# Paradoxical intrathymic positive selection in mice with only a covalently presented agonist peptide

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**The Y-A*e* mAb and the 1H3.1 $\alpha\beta$ T cell antigen receptor (TCR) are both specific for the I-E$\alpha$52–68 peptide bound to the I-A$^b$ major histocompatibility complex (MHC) class II molecule. Antigen-presenting cells (APCs) from I-A$^{b+}$ mice with a natural or transgenic (Tg) I-E$\alpha$ chain activate mature 1H3.1 T cells and cause the deletion of 1H3.1 TCR Tg thymocytes. However, 1H3.1 T cells were neither activated nor inactivated by confrontation with APCs from I-Ab-Ep mice in which I-A$^b$ molecules are occupied only by the covalently associated E$\alpha$52–68 peptide. Instead, immature 1H3.1 TCR Tg thymocytes were efficiently positively selected into the CD4 lineage in the I-Ab-Ep thymus. This selection relied on specific recognition of the E$\alpha$52–68/I-A$^b$ complex because it was blocked by Y-A*e*. 1H3.1 TCR Tg T cells maturing in the I-Ab-Ep thymus efficiently populated the periphery, displayed a naive phenotype, and were specifically reactive to the E$\alpha$52–68 peptide or to I-A$^{b+}$I-E$\alpha^+$ APCs, indicating that 1H3.1 T cells were not antagonized in I-Ab-Ep mice. The data identify major histocompatibility complex class II molecules with only a covalently attached self-peptide as a ligand for *in vivo* positive selection of T cells specific for the same peptide.**

The maturation of conventional $\alpha\beta$ T cells relies on the interaction of their T cell antigen receptor (TCR) with self-major histocompatibility complex (MHC) molecules presenting self-peptides in the thymus. This interaction either rescues immature thymocytes from apoptosis and signals them to complete their maturation (positive selection), or causes their deletion by precipitating apoptosis (negative selection). These opposed outcomes ensure the generation of a highly diverse mature TCR repertoire that is tolerant to virtually all thymic self-determinants (1–4). The recognition of self-peptide/self-MHC complexes is inherent to negative selection. In contrast, the nature of the TCR–MHC interaction involved in positive selection has been a matter of discussion (5–7). Early studies using natural mutations affecting MHC class I amino acid residues important for peptide binding but not for TCR–MHC interaction (8, 9) and *in vitro* studies using fetal thymic organ culture (FTOC) from wild-type or TCR transgenic (Tg) MHC class I deficient mice (10–15) indicated that self-peptide recognition does have a role in the positive selection of CD8$^+$ T cells. For a given TCR, positive selection can be supported by peptides with minor or no change compared with the antigenic peptide. However, CD8$^+$ T cells selected in the presence of agonist peptides displayed an altered coreceptor expression level and were functionally deficient (16).

The role of self-peptides in the positive selection of MHC class II-restricted T cells was studied mostly by using mice with a defect in the antigen processing and/or presentation pathway. Mice deficient in the peptide exchange factor H-2 M (H-2M$\alpha^{-/-}$; refs. 17–19) and mice expressing only the E$\alpha$52–68/I-A$^b$ complex (I-Ab-Ep; refs. 20 and 21) revealed that when thymic stromal cells express one or a few peptide/MHC class II complexes, only a quarter to a half of the normal CD4$^+$ T cell numbers is detected in the periphery. Many of these CD4$^+$ T cells indeed differ from normal CD4$^+$ T cells because they react to syngeneic antigen-presenting cells (APCs). In addition, seven distinct CD4$^+$ T cell specificities that develop within a wild-type thymus fail to do so in H-2M$\alpha^{-/-}$ mice (22–26). Maturation of CD4$^+$ T cells is also impaired in mice with an altered self-peptide repertoire presented by MHC class II molecules on thymic epithelial cells (cathepsin L$^{-/-}$; ref. 27) or mice where a single peptide occupies more than 95% of all MHC class II molecules (28). The restriction of the parental $\alpha$-chain junction in mature thymocytes from single $\beta$-chain TCR Tg mice (29) and the blockade of positive selection of CD4 T cells by peptide/MHC class II specific mAbs *in vivo* (26, 30) provide additional evidence for the peptide specificity of positive selection of CD4 T cells. Despite the fact that fewer CD4$^+$ T cells are positively selected in I-Ab-Ep and H-2M$\alpha^{-/-}$ mice, they displayed an apparently diverse TCR repertoire, indicating that positive selection may be more promiscuous than suggested for CD8 T cells. The adenovirus-mediated delivery of invariant chain (Ii)-peptide-fusion proteins in the thymus also revealed that whereas the selection of the moth cytochrome *c* (MCC)-specific TCR repertoire was peptide-specific, positive selection seemed promiscuous. MCC-specific T cells could be positively selected by the MCC peptide itself, close analogs, or unrelated peptides (31, 32).

Thus, whereas many lines of evidence now support the importance of self-peptide/self-MHC complex recognition in positive selection, the degeneracy of such recognition, the structural relationship between the selecting and the antigenic ligands and their potential physiological consequences remain less well delineated. Here, we report that the continuous *in vivo* expression of I-A$^b$ molecules presenting only the covalently associated E$\alpha$52–68 peptide supports the intrathymic positive selection and the peripheral persistence of E$\alpha$52–68/I-A$^b$ complex-specific naive T cells.

