-Aβ−/− 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 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 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 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 persisted and slowly cycled in wild-type recipients.
Mutant mice lacking the cathepsin L protease 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 (CD8αβ, CD4+ CD8− or CD4+ CD8+) peripheral αβ 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 αβ 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-2Mα−/−/− thymic microenvironment did not support positive selection of 1H3.1 TCR αβ Tg thymocytes, we found that persistence of mature naive 1H3.1 TCR Tg CD4+ T cells is impaired in irradiated H-2Mα−/−/− 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 CD8α T cells (42).

In this study, we show that the self-peptide(s) involved in positive selection of the Eo32–68-I-Aβ-specific 1H3.1 TCR Tg thymocytes forms peptide–I-Ab complex(es) structurally related to the Eo32–68-I-Aβ complex. We then used a distinct MHC class II-restricted T cell specificity (the Eoα β 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-Ab−/− mice (MHC class II-deficient) (38) were purchased from Taconic Farms (Germantown, NY). The 1H3.1 (Vα21-Jα21/ Vβ6-Dβ1-Jβ2.6) and Eo6 (Vα33.1-Jα33.1/Vβ5-Dβ1-Jβ2.1) TCR Tg mice were generated in this laboratory using pTα-Jα (43) -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 Y33P (mouse IgG2a, anti-I-Aβ) 14.8 (rat IgG2b, anti-CD45RA/B220), 53-6.2 and 2.43 (both rat IgG2b and anti-CD8) mAbs to remove APCs and CD8+ T cells. Cells were resuspended in three times, and resuspended in normal saline (5% or 20%). mAbs were given i.v. into the retro-orbital plexus of the eye using 5–6 x 106 cells/200 μl/mouse unless otherwise indicated. The 6- to 10-wk-old recipient animals used were sublethally irradiated (600 rad, 137Cs source; Yale University 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 1 x 107 cells/ml using 2 μM 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 x 106 cells were incubated in microtiter U-bottom plates with saturating concentrations of labeled mAb in 20 μl for 30 min on ice. Cells were washed twice and analyzed immediately after fixation. The mAbs used were anti-Vβ6-FITC (clone RR4-7), anti-β2-microglobulin (H-2Kb, H-2Db, H-2M), anti-I-A b (47), anti-I-A b-PE (H5-579), anti-I-C57BL/6-PE (MEL14), anti-I-Ea-bi (21-2), anti-CD45RB (16A), and anti-CD8 (R3-682) from eBioscience (San Diego, CA). FITC (53-6.7) from Life Technologies (Gaithersburg, MD); and anti-CD4-quantum red (H129.19) from Sigma (St. Louis, MO). The Y33P mouse IgG2a, anti-I-Aβ) 45, Y17 (mouse IgG2b, anti-I-Eα) (46), 25.9.17 (mouse IgG2a, anti-I-Aβ) (47), Y-Ae (mouse IgG2b, anti-Aβ+ Ea) (48), 10.2.16 (mouse IgG2a, anti-I-Aβ) (49), GK1.5 (rat IgG2b, anti-I-C44), 53-6.72 and 2.43 (both rat IgG2b, anti-CD8), and 14.8 (rat IgG2b, anti-CD45RA/BD) mAbs were affinity purified in the laboratory using standard affinity chromatography. 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-Ae to bind I-Ab molecules loaded with variants of the Eo32–68 peptide, C57BL/6 splenocytes were incubated 4 h at 37°C using 50 μg/ml of peptide in complete media. The cells were washed twice and stained for B220 and Y-Ae 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 x 10-3 M 2-ME (Bio-Rad, Richmond, CA), 2 mM L-glutamine, and 50 μg/ml gentamicin (Life Technologies). Depending on the experiment, T cells (30–50 x 10³/well) were stimulated using irradiated B6 splenocytes as APCs (3 x 10⁵ or less/well, 2000 rad) plus serial dilutions of synthetic Eo32–68 peptide (AS-PEAQGLANIAVDKA; single-letter amino acid code) or its variants in a total volume of 150 μl. The plates were centrifuged in duplicate wells, and 1 μCi of [3H]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 μg/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 Eo32–68 structurally related self-peptide(s) as a major contributor(s) to the maturation of the Eo32–68-I-Ab-specific 1H3.1 TCR Tg thymocytes

