

# Highly Restricted T Cell Repertoire Shaped by a Single Major Histocompatibility Complex–Peptide Ligand in the Presence of a Single Rearranged T Cell Receptor $\beta$ Chain

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## Summary

The T cell repertoire is shaped by positive and negative selection of thymocytes through the interaction of  $\alpha/\beta$ -T cell receptors (TCR) with self-peptides bound to self-major histocompatibility complex (MHC) molecules. However, the involvement of specific TCR-peptide contacts in positive selection remains unclear. By fixing TCR- $\beta$  chains with a single rearranged TCR- $\beta$  irrelevant to the selecting ligand, we show here that T cells selected to mature on a single MHC-peptide complex express highly restricted TCR- $\alpha$  chains in terms of V $\alpha$  usage and amino acid residue of their CDR3 loops, whereas such restriction was not observed with those selected by the same MHC with diverse sets of self-peptides including this peptide. Thus, we visualized the TCR structure required to survive positive selection directed by this single ligand. Our findings provide definitive evidence that specific recognition of self-peptides by TCR could be involved in positive selection of thymocytes.

Key words: positive selection • single major histocompatibility complex-peptide complex • single rearranged T cell receptor  $\beta$  chain • T cell repertoire • transgenic knockout mice

Although the diversity of  $\alpha/\beta$ -TCR theoretically reaches  $10^{15}$  by random rearrangement of five gene segments (V $\alpha$ , J $\alpha$ , V $\beta$ , D $\beta$ , and J $\beta$ ) and random nucleotide addition (1), mature T cells express highly selected TCR in that they exhibit tolerance to self-antigenic peptides and restriction by self-MHC molecules. This mainly results from two reciprocal selection processes, positive and negative selection, acting during T cell development in the thymus. Positive selection is the process that induces differentiation of CD4<sup>+</sup>CD8<sup>+</sup> immature thymocytes into CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>-</sup> mature thymocytes that mount immune response on foreign antigenic peptides bound to self-MHC molecules in the periphery, only when their TCR recognize self-MHC class I or class II molecules in the thymic environment (2–7). On the other hand, negative selection is the process that eliminates immature thymocytes bearing TCR specific for self-peptides bound to self-MHC molecules (8–12).

Although it is widely accepted that self-peptides play a central role in negative selection, the role of self-peptides in positive selection has been the subject of considerable debate (13, 14). This issue was first directly addressed for positive selection of CD8<sup>+</sup> T cells using fetal thymic organ cultures derived from mutant mouse strains where a partic-

ular MHC class I-peptide complex is expressed by exogenously adding a given peptide to the culture (15–19). More recently, several groups developed in vivo experimental systems focusing on the role of self-peptides in positive selection of CD4<sup>+</sup> T cells, by creating mouse strains that express MHC class II molecules predominantly occupied with a single peptide (20–24). The conclusions deduced from these in vitro and in vivo studies for positive selection of CD8<sup>+</sup> or CD4<sup>+</sup> T cells are largely in agreement: whereas limited numbers of self-peptides bound to a given MHC molecule promoted the positive selection of T cells expressing diverse sets of TCR- $\alpha/\beta$  with no obvious structural features, this complex could not be a positively selecting ligand for T cells expressing a particular transgenic TCR- $\alpha/\beta$  that is selected to mature on the same MHC molecule with a normal array of self-peptides (25–28). This might reflect the weak but specific recognition of selecting peptides by TCR- $\alpha/\beta$  in positive selection. However, experiments using TCR- $\alpha/\beta$  transgenic mice could not exclude the possibility that side chains of the peptides interfere with the interaction of the analyzed TCR- $\alpha/\beta$  with MHC molecule that would be essentially required for positive selection (29). In this respect, it would be important to