## Methods

**Animals.** C57BL/6 (B6), SJL, B10.BR-H2$^{k2}$ H2-T18$^a$/SgSnJ (B10.BR), and B10.A-H2$^{i5}$ H2-Tl8$^a$(5R)SgSnJ (5R) mice were obtained from The Jackson Laboratory. The 1H3.1 TCR Tg mice (V$\alpha$1/V$\beta$6) are described elsewhere (33). The I-A$^b$-Ep mice (20) were a gift of P. Marrack and J. Kappler (Howard Hughes Medical Institute, National Jewish Medical and Research Center, Denver, CO). The I-E$\alpha^d$ Tg 107.1 (107 Tg) mice were generated by R. A. Flavell (Howard Hughes Medical Institute, Yale University, New Haven, CT; ref. 34).

**mAb Treatment of Newborn Mice.** Newborn mice were genotyped by PCR on day 1 by using tail genomic DNA. Starting on day 2, animals were intraperitoneally injected with 50 $\mu$g of purified Y17 or Y-A*e* mAb (both mouse IgG2a) diluted in 50 $\mu$l of normal saline every 2 days for 2 weeks. On day 13 to 15, mice were killed, the thymus and spleen were removed, and single-cell suspensions

Abbreviations: TCR, T cell antigen receptor; APC, antigen presenting cell; Tg, transgenic; Ii, invariant chain; LPS, lipopolysaccharide; DCs, dendritic cells; BM, bone marrow; MIIC, MHC class II compartment.

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were prepared by using standard procedures for phenotypic analysis.

**mAbs, Immunostaining, and Flow Cytometry.** Fluorescent-labeled mAbs were used for multicolor staining. Briefly, $0.2 \times 10^6$ cells were incubated in microtiter U-bottom plates with a saturating concentration of labeled mAb in 20 $\mu$l for 30 min on ice. Cells were washed twice and analyzed immediately. For two-step staining, cells were incubated first with purified mAbs in PBS 2% (vol/vol) FCS/0.1% $NaN_3$, followed by an $F(ab')_2$ fragment of goat anti-mouse Ig-FITC conjugate (Sigma). The mAbs used were anti-V$\beta$6-FITC (clone RR4–7), anti-V$\alpha$2,3.2,8,11-FITC (B20.1, RR3–16, B21.14, RR8–1), anti-CD45R/B220-phycoerythrin (PE) (RA3–6B2), anti-CD86/B7–2-biotin (GL1) from PharMingen, anti-CD8$\alpha$-PE/FITC (53–6.7) from Life Technologies (Grand Island, NY), and anti-CD4-quantum red (H129.19) from Sigma. The Y3JP (mouse IgG2a, anti-I-$A^b$), 25–9-17 (mouse IgG2a, anti-I-$A^b$), Y-A*e* (mouse IgG2b, anti-$A^b$+E$\alpha$52–68), Y17 (mouse IgG2b, anti-I-E), 53–6.72 and 2.43 (both rat IgG2b, anti-CD8) and 14.8 [rat IgG2b, anti-CD45RA(B220)] mAbs were affinity-purified from hybridoma supernatants. Lipopolysaccharide (LPS) blasts were obtained by treating splenocytes with LPS (*Escherichia coli* 0111:B4; Sigma) for 2 days in culture. A FACScan flow cytometer and the CELLQUEST software, both from Becton Dickinson, were used to collect and analyze the data. Nonviable cells were excluded by using forward- and side-scatter electronic gating.

**Functional Assays.** For proliferation assay, T cells were cultured in U-bottom 96-well plates for 3 days at 37°C in Click's media (EHAA, Irvine Scientific) supplemented with 5% (vol/vol) FCS, $5 \times 10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine, and 50 $\mu$g/ml gentamicin. In some cases, $CD4^+$ T cells were enriched to 90/95% by depletion of $CD8^+$ and MHC class $II^+$ cells with anti-CD8, anti-B220, and anti-I-$A^b$ mAbs. T cells ($30–50 \times 10^3$ cells per well) were stimulated by using irradiated B6 splenocytes ($2.5 \times 10^5$ per well) plus serial dilutions of E$\alpha$52–68 peptide (ASFEAQGALANIAVDKA) in 150 $\mu$l of medium. The cells were incubated in duplicate wells and 1 $\mu$Ci (1 Ci = 37 GBq) of [$^3$H]thymidine per well was added to the culture during the last 12 hr. The plates then were harvested and counts per minute were determined. For inhibition experiments, purified mAbs were sterile-filtered and added to cultures (3–5 $\mu$g/ml final).

**Derivation of Dendritic Cells (DCs) from Bone Marrow (BM) Progenitors and Reprocessing of Antigens from Apoptotic B Cells Engulfed by Immature DCs.** DCs were generated *in vitro* from BM progenitors in complete RPMI medium 1640 supplemented with 1% recombinant granulocyte/macrophage colony-stimulating factor as described (33, 35, 36). Apoptotic B cells were derived by culturing splenocytes with 10 $\mu$g/ml LPS for 5 days in complete media. Apoptotic B cells were loaded on immature (day 5–6) DCs 24 hr before stimulation experiments as described (36, 37).