The 1H3.1 αβ TCR is specific for the 52–68 portion of the I-Ea chain complexed to I-Ab MHC class II molecules (50, 51). Such a complex is also specifically recognized by the Y-Ae mAb (48, 50–52). Thus, APCs from I-Ab/I-Eα+ mice (e.g., B10.A (5R))

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stain positively for Y-Ae and activate 1H3.1 TCR Tg T cells in a Y-Ae-inhibitable manner. Conversely, APCs from I-A<sup>B</sup>+/I-E<sup>a</sup> mice (e.g., C57BL/6) (53) stain negatively for Y-Ae 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<sup>a</sup> expression is due to an endogenous functional I-E<sup>a</sup> gene (1H3.1 TCR Tg B10.A (5R)) or is driven by a transgene (1H3.1 TCR/I-E<sup>a</sup> double Tg) (54) (our unpublished observations). We previously observed that neonatal injection of Y-Ae mAb was able to specifically interfere with the process of intrathymic deletion in 1H3.1 TCR/I-E<sup>a</sup> double-Tg mice (54); a low, but significant, fraction of V<beta>6 high CD4<sup>+</sup>CD8<sup>-</sup> thymocytes was rescued in the presence of Y-Ae, and E<sub>α<sub>52–68</sub>responsive T cells were detectable in the periphery. It was also striking to note that the Y-Ae 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-Ae. Thus, in the presence of Y-Ae, 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<sup>B</sup>+/I-E<sup>a</sup>) mice repeatedly treated with Y-Ae have an impaired capacity to respond to E<sub>α<sub>52–68</sub></sub> upon immunization (A. Y. Rudensky, C. E. Grubin, and C. A. Janeway, Jr., unpublished observations) (55). This result suggested that positive selection of CD4<sup>+</sup> T cells able to respond to E<sub>α<sub>52–68</sub></sub> involves self-peptides that are sufficiently structurally related to E<sub>α<sub>52–68</sub></sub> to be recognized by Y-Ae in the context of I-A<sup>B</sup>. To directly test this possibility, we injected newborn C57BL/6 1H3.1 TCR Tg mice with Y-Ae 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-Ae isotype (IgG2b). Fig. 1A shows that after 2 wk of treatment, the fraction of V<beta>6<sup>high</sup> thymocytes, that is, the population of cells that are beyond the stage of positive selection, is drastically reduced by the administration of Y-Ae. V<beta>6 distribution was virtually unchanged by Y-17 mAb treatment, indicating that the effect seen in the presence of Y-Ae is specific (Fig. 1A, bottom panels). In addition, the administration of isotype-matched 10.2.16 mAb, which reacts to many I-A<sup>B</sup> molecules but not to I-A<sup>B</sup>, had no effect (data not shown). Y-Ae mAb treatment also resulted in increased thymic cellularity. Consequently, the number of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes was augmented; in the experiment depicted in Fig. 1A, numbers of CD4<sup>+</sup>CD8<sup>-</sup> cells were: saline, 32.4 × 10<sup>6</sup>; Y-Ae, 81.84 × 10<sup>6</sup>; and Y17, 34.08 × 10<sup>6</sup>. These observations are consistent with an accumulation of immature thymocytes at the TCR<sup>low</sup>CD4<sup>+</sup>CD8<sup>-</sup> 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-Ae the fraction as well as the absolute number of thymocyte subpopulations were virtually unchanged compared with those in vehicle- or Y17-treated mice (Fig. 1B). The fact that a normal number of mature CD4<sup>+</sup> thymocytes is present in Y-Ae-treated non-Tg mice indicates that the inhibitory effect on positive selection of 1H3.1 TCR Tg thymocytes in vivo cannot be explained by depletion of thymic stromal cells. It also indicates that Y-Ae 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<sup>B</sup><sup>+</sup>/I-E<sup>a</sup> 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<sup>B</sup> molecules. Therefore, the inhibition of positive selection in Y-Ae-treated 1H3.1 TCR Tg mice indicates that Y-Ae effectively reacts to a C57BL/6 self-peptide(s) presented in the context of I-A<sup>B</sup>. Thus, H-2M<sup>+</sup>-dependent (25) self-peptide–I-A<sup>B</sup> complexes able to be recognized by Y-Ae represent major contributors to positive selection of the E<sub>α<sub>52–68</sub>–I-A<sup>B</sup></sub> complex-specific 1H3.1 TCR Tg thymocytes in C57BL/6 (I-A<sup>B</sup>+/I-E<sup>a</sup>−) mice. Presumably such complexes contain self-peptides with structural characteristics related to those of the E<sub>α<sub>52–68</sub></sub> peptide.