assess whether specific TCR-peptide contacts are involved in positive selection, under physiological conditions where developing thymocytes express diverse sets of TCR- $\alpha/\beta$ . Sant'Angelo et al. (30) recently addressed this issue by analyzing a particular V $\alpha$ -J $\alpha$  segment in mice lacking H-2M (H-2M<sup>0/0</sup>)<sup>1</sup> that catalyzes the dissociation of invariant chain-derived class II-associated peptide, CLIP, in the presence of a single rearranged TCR- $\beta$  chain. Although this study has shown that alternation of self-peptide repertoire affects CDR3 length of the selected TCR- $\alpha$  repertoire, no remarkable bias for amino acid composition of their CDR3 loops could be deduced. This might be a general feature of a positively selected T cell repertoire. Alternatively, this may be the result of the heterogeneity of selecting peptides, because it has been shown that peptides other than CLIP are bound to I-A<sup>b</sup> molecules and contribute to the positive selection of CD4<sup>+</sup> thymocytes in H-2M<sup>0/0</sup> mice (26). In addition, it remains unclear from this study whether self-peptides involved in positive selection would affect variable gene segments of the selected TCR repertoire and this needs to be known if one is to better understand TCR-MHC-peptide interaction in positive selection process.

To clarify specific TCR-peptide contacts in positive selection, we analyzed the structure of TCR- $\alpha$  chains expressed on CD4<sup>+</sup>CD8<sup>-</sup> thymocytes selected to mature on a single ligand, I-A<sup>b</sup> molecule covalently bound to E $\alpha$ -derived peptide (E $\alpha$ 52-68), in the presence of a single rearranged TCR- $\beta$  chain with irrelevant specificity for this ligand. Since positive selection is thought to be mediated through low affinity interaction of TCR- $\alpha/\beta$  with MHC-peptide ligands (31), introduction of the TCR- $\beta$  chain irrelevant to I-A<sup>b</sup>-E $\alpha$ 52-68 complex would give us a better chance to reveal structural features of the associated TCR- $\alpha$  chains selected by this ligand. Therefore, we have selected the 2B4 TCR- $\beta$  chain derived from the TCR- $\alpha/\beta$  specific for moth cytochrome c peptide bound to I-E<sup>k</sup> or I-E<sup>b</sup> molecules (32). By comparing the TCR- $\alpha$  repertoire shaped by I-A<sup>b</sup>-E $\alpha$ 52-68 complex with that by I-A<sup>b</sup> molecules with a normal array of self-peptides, including E $\alpha$ 52-68, in the presence 2B4 TCR- $\beta$  chain, we demonstrate here that expression of TCR- $\alpha$  chains with both particular V $\alpha$  segments and amino acid residue in their CDR3 loops is required to survive positive selection by this single ligand. These findings provide definitive evidence that specific TCR-peptide interaction could be involved in the positive selection of thymocytes.

<sup>1</sup>Abbreviations used in this paper:  $\beta$ 2<sup>0/0</sup>, mice lacking  $\beta$ 2-microglobulin; B2L or B2H, mouse lines expressing I-A<sup>b</sup> chain covalently bound to E $\alpha$ 52-68; B6, C57BL/6 mice; CLIP, invariant chain-derived class II-associated peptide; DKO, mice lacking endogenous I-A<sup>b</sup> and invariant chains; E $\alpha$ 52-68, E $\alpha$ -derived peptide; E $\alpha$ -B6, C57BL/6 mice transgenic for E $\alpha$ ; H-2M<sup>0/0</sup>, mice lacking H-2M expression; TKO, mice lacking endogenous I-A<sup>b</sup>, invariant chain, and  $\beta$ 2-microglobulin; TKO/2B4 $\beta$ , B2L TKO/2B4 $\beta$ ,  $\beta$ 2<sup>0/0</sup>/2B4 $\beta$ , E $\alpha$ -B6/2B4 $\beta$ ; TKO, B2L TKO,  $\beta$ 2<sup>0/0</sup> and E $\alpha$ -B6 mice expressing 2B4 TCR- $\beta$  chain.