## Results

**Encounter with APCs Expressing a Covalent Configuration of the E$\alpha$52–68/I-$A^b$Complex Neither Activates nor Inactivates the E$\alpha$52–68/I-$A^b$Complex-Specific 1H3.1 T Cells.** The 1H3.1 TCR and the Y-A*e* mAb both react to the 52–68 fragment of the I-E$\alpha$ chain presented in the context of I-$A^b$ molecules (38–40). 1H3.1 TCR Tg T cells are induced, in a manner to inhibit Y-A*e*, to proliferate in response to E$\alpha$52–68 peptide plus B6 (I-$A^{b+}$/I-E$\alpha^-$) APCs or to 5R (I-$A^{b+}$/I-E$\alpha^+$) splenocytes that naturally express the E$\alpha$52–68/I-$A^b$ complex (33). Thus, the 1H3.1 TCR reacts to E$\alpha$52–68/I-$A^b$ complexes assembled either extracellularly or intracellularly. Whereas they develop normally in B6 mice (33), 1H3.1 TCR Tg T cells undergo a drastic intrathymic deletion in 5R mice or in I-E$\alpha$ Tg B6 mice. We thought to compare the activation of naive 1H3.1 TCR T cells by 5R APCs where the E$\alpha$52–68/I-$A^b$ complex represents 10–12% of all surface peptide/MHC class II complexes (41) vs. APCs from I-Ab-Ep mice where the E$\alpha$52–68 peptide is loaded onto 100% of I-$A^b$ molecules (20). The single peptide presentation is caused by the expression of a transgene (I-Ab-Ep) where the E$\alpha$52–68 sequence is fused to the I-$A^b\beta$ sequence (hereafter called the covalent configuration of the E$\alpha$52–68/I-$A^b$ complex; ref. 42), as well as to the deficiency of both Ii ($Ii^{-/-}$) and I-$A^b\beta$ chain (I-$A^b\beta^{-/-}$; ref. 20).

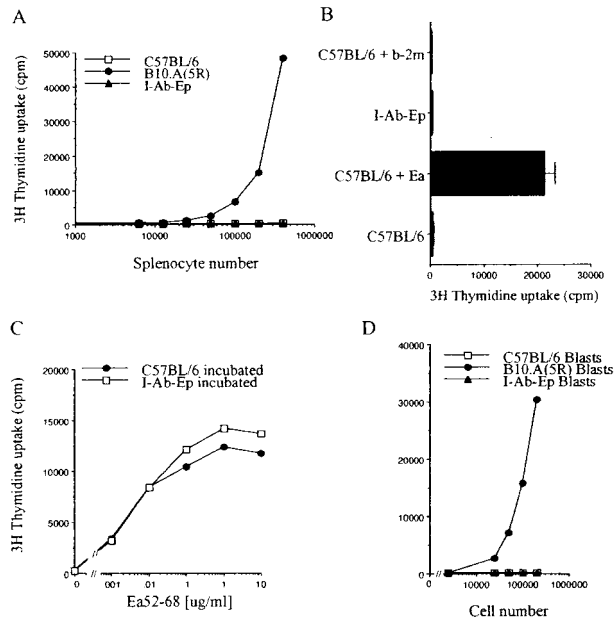


**Fig. 1.** Mature 1H3.1 TCR Tg T cells are neither activated nor inactivated by I-Ab-Ep APCs. (*A*) Naive 1H3.1 TCR Tg T cells do not proliferate in response to I-Ab-Ep splenocytes. 5R and B6 splenocytes were used as controls. (*B*) The original 1H3.1 T hybridoma does not react to I-Ab-Ep splenocytes. The E$\alpha$52–68 (Ea) and $\beta$2-microglobulin 48–58 (b-2m) peptides plus B6 APCs were used as positive and negative controls. (*C*) Naive 1H3.1 T cells exposed to I-Ab-Ep APCs do not show signs of anergy. 1H3.1 TCR Tg T cells were incubated overnight with B6 or I-Ab-Ep purified B cells; the cultures then were enriched for $CD4^+$ T cells and challenged with E$\alpha$52–68 peptide plus B6 splenocytes. (*D*) LPS-induced I-Ab-Ep B cell blasts do not induce proliferation of naive 1H3.1 TCR Tg T cells. Data are representative of eight (*A*), five (*B*), and two (*C* and *D*) experiments, respectively.

Although multiple E$\alpha$52–68/I-$A^b$ complex-specific T cells specifically react to I-Ab-Ep APCs (20, 21), naive 1H3.1 TCR Tg T cells neither produce IL-2 nor proliferate in response to I-Ab-Ep splenocytes (Fig. 1*A*). The original 1H3.1 hybrid also failed to respond (Fig. 1*B*). Because 1H3.1 T cells react to 107 Tg (B6 I-E$\alpha$ Tg) $B220^+$ splenocytes which stain brighter for Y-A*e* than I-Ab-Ep $B220^+$ cells (data not shown) and to minute amounts of the E$\alpha$52–68 peptide that do not generate a visualizable Y-A*e* signal on B6 APCs (33), the lack of response to I-Ab-Ep APCs is not caused either by the expression of too many or of too few Y-A*e* epitopes. We then considered anergy as a possible mechanism for the unresponsiveness of 1H3.1 T cells. Exogenous IL-2 had no effect on 1H3.1 T cells exposed to I-Ab-Ep splenocytes (data not shown). We then purified naive 1H3.1 TCR Tg $CD4^+$ T cells, exposed them to B6 or I-Ab-Ep purified B cells, repurified 1H3.1 T cells, and stimulated them with E$\alpha$52–68 and B6 APCs. Lack of costimulation was not an explanation for the very similar response in both cases (Fig. 1*C*) because LPS-induced I-Ab-Ep blasts, which express a high level of B7 molecules (data not shown), were not stimulatory (Fig.