**Plasticity in Y-Ae reactivity**

To estimate the capacity of Y-Ae to recognize a range of diverse peptide–I-A<sup>B</sup> complexes, we analyzed the recognition of several length variants as well as mutants of E<sub>α<sub>52–68</sub></sub> by Y-Ae and the 1H3.1 TCR using immunostaining and proliferation assay (Fig. 3). Several irrelevant peptides able to bind I-A<sup>B</sup> molecules (56) were used as a negative control: β<sub>2m</sub>58–58, CD22<sub>25–29</sub>, and LDL receptor 486–501. As expected, these control peptides did not generate Y-Ae signal or proliferative T cell response. Y-Ae was able to accommodate virtually all truncations tested at both the NH<sub>2</sub> and COOH termini as well as elongation at the NH<sub>2</sub> terminus (Fig. 3A). We then tested E<sub>α</sub> peptide variants carrying point mutations. Since 1H3.1 T cells react well to the 56–64 truncated form of the peptide, the I-A<sup>B</sup> 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. 3B). Although the panel of mutants tested was limited, we observed that recognition by Y-Ae 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-Ae, while such changes completely extinguished the 1H3.1 T cell proliferative response (Fig. 3B). In addition, changes at positions 54 and 55 did not reduce the Y-Ae 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-Ae 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<sup>B</sup> (data not shown). Thus, it appears that Y-Ae 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-Ae recognition to I-A<sup>B</sup> molecules.

**Deficient positive selection of Ea6 TCR Tg thymocytes confronted with a restricted self-peptide complexity**

To further assess 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 Ea6 αβ TCR (V<sub>α<sub>23</sub></sub>–V<sub>β<sub>6</sub></sub>) specifically recognizes the E<sub>α<sub>52–66</sub>–I-A<sup>B</sup></sub> complex, but not the E<sub>α<sub>52–68</sub>–I-A<sup>B</sup></sub> complex recognized by the 1H3.1 αβ TCR (V<sub>α<sub>5</sub></sub>–V<sub>β<sub>6</sub></sub>) (57). Ea6 TCR αβ Tg mice (C. Viret and C. A. Janeway, Jr., unpublished observations) were bred to H-2M<sup>α<sup>−/−</sup></sub>/M<sup>−/−</sup> mice to generate Ea6 TCR Tg H-2M<sup>α<sup>−/−</sup></sub>-deficient mice. Results from immunostaining and flow cytometric analysis of lymphoid organs (Fig. 4A) show that the positive selection of immature Ea6 TCR Tg thymocytes is clearly altered in the absence of H-2M heterodimers; compared with TCR Tg H-2M<sup>α<sup>−/−</sup></sub> thymus (middle panels), a higher number of CD4<sup>+</sup>CD8<sup>-</sup> (double-positive) thymocytes appears in the TCR Tg H-2M<sup>α<sup>−/−</sup></sub> thymus (bottom panels; TCR Tg, 41.4 × 10<sup>6</sup>; TCR Tg H-2Mα<sup>−/−</sup>, 67.4 × 10<sup>6</sup>), and fewer mature CD4<sup>+</sup>CD8<sup>-</sup> thymocytes are detected (TCR Tg, 32.8 × 10<sup>6</sup>; TCR Tg H-2Mα<sup>−/−</sup>, 10.2 × 10<sup>6</sup>). In accordance, the V<beta>6<sup>high</sup> thymocyte fraction is severely reduced in the TCR Tg H-2Mα<sup>−/−</sup> thymus (Fig. 4A, central histograms). In the periphery, some V<beta>6<sup>+</sup> T cells accumulated in the TCR Tg H-2Mα<sup>−/−</sup> spleen and lymph nodes (Fig. 4B, mid-
dle and bottom panels), but the majority were expressing the CD8 coreceptor. This CD8 skewing is also visible in the thymic compartment (Fig. 4A, lower right panel). These observations indicated that immature thymocytes carrying Ea6 αβ TCR specificity do not efficiently develop in the H-2M\(^{a2}\) 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 Ea6 TCR αβ Tg CD4\(^{+}\) T cells in irradiated syngeneic recipients**