## Materials and Methods

**Mice.** B2L mice that express the I-A<sup>b</sup> chain covalently bound to E $\alpha$ 52-68 but lack endogenous I-A<sup>b</sup> and invariant chains (B2L DKO) have been described (24). B2L DKO mice were crossed with  $\beta$ 2-microglobulin-deficient mice carrying H-2<sup>b</sup> haplotype ( $\beta$ 2<sup>0/0</sup>; Jackson Laboratories, Bar Harbor, ME), and B2L mice lacking the endogenous I-A<sup>b</sup> chain, invariant chain, and  $\beta$ 2-microglobulin were developed (B2L TKO). To develop B2L TKO/2B4 $\beta$  mice, we crossed B2L TKO with mice transgenic for 2B4 TCR- $\beta$  chain that were maintained of C57BL/6 (B6) background (H-2<sup>b</sup>) in our facility (33).  $\beta$ 2<sup>0/0</sup>/2B4 $\beta$  or E $\alpha$ -B6/2B4 $\beta$  mice were developed by crossing TKO/2B4 $\beta$  mice with  $\beta$ 2<sup>0/0</sup> or E $\alpha$  transgenic B6 (E $\alpha$ -B6) mice (34), respectively.

**Antibodies.** The following mAbs were purchased from PharMingen (San Diego, CA): FITC-anti-CD8 (53-6.7); PE-anti-CD4 (RM4-5); biotinylated anti-NK1.1 (PK136); purified anti-CD24 (HSA, J11D); FITC-anti-TCR V $\alpha$ 2 (B20.1); V $\alpha$ 3.2 (RR3-16); V $\alpha$ 8 (B21.14); FITC-anti-TCR V $\beta$ 2 (B20.6); V $\beta$ 4 (KT4); V $\beta$ 5 (MR9-4); V $\beta$ 6 (RR4-7); V $\beta$ 7 (TR310); V $\beta$ 8 (MR5-2); V $\beta$ 9 (MR10-2); V $\beta$ 10 (B21.5); V $\beta$ 11 (RR3-15); V $\beta$ 12 (MR11-1); V $\beta$ 13 (MR12-3); V $\beta$ 14 (14-2); and biotinylated anti-TCR V $\beta$ 3 (KJ25). The mouse IgM mAb specific for CD8 used for the killing experiments were purchased from Meiji Institute of Health Science (Tokyo, Japan).

**Flow Cytometry and Cell Sorting.** Single cell suspensions of thymocytes were prepared from mice 6-7 wk old and stained with FITC-anti-CD8, PE-anti-CD4, and biotinylated anti-NK1.1 or biotinylated anti-TCR V $\beta$ 3 mAb followed by streptavidin-Cy-Chrome (PharMingen). To assess the expression of TCR V $\alpha$  or V $\beta$  on CD4<sup>+</sup>CD8<sup>-</sup> thymocytes, thymocytes were incubated with anti-CD8 and anti-HSA IgM mAbs, followed by rabbit complement to remove immature thymocytes and CD4<sup>-</sup>CD8<sup>+</sup> thymocytes. Viable cells recovered using Lympholyte-M (Cedarlane, Ontario, Canada) were stained with PE-anti-CD4 and FITC-anti-TCR V $\alpha$  or V $\beta$  mAb, with or without biotinylated anti-TCR V $\beta$ 3 followed by streptavidin-Cy-Chrome. Analyses were done on a FACScan<sup>®</sup> (Becton Dickinson, Mountain View, CA). For cell sorting, CD4<sup>+</sup>CD8<sup>-</sup> thymocytes were enriched as described above and were stained with PE-anti-CD4 and FITC-anti-CD8, with or without biotinylated anti-TCR V $\beta$ 3 mAb followed by streptavidin-Cy-Chrome. CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>+</sup>CD8<sup>-</sup>V $\beta$ 3<sup>hi</sup> thymocytes (1-2  $\times$  10<sup>5</sup>) were sorted out using EPICS ELITE<sup>®</sup> (Coulter Corp., Hialeah, FL) or FACS<sup>®</sup> Vantage (Becton Dickinson) and were immediately placed in liquid nitrogen. All reagents were used at 10  $\mu$ g/ml.