1*D*). Therefore, I-Ab-Ep APCs do not seem to induce anergy in naive 1H3.1 TCR Tg T cells.

Finally, all of the I-Ab-Ep APC-responsive T hybrids we have tested (5/5) were CD4-dependent; they did not respond to E$\alpha$52–68 loaded onto APCs with mutant I-A$^b\beta$ chains unable to interact with CD4 (ref. 43 and data not shown). Such requirement suggests that they coengage CD4 when reacting to I-Ab-Ep APCs and, therefore, that the ability of the I-Ab-Ep protein to interact with CD4 is intact. Thus, the inability of 1H3.1 T cells to respond to I-Ab-Ep APCs probably does not result from a failure to establish a productive CD4–I-A$^b$ interaction. Collectively, the data indicate that the E$\alpha$52–68/I-A$^b$ complex-specific 1H3.1 T cells are neither activated nor inactivated by encounter with I-Ab-Ep splenocytes. Theses results seem to be in contradiction with our earlier finding that adoptively transferred naive 1H3.1 TCR Tg T cells undergo a strong expansion into sublethally irradiated I-Ab-Ep hosts (25).

**Positive Selection of 1H3.1 TCR Tg Thymocytes in Mice with Expression of I-A$^b$Molecules Presenting Only the Covalently Attached E$\alpha$52–68 Peptide.** Because immature T cells are more sensitive than mature T cells to stimulation through their TCR (44, 45), we asked whether 1H3.1 TCR Tg thymocytes would undergo deletion in the I-Ab-Ep thymus. Remarkably, the analysis of lymphoid organs from 1H3.1 TCR Tg I-Ab-Ep (1H3.1 TCR $\alpha\beta$ Tg I-Ab-Ep Tg Ii$^{-/-}$ I-A$^b\beta^{-/-}$) mice (Fig. 2) revealed a profile that was fully consistent with positive selection. Despite the fact that the thymic cellularity was lower than that of normal 1H3.1 TCR Tg mice (80–100 $\times$ 10$^6$ vs. 110–130 $\times$ 10$^6$), it was definitely distinct from that of 5R 1H3.1 TCR Tg mice, which show a drastic intrathymic deletion (3–5 $\times$ 10$^6$). The V$\beta$6 expression was similar to that of 1H3.1 TCR Tg mice: a large population of cells (virtually all of them were CD4$^+$CD8$^-$) were V$\beta$6$^{high}$ (Fig. 2*A*). In the periphery, a large number of V$\beta$6$^+$ cells was detected in the spleen and lymph nodes and, as with normal 1H3.1 TCR Tg mice, these cells were mainly CD4$^+$ with only a few CD8$^+$. The TCR- and CD4-expression levels were identical to those observed in normal 1H3.1 TCR Tg mice (Fig. 2*B*). V$\beta$6$^+$CD4$^-$CD8$^-$ peripheral cells that accumulate in 1H3.1 TCR Tg/I-A$^{b+}$/I-E$\alpha^+$ mice (33) were virtually absent. Furthermore, immunostaining with anti-V$\beta$ and anti-V$\alpha$ mAbs showed that allelic exclusion was as efficient as in normal 1H3.1 TCR Tg mice (data not shown). As expected, B220$^+$ cells from 1H3.1 TCR Tg I-Ab-Ep mice were Y3JP$^+$ and Y-A*e*$^+$, but they stained negatively with the 25.9.17 mAb which reacts to multiple peptide/I-A$^b$ complexes but not to the E$\alpha$52–68/I-A$^b$ complex (ref. 46 and data not shown). Only the reintroduction of Ii, which leads to disruption of the covalent complex (42), resulted in deletion of 1H3.1 thymocytes (data not shown). Thus, in mice where thymic stromal cells continuously express I-A$^b$ molecules presenting only the covalently associated E$\alpha$52–68 peptide, 1H3.1 TCR Tg thymocytes are positively selected in an efficient manner in the proper coreceptor lineage and signaled to populate the periphery.

**Recognition of the E$\alpha$52–68/I-A$^b$Complex Directs the Intrathymic Positive Selection of 1H3.1 T Cells in I-Ab-Ep Mice.** In I-Ab-Ep mice, the evidence that 1H3.1 TCR Tg thymocytes are positively selected on the E$\alpha$52–68/I-A$^b$ complex itself is twofold. First, we know that CD4$^+$ 1H3.1 T cells do not mature in the thymus of 1H3.1 TCR Tg MHC class II-deficient (I-A$^b\beta^{-/-}$) mice (26). Therefore, neither classical nor nonclassical MHC class I molecules can drive the positive selection of 1H3.1 TCR Tg thymocytes. Second, positive selection of 1H3.1 TCR Tg thymocytes in I-Ab-Ep mice can be inhibited by the E$\alpha$52–68/I-A$^b$ complex-specific Y-A*e* mAb but not the isotype-matched Y17 mAb (Fig. 3). Such positive selection does not rely solely on a peptide-independent interaction with I-A$^b$ or on a tickling received through CD4 because in H-2M$\alpha^{-/-}$ mice, which display a normal level of I-A$^b$ molecules with intact CD4-binding sites but a highly restricted self-peptide diversity, positive selection of 1H3.1 thymocytes is deficient (25). Thus, positive selection of 1H3.1 T cells in I-Ab-Ep mice is driven by the process of self-peptide/self-MHC class II complex recognition known to operate in the maturation of conventional CD4$^+$ $\alpha\beta$ T cells (47–50). In this instance, positive selection of E$\alpha$52–68/I-A$^b$-specific T cells paradoxically relies on the recognition of the E$\alpha$52–68/I-A$^b$ complex.