We previously observed that persistence of adoptively transferred 1H3.1 TCR αβ 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 cells in such experimental conditions. We adoptively transferred purified naive Ea6 TCR Tg CD4\(^{+}\)RAG-1\(^{-/-}\) T cells into irradiated normal C57BL/6 vs I-A\(^{b}\) \(^{-/-}\) and H-2M\(^{a}\) \(^{-/-}\) 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\(^{b}\)αβ and H-2M αβ heterodimers (i.e., I-A\(^{b}\) molecules presenting a normal array of self-peptides) are required for persistence of mature naive Ea6
TCR αβ Tg CD4+ T cells in secondary lymphoid organs of irradiated C57BL/6 (B6) recipients (Table I). Using CFSE-labeled naive Ea6 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-Ab complexes because it is not observed in recipient mice with a restricted self-peptide repertoire (see H-2M2 mice). 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% CD44low, 93–95% CD45RBhigh/CD62Lhigh, 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. 6A). 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 CD41 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. 6A) expression level was virtually unmodified, and CD25 and CD69 expressions remained negative (data not shown). Only CD49d expression appeared slightly increased during expansion (Fig. 6A). 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. 6B). 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-Ab molecules but a severely restricted self-peptide complexity (the H-2M2 mice) revealed a central role for self-peptide(s) recognition for positive selection of immature 1H3.1 T cells. The fact that the Y-Ae mAb, which, like an αβ TCR, interacts with both the peptide and polymorphic residues of I-Ab (such as I-Aaα2 and I-Abα5–67) (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 Eaα5–67. This result is consistent with the previous observation that 1H3.1 TCR Tg thymocytes were positively selected in I-Aa1 mice but not in I-Aa−/− mice (47). Because the Ea2 gene does not contain I-A subregion sequences (47), these results indicate that the Ea2 gene encodes a self-peptide(s) distinct from those encoded by the Ea1 gene. The observation that the Ea1 gene also encodes a self-peptide(s) that interferes with positive selection of 1H3.1 TCR Tg thymocytes (47) suggests that the Ea1 gene encodes multiple self-peptides that are structurally related but distinct from those encoded by the Ea2 gene.
FIGURE 3. Plasticity in E$^{52–68}$-I$^{a}$b recognition by Y$^{a}$-TCRs. Reactivity of the Y$^{a}$- and 1H3.1-synonymous immune receptors to distinct length variants (A) and mutants (B) of the E$^{a}$ peptide. The β2-microglobulin (β2m)$^{m}$, CD223, and LDL receptor 486–501 I$^{a}$-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 variants ($^{\text{m}}$). With the observation that Y$^{a}$-treated C57BL/6 mice have an impaired ability to mount a specific response upon immunization with E$^{52–68}$ (55), however, under the experimental conditions used by Rudensky (55), it remained possible that a residual amount of Y$^{a}$-TCR could interfere with the in situ priming of naive T cells. Our observation that Y$^{a}$-TCR interferes with positive selection of 1H3.1 T cells involves the in situ priming of naive T cells. Interestingly, in a distinct experiment, 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$^{52–68}$-binding peptide that do not generate a visualizable Y$^{a}$-TCR signal can efficiently activate 1H3.1 T cells (54).