**Mixed Lymphocyte Reaction.** Lymph node CD4<sup>+</sup> T cells were prepared by eliminating CD8<sup>+</sup> T cells and B cells from lymph node cells using anti-CD8 antibody followed by immunomagnetic beads coated with anti-Rat IgG antibody and those coated with anti-mouse IgG antibody (both from DYNAL, Oslo, Norway). The lymph node CD4<sup>+</sup> T cells (1  $\times$  10<sup>5</sup> cells/well) were cultured with irradiated spleen cells (1  $\times$  10<sup>6</sup> cells/well) for 80 h and 1  $\mu$ Ci of [<sup>3</sup>H]thymidine was added during 16 h of the culture.

**Anchored PCR.** The following oligonucleotides were used: PC $\alpha$ F1, TTGGGAGTCAAAGTCCGGTGAAC; C $\alpha$ out, AACAGGCAGAGGGTGTCTGTC; C $\alpha$ in, CTGAGACCGAGGATCTTTTAAAC; AP, GGCCACGCGTCCGACTAGTACGGGIGGGIIGG GIIG; AUAP, GGCCACGCGTCCGACTAGTAC.

Total cellular RNA was extracted from the sorted thymocytes using ISOGEN in the presence of 1  $\mu$ l of Ethachinmate that facilitates the recovery of small amounts of nucleotides (both from Nippon Gene, Tokyo, Japan). The first strand cDNA synthesis

primed with TCR  $\alpha$ -specific antisense oligonucleotide, PC $\alpha$ F1, was carried out using SuperScript<sup>TM</sup> II reverse transcriptase (GIBCO BRL, Gaithersburg, MD) at 50°C. After treatment with RNase H and RNase I at 37°C for 30 min, the first strand cDNA was purified from unincorporated dNTPs and PC $\alpha$ F1 and was subjected to homopolymeric tailing using terminal deoxynucleotidyl transferase and dCTP. With this dC-tailed cDNA, several PCR were carried out using  $\alpha$ out and AP primers, under the following condition: 94°C (2 min), 1 cycle; 94°C (1 min), 55°C (1 min), and 72°C (2 min), 30 cycles; 72°C (5 min), 1 cycle. Then, the first PCR products were mixed and subjected to the second PCR using  $\alpha$ in and AUAP primers in the presence of TaqStart antibody (CLONTECH Laboratories, Inc., Palo Alto, CA), under the following condition: 94°C (2 min), 1 cycle; 94°C (1 min), 57°C (1 min), and 72°C (2 min), 30 cycles; 72°C (5 min), 1 cycle. When cDNA without dC-tailing was used, no visible band was obtained after the second PCR (data not shown).