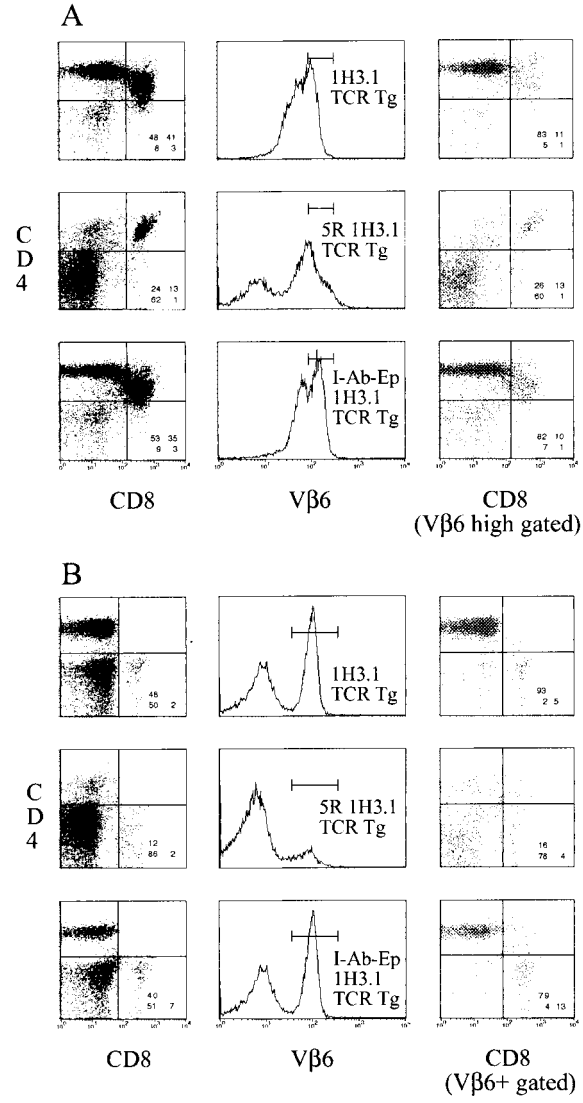


**Fig. 2.** 1H3.1 TCR Tg thymocytes are positively selected in mice with only the covalently presented E$\alpha$52–68 peptide. Phenotypic analysis of thymocytes (*A*) and splenocytes (*B*) from 1H3.1 TCR Tg (I-A$^{b+}$/I-E$\alpha^-$), 1H3.1 TCR Tg 5R (I-A$^{b+}$/I-E$\alpha^+$), and 1H3.1 TCR Tg I-Ab-Ep mice. The central panels show the V$\beta$6 histograms (bars indicate gates); the left and right panels show the CD4 and CD8 distribution, respectively, with and without electronic gating on the V$\beta$6$^{high}$ (*A*) or V$\beta$6$^+$ (*B*) cells. The percentages of cells in each quadrant are indicated in the lower right quadrant. The profiles are representative of seven stains. In the depicted experiment, the cellularity was TCR Tg, 142.6 $\times$ 10$^6$; TCR Tg 5R, 5.5 $\times$ 10$^6$; and TCR Tg I-Ab-Ep, 112 $\times$ 10$^6$.

**Naive T Cell Phenotype and Intact Functionality of Peripheral T Cells from 1H3.1 TCR Tg I-Ab-Ep Mice.** Besides the fact that peripheral CD4$^+$V$\beta$6$^+$ cells showed normal expression levels of TCR and

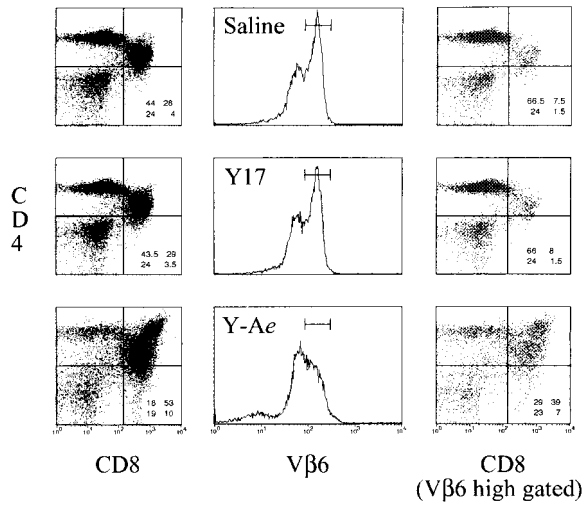


**Fig. 3.** Intrathymic positive selection of 1H3.1 TCR Tg thymocytes in I-Ab-Ep mice is driven by recognition of the Eα52–68/I-A$^b$ complex. Phenotypic analysis of thymocytes from mAb-treated mice. 1H3.1 TCR Tg I-Ab-Ep newborn mice were injected with the Y-Ae mAb, the isotype-matched Y17 mAb, or vehicle (saline). Note the accumulation of true CD4$^+$CD8$^+$ thymocytes in the presence of Y-Ae treatment. The profiles are organized as in Fig. 2. In this experiment, the thymic cellularity was saline-injected, 83.5 × 10$^6$; Y-Ae-injected, 139.5 × 10$^6$; and Y17-injected, 78.5 × 10$^6$.