In C57BL/6 mice the presentation of an E$^{52–68}$-independent, Y$^{a}$-TCR-relevant epitope(s) appears to allow both positive (this report and Ref. 55) and negative (54) intrathymic selection of CD4$^{+}$ T cells. Therefore, such epitopes are likely most constitutively expressed on both thymic epithelial cells and bone marrow-derived cells. Since in these two cell types, the lysosomal degrada-
tion of proteins involves distinct members of the cystein

protease family (cathepsins L and S, respectively) (65), we con-
clude that the generation of a peptide fragment(s) able to confer Y$^{a}$-TCR binding to I$^{a}$b complex(es) expressed in unmanipulated C57BL/6 mice is unlikely to involve the E$^{52–68}$ peptide itself for two reasons. First, using a walking RT-PCR we failed to detect any part of the transcript region encoding the E$^{52–68}$ sequence in C57BL/6 APCs (54). Second, while mature C57BL/6 dendritic cells are clearly Y$^{a}$-TCR$,^*$, they do not activate 1H3.1 T cells. This is in sharp contrast with the fact that the loading of C57BL/6 spleno-
cytes with doses of the E$^{52–68}$-binding peptide that do not generate a visualizable Y$^{a}$-TCR signal can efficiently activate 1H3.1 T cells (54).

The analysis of Ea6 TCR Tg H-2M$^a$/~ 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 Ea6 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 Ea6 TCR Tg thymocytes was mirrored in the periphery for the survival of mature Ea6 TCR Tg T cells; the persistence of naive Ea6 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 Ea6 TCR Tg T cells are unable to support the peripheral main-
tenance of naive Ea6 TCR Tg T cells. Interestingly, in a distinct
FIGURE 4. Deficient positive selection of the Eαβ TCR Tg immature thymocytes in the H-2Mα−/− thymic microenvironment. A, FACS analysis of thymic cell suspensions prepared from 4.5-wk-old Eαβ TCR Tg H-2Mα+/+ and H-2Mα−/− littermate mice. A TCR Tg-negative littermate is included. The central panels show the Vβ6 histogram. The left and right panels show the CD4/CD8 distribution, respectively, without and with electronic gating on the Vβ6high thymocyte population. Quadrant statistics are indicated. In the experiment depicted the cellularity was: TCR Tg H-2Mα+/+, 86.4 × 10⁶; and TCR Tg H-2Mα−/−, 93.6 × 10⁶. B, FACS analysis of spleen cell suspensions. The right panels indicate the CD4/CD8 distribution after electronic gating on the Vβ6− 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.
of adoptively transferred Eαβ Tg T cells. The peripheral low expansion of mature CD4+ T cells involves the Ras mitogen-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 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 Eαβ 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

The self-peptide(s)-dependent persistence of Eαβ Tg Tg CD4+ 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 measurable 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 αβ 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

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 × 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 αβ TCR was detected using the Cβ-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, enumerated, and stained for CD4 and Vβ6 before analysis by flow cytometry. The absolute numbers were calculated using the formula: (cellularity × percentage CD4+ Vβ6 cells)/100.

Table 1. Representative numbers of CD4+ Vβ6+ T cells recovered from the spleen of irradiated normal C57BL/6, I-Aβ−/−, and H-2Mα−/− mice after adoptive transfer of Eαβ TCR Tg CD4+ Vβ6+ T cells

<table>
<thead>
<tr>
<th>Day</th>
<th>C57BL/6</th>
<th>I-Aβ−/−</th>
<th>H-2Mα−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.34 × 10^6</td>
<td>0.24 × 10^6</td>
<td>0.26 × 10^6</td>
</tr>
<tr>
<td>8</td>
<td>2.05 × 10^6</td>
<td>0.04 × 10^6</td>
<td>0.1 × 10^6</td>
</tr>
</tbody>
</table>

A total of 5–6 × 10^6 Eαβ Tg Tg CD4+ 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 Vβ6 before analysis by flow cytometry. The absolute numbers were calculated using the formula: (cellularity × percentage CD4+ Vβ6 cells)/100.
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 TCRhigh 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−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 Eos52−/− mice structurally related, endogenous, self-peptide(s) contributes to positive selection of the Eos52−/−I-Aβ 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 adaptively transferred Eos6 TCR Tg T cells to 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