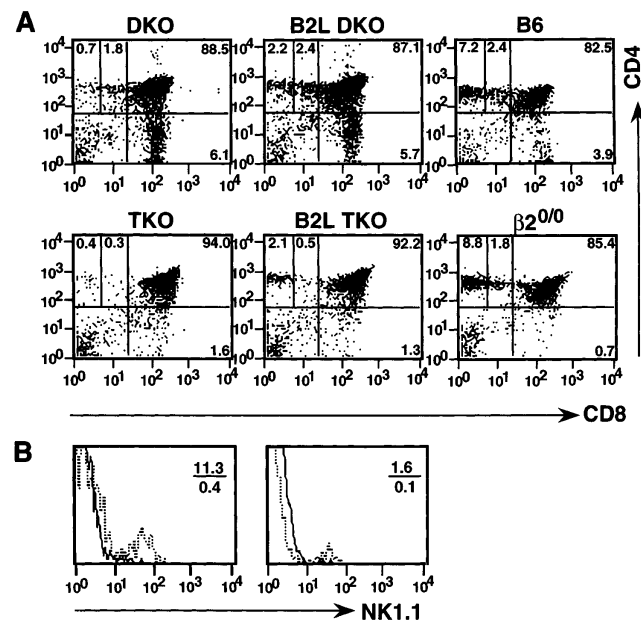
**Cloning and Sequencing of Anchored PCR Products.** The mixture of the second PCR products was ligated to pGMT-T Easy Vector (Promega Corporation, Madison, WI) and was used for the subsequent transformation of DH5 $\alpha$ . Each colony was picked up and cultured in LB medium (100  $\mu$ l) at 37°C for 5 h using 96-well U bottom plates, then the supernatant was subjected to PCR using AUAP and  $\alpha$ in2 (GAGACCGAGG ATCTTTTAACT) primers, under the following condition: 94°C (30 s), 1 cycle; 95°C (30 s) 68°C (3 min), 25 cycles. After removal of excess primers and dNTPs using centricon 100 (Amicon, Inc., Beverly, MA), the purified PCR products were labeled with dye terminator cycle sequencing using C $\alpha$ seq primer (AGACCGAGGATCTTTTAACT) and were analyzed on an ABI PRISM<sup>TM</sup> 377 DNA sequencer (Perkin-Elmer Corporation, Foster City, CA) according to standard protocols.

## Results

**CD4<sup>+</sup> T Cell Differentiation Directed by the I-A<sup>b</sup>-E $\alpha$ 52-68 Complex in Mice Lacking Endogenous MHC Class I and Class II Expressions.** Earlier, we had reported three lines of transgenic mice that had been developed by introducing the gene encoding I-A $\beta^b$  chain covalently bound to E $\alpha$ 52-68 into mice lacking both endogenous I-A $\beta^b$  and invariant chains (DKO; reference 24). Evidence that the I-A<sup>b</sup> molecule covalently bound to E $\alpha$ 52-68 is expressed in these transgenic lines as a single species was obtained by complete inhibition by the mAb specific for I-A<sup>b</sup>-E $\alpha$ 52-68 complex of staining with an anti-I-A<sup>b</sup> reagent, the inability of transgenic spleen cells to present other I-A<sup>b</sup>-binding peptides, and the robust proliferative response of transgenic CD4<sup>+</sup> T cells to wild-type I-A<sup>b</sup> molecules with a normal array of self-peptides (24). In addition, a similar transgenic mouse line developed by Ignatowicz et al. (20) has been shown by the detailed analysis to present no other detectable self-peptides (26). However, we have found that CD4<sup>+</sup>CD8<sup>-</sup> thymocytes in these transgenic mice include NK1.1<sup>+</sup> thymocytes that are selected by nonclassical MHC class I molecules such as CD1 (35, 36). To purify CD4<sup>+</sup>CD8<sup>-</sup> thymocytes selected to mature on I-A<sup>b</sup>-E $\alpha$ 52-68 complex from those selected by nonclassical and probably classical MHC class I molecules, we introduced null mutation of  $\beta$ 2-microglobulin required for MHC class I expression into

B2L DKO mice that expressed I-A<sup>b</sup>-E $\alpha$ 52-68 complex in the thymus at an intermediate level and showed most effective CD4<sup>+</sup> T cell differentiation among three lines (24). Finally, B2L transgenic mice or nontransgenic mice lacking endogenous I-A $\beta^b$ , invariant chain, and  $\beta$ 2-microglobulin (B2L TKO and TKO) were developed and used in the present study.