CD4 (Fig. 2), most of them were CD44$^{low}$ and CD62L$^{high}$ (Fig. 4). Surface expression of the CD25 and CD69 activation markers was undetectable (data not shown). These features are most consistent with a naive T cell phenotype (51), indicating that

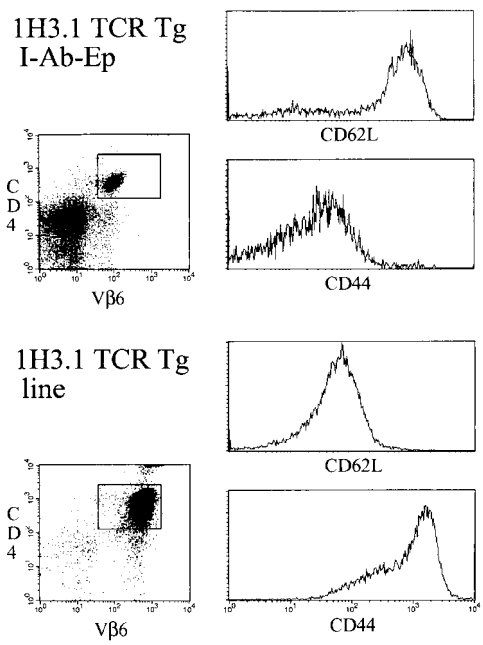


**Fig. 4.** Vβ6$^+$CD4$^+$ peripheral cells from 1H3.1 TCR Tg I-Ab-Ep mice display a naive T cell phenotype. Splenocytes from 4-week-old 1H3.1 TCR Tg I-Ab-Ep mice were triple-stained for CD4, Vβ6, and either CD62L or CD44 molecules. CD44 and CD62L surface expression are represented as histograms after electronic gating on Vβ6$^+$CD4$^+$ cells. 1H3.1 TCR Tg T cells maintained *in vitro* by specific stimulation were used as a nonnaive control. The data are representative of seven mice analyzed.

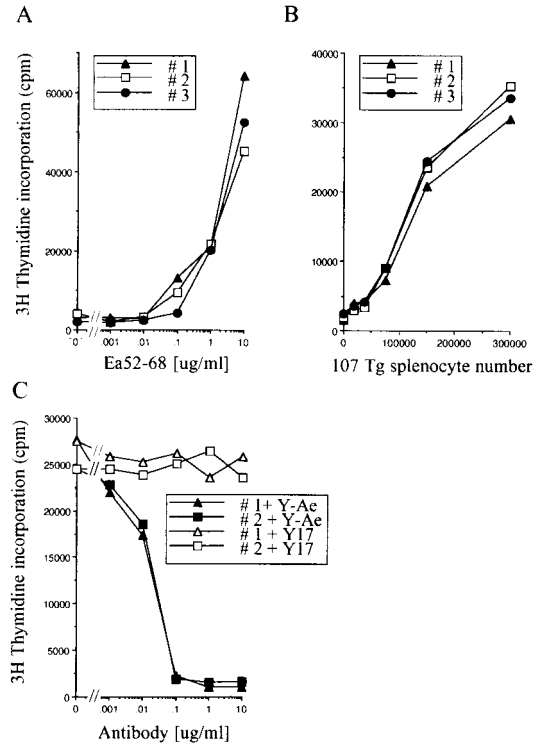


**Fig. 5.** Peripheral T cells from 1H3.1 TCR Tg I-A$^b$-Ep mice specifically respond to the Eα52–68/I-A$^b$ complex. (*A*) Proliferative response of freshly isolated lymph-node cells from 1H3.1 TCR Tg I-A$^b$-Ep mice; Eα52–68 peptide presented by B6 APCs. Three mice (#1, #2, and #3) were analyzed in this experiment. (*B*) Reactivity to B6 APCs that constitutively express the Y-Ae epitope caused by an I-Eα a transgene (107 Tg splenocytes). (*C*) The proliferative response to 107 Tg APCs is inhibited by Y-Ae but not by Y17. The response to Eα52–68 peptide is inhibited by Y-Ae as well (data not shown). Results are representative of three experiments.

these cells are not activated by the continuous *in vivo* confrontation of I-Ab-Ep APCs. To assess their functionality, we stimulated CD4$^+$Vβ6$^+$ cells with noncovalent forms of the Eα52–68/I-A$^b$ complex. Both Eα52–68-loaded B6 splenocytes and B6 I-Eα Tg (107 Tg) splenocytes were able to induce proliferation of naive Vβ6$^+$CD4$^+$ T cells from 1H3.1 TCR Tg I-Ab-Ep mice (Fig. 5 *A* and *B*). This reactivity is unequivocally specific because it is inhibited in a dose-dependent manner by Y-Ae but not Y17 (Fig. 5*C*). Thus, 1H3.1 TCR Tg T cells that mature *in vivo* in the I-Ab-Ep thymus are not antagonized but rather remain naive and retain the capacity to specifically react to noncovalent configurations of the Eα52–68/I-A$^b$ complex.

**Mature but Not Immature I-Ab-Ep DCs Derived from BM Progenitors Specifically Activate 1H3.1 T Cells.** In APCs expressing the I-Ab-Ep construct, the Eα52–68/I-A$^b$ complexes assemble in the endoplasmic reticulum and egress rapidly from it to reach the cell surface (52). These complexes are present in multivesicular MHC class II compartment (MIICs), but unlike endogenously assembled Eα52–68/I-A$^b$ complexes from I-A$^{b+}$/I-Eα$^+$ APCs or endocytosed antigens (53) do not reach denser compartments such as multilaminar or intermediate MIICs (52) which contain high levels of cathepsin proteases and H-2 M (54). To examine whether forcing Eα52–68/I-A$^b$ complexes from I-Ab-Ep APCs to access dense MIICs would modify their properties, we loaded apoptotic I-Ab-Ep B cells onto DCs derived from BM progenitors. At their immature stage, DCs are highly phagocytic and have the capacity to extract peptides from engulfed apoptotic

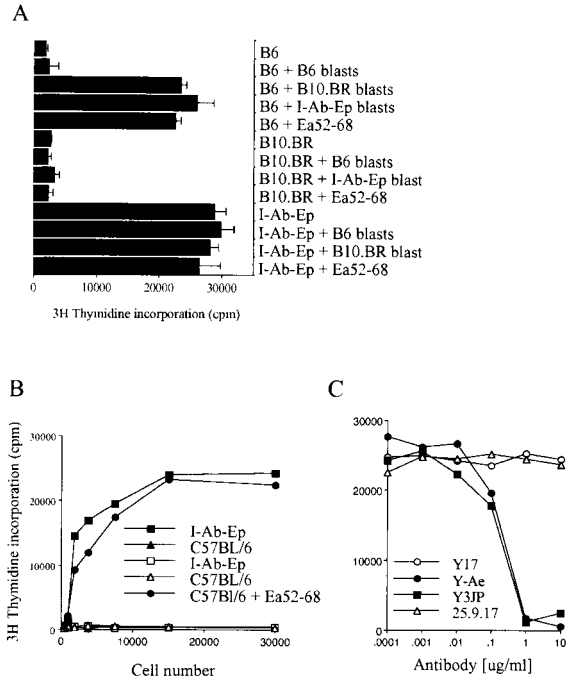


**Fig. 6.** Mature, but not immature, I-Ab-Ep DCs derived from BM progenitors specifically activate 1H3.1 T cells. (*A*) Day 5 granulocyte/macrophage colony-stimulating factor-driven BM cultures from B6 ($A^{b+}/E\alpha^{-}$), B10.BR ($A^{b-}/E\alpha^{+}$), and I-Ab-Ep mice were loaded with apoptotic LPS-blasts and used to stimulate 1H3.1 TCR Tg T cells in a proliferation assay. The values represent the mean ± SD of duplicate cultures. The data are representative of four experiments. (*B*) Unmanipulated, fully mature (day 8–9) I-Ab-Ep DCs, but not immature (day 5–6) DCs, induce 1H3.1 TCR Tg T cells to proliferate. Closed symbols, mature DCs; open symbols, immature DCs. Similar results were obtained by using total cells from BM culture or sorted DCs (data not shown). The values are representative of four experiments. (*C*) Y-Ae and Y3JP, but not Y17 or 25.9.17, inhibit the proliferative response of 1H3.1 TCR Tg T cells to mature I-Ab-Ep DCs. The data are representative of three experiments.

cells before presentation on MHC class II molecules at the mature stage (36, 37, 55). As expected, B6 ($I\text{-}A^{b+}/I\text{-}E\alpha^{-}$) DCs loaded with both I-Ab-Ep and B10.BR ($I\text{-}A^{b-}/I\text{-}E\alpha^{+}$) but not with B6 apoptotic B cells were able to activate 1H3.1 TCR Tg T cells (Fig. 6*A*). Surprisingly, I-Ab-Ep DCs were able to cause activation without loading. Such effect was observed for day 8–9 I-Ab-Ep BM cultures (i.e., mature DCs) but not for I-Ab-Ep cells from day 5–6 cultures (i.e., immature DCs; Fig. 6*B*). Finally, mAb-blocking experiments showed that this activation is specific (Fig. 6*C*). These results indicate that forcing $E\alpha 52\text{–}68/I\text{-}A^{b}$ complexes from I-Ab-Ep APCs to traffic through the endocytic pathway allows the extraction of Eα52–68 and the assembly of noncovalent complexes. The data also show that mature I-Ab-Ep DCs specifically activate 1H3.1 T cells. This activity most likely relies on the same phenomenon: highly phagocytic immature I-Ab-Ep DCs have the capacity to engulf apoptotic neighboring I-Ab-Ep cells and to extract Eα52–68 before presentation at their mature stage. The fact that mature, but not immature, DCs from B6 + B10.BR (i.e., $I\text{-}A^{b+}/I\text{-}E\alpha^{-}$ + $I\text{-}A^{b-}/I\text{-}E\alpha^{+}$) but not B6 + SJL (i.e., $I\text{-}A^{b+}/I\text{-}E\alpha^{-}$ + $I\text{-}A^{b-}/I\text{-}E\alpha^{-}$) mixed BM cultures specifically induced 1H3.1 TCR Tg T cells to proliferate supports this notion (data not shown).

## Discussion

In contrast with multiple $E\alpha 52\text{–}68/I\text{-}A^{b}$-specific T hybridomas (20, 21), 1H3.1 T cells do not react *in vitro* to I-Ab-Ep splenocytes on which all of the $I\text{-}A^{b}$ molecules present Eα52–68 covalently attached to $I\text{-}A^{b}$. However, 1H3.1 T cells do recognize the $E\alpha 52\text{–}68/I\text{-}A^{b}$ complex on I-Ab-Ep MHC class $II^{+}$ cells because the I-Ab-Ep thymus supports positive selection of 1H3.1 TCR Tg thymocytes *in vivo*. That is, recognition of the covalent configuration of the $E\alpha 52\text{–}68/I\text{-}A^{b}$ complex delivers an anti-apoptotic signal to 1H3.1 TCR Tg thymocytes. The fact that naive functional T cells are abundant in the periphery of 1H3.1 TCR Tg I-Ab-Ep mice suggests that recognition of the $E\alpha 52\text{–}68/I\text{-}A^{b}$ complex on I-Ab-Ep APCs also provides the survival signal necessary for peripheral persistence (48, 56) and shows that these cells are not antagonized.

The fine mechanism underlying this phenotype is unclear. We can formulate at least three hypotheses to explain it. First, the presence of the flexible linker could induce a subtle conformational change in the $E\alpha 52\text{–}68/I\text{-}A^{b}$ complex. Such an altered conformation could allow the activation of several specific T hybrids (but not of 1H3.1 T cells). Indeed, both N- and C-terminal extensions of the bound peptides have been shown to have an impact on the conformation of MHC class II molecules (57). Such an effect may not be caused by a direct linker–TCR interference because the crystal structure of $I\text{-}E^{k}$ molecules with covalently attached peptides indicates that this linker does not protrude up from the complex (58). Second, the conformation of $E\alpha 52\text{–}68/I\text{-}A^{b}$ complexes may be altered by the inappropriate environment where they are formed. In I-Ab-Ep mice, $E\alpha 52\text{–}68/I\text{-}A^{b}$ complexes are assembled in the endoplasmic reticulum (52) as opposed to the MIIC compartments; that is, in a significantly less-acidic context. One can imagine that in these two different cases, distinct conformations are possible, and that the 1H3.1 TCR is sensitive to such differences. Third, the activation of 1H3.1 T cells by the $E\alpha 52\text{–}68/I\text{-}A^{b}$ complex may depend on the corecognition of self-peptide/$I\text{-}A^{b}$ complexes. This hypothesis (59) tries to explain the apparent contrast between the fact that few antigenic-peptide/MHC complexes are required (0.03–0.1% of surface MHC class II molecules) for the activation of $CD4^{+}$ T cells (60, 61) and the results of a study of the APC–$CD4^{+}$ T cell interaction that reveals a large central domain occupied by supramolecular activation complexes (c-SMACs) made only of TCRs contacting peptide/MHC class II complexes and surrounded by a ring of interacting adhesive molecules (p-SMACs; ref. 62). In addition to antigenic peptide/MHC class II complexes, the large c-SMAC domain may incorporate self-peptide/MHC complexes, which could participate in activation through a phenomenon discovered during a BIAcor (biomolecular interaction analysis) surface plasmon resonance analysis conducted at 37°C (63). This study revealed a biphasic binding kinetics of the OT-1 TCR to its cognate ligand at physiological temperature. The kinetics fits best with a model where binding of a TCR to its ligand is followed by a dimerization event in which a second TCR binds to the existing TCR–peptide/MHC complex. This effect occurs only after an initial monomeric binding event, suggesting that a conformational change of the bound TCR triggers binding to unmodified TCRs. In an APC–T cell interaction context, TCRs recognizing agonist peptide/MHC complexes may undergo a conformational change that allows their dimerization with unmodified TCRs that contact self-peptide/self-MHC complexes. Then, this may allow coreceptor recruitment and lead to the aggregation seen in the c-SMAC domain upon T cell activation. Thus, recognition of the $E\alpha 52\text{–}68/I\text{-}A^{b}$ complex in the absence of self-peptide/self-MHC complexes may preclude 1H3.1 TCR multimerization and consequently 1H3.1 T cell activation. Indeed, TCR conformational change (64) also may be involved in hypotheses 1 and 2; an altered conformation of $E\alpha 52\text{–}68/I\text{-}A^{b}$ complexes may still allow TCR-mediated delivery of a minimal signal but preclude conformational change in the 1H3.1 TCR and, therefore, the deletion/activation of 1H3.1 T cells.

Unlike I-Ab-Ep splenocytes and immature DCs, mature I-Ab-Ep DCs derived from BM progenitors were able to specifically stimulate 1H3.1 T cells. This is explained best by the reprocessing of E$\alpha$52–68/I-A$^b$ complexes extracted from neighboring apoptotic cells. Upon endocytosis, the complexes can reach dense MIICs where the linker can be excised, and complexes carrying free E$\alpha$52–68 peptide and other peptides can be formed. Such reprocessing provides an explanation for the strong expansion of transferred 1H3.1 T cells into irradiated I-Ab-Ep hosts (25). Sublethal irradiation of I-Ab-Ep mice certainly causes a massive apoptosis of B cells; such cells can be disposed of by phagocytic APCs that can reprocess the complexes.

In conclusion, our observations demonstrate that the E$\alpha$52–68/I-A$^b$ complex-specific 1H3.1 T cells are not subjected to negative selection but, paradoxically, undergo positive selection *in vivo* in the I-Ab-Ep thymus where all of the I-A$^b$ molecules continuously present the covalently bound E$\alpha$52–68 peptide. Such positive selection directly relies on the conventional process of self-peptide/self-MHC complex recognition. Finally, peripheral T cells from 1H3.1 TCR Tg I-Ab-Ep mice are not antagonized but rather display a naive phenotype and are specifically reactive to the E$\alpha$52–68 peptide bound to I-A$^b$. Thus, the particular configuration of a bound agonist peptide expressed throughout the body is able to positively select and ensure the persistence of functional T cells specific for the same peptide.

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