Although no definite CD4<sup>+</sup>CD8<sup>-</sup> thymocytes were observed in TKO mice lacking MHC class I and class II expression, significant numbers of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes were selected to mature on a single I-A<sup>b</sup>-E $\alpha$ 52-68 complex in B2L TKO, reaching a level of  $\sim$ 25% of that seen in mice that express wild-type I-A<sup>b</sup> molecules but lack  $\beta$ 2-microglobulin expression ( $\beta$ 2<sup>0/0</sup>; Fig. 1 A). Although the proportion of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes in B2L TKO was comparable to that in B2L DKO, CD4<sup>+</sup>CD8<sup>int</sup> thymocytes markedly decreased in B2L TKO. Such a decrease, compared with DKO or C57BL/6 (B6) mice, was also found in TKO and  $\beta$ 2<sup>0/0</sup> mice. These observations are consistent with the previous report (37), supporting the model that



**Figure 1.** CD4<sup>+</sup> T cell differentiation in B2L TKO and B2L DKO mice. (A) Thymocytes were prepared from six different lines (DKO, B2L DKO, B6, TKO, B2L TKO, and  $\beta$ 2<sup>0/0</sup>) at 6–7 wk old and analyzed for CD4 and CD8 expression. The percentages of CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>int</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> thymocytes are indicated. The data represent at least three independent experiments. The mean  $\pm$  one SD for the percentage of CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>int</sup> thymocytes in each line are as follows: DKO, 0.7  $\pm$  0.1% and 1.9  $\pm$  0.1%; B2L DKO, 2.0  $\pm$  0.4% and 2.5  $\pm$  0.4%; B6, 7.2  $\pm$  0.5% and 2.8  $\pm$  0.3%; TKO, 0.4  $\pm$  0.1% and 0.3  $\pm$  0.0%; B2L TKO, 2.0  $\pm$  0.3% and 0.4  $\pm$  0.1%;  $\beta$ 2<sup>0/0</sup>, 8.3  $\pm$  0.5% and 1.7  $\pm$  0.0%. (B) The expression of NK1.1 on CD4<sup>+</sup>CD8<sup>-</sup> thymocytes is compared between B2L DKO (left, dotted line) and B2L TKO (left, solid line) or between B6 (right, dotted line) or  $\beta$ 2<sup>0/0</sup> (right, solid line). The percentage of CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>+</sup> thymocytes in mice with or without MHC class I expression are indicated above or below the line, respectively. For each line, at least two mice were analyzed. The each value for the percentage of CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>+</sup> thymocytes to total CD4<sup>+</sup>CD8<sup>-</sup> thymocytes was as follows: B2L DKO, 11.3, 7.9, 8.9; B2L TKO, 0.4, 0.8, 0.7; B6, 1.6, 2.1;  $\beta$ 2<sup>0/0</sup>, 0.1, 0.1.

CD4<sup>+</sup>CD8<sup>int</sup> population represents developing thymocytes to CD4<sup>+</sup>CD8<sup>+</sup> phenotype as well as those to CD4<sup>+</sup>CD8<sup>-</sup> phenotype (38). When NK1.1 expression was analyzed in B2L DKO, around 10% of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes expressed this surface marker. However, as we expected, CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>+</sup> T cells were scarcely observed in the thymus from B2L TKO (Fig. 1 B).

*CD4<sup>+</sup>CD8<sup>-</sup> Thymocytes Selected to Mature on the I-A<sup>b</sup>-E $\alpha$ 52-68 Complex Express Diverse TCR- $\alpha$  Chains with No Obvious Structural Features.* In an attempt to assess the role of self-peptides in shaping a mature T cell repertoire, we first compared the TCR V $\beta$  usages in CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from B2L TKO with those from  $\beta$ 2<sup>0/0</sup> mice, using a panel of mAbs. Although the proportions of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes expressing TCR V $\beta$ 4, 12, and 14 slightly increased in B2L TKO (V $\beta$ 4, 12.7  $\pm$  0.5% vs. 8.3  $\pm$  0.3%; V $\beta$ 12, 3.7  $\pm$  0.5% vs. 2.7  $\pm$  0.1%; V $\beta$ 14, 12.7  $\pm$  0.7% vs. 10.2  $\pm$  0.2%), other TCR V $\beta$  were similarly expressed on CD4<sup>+</sup>CD8<sup>-</sup> thymocytes in these lines (data not shown).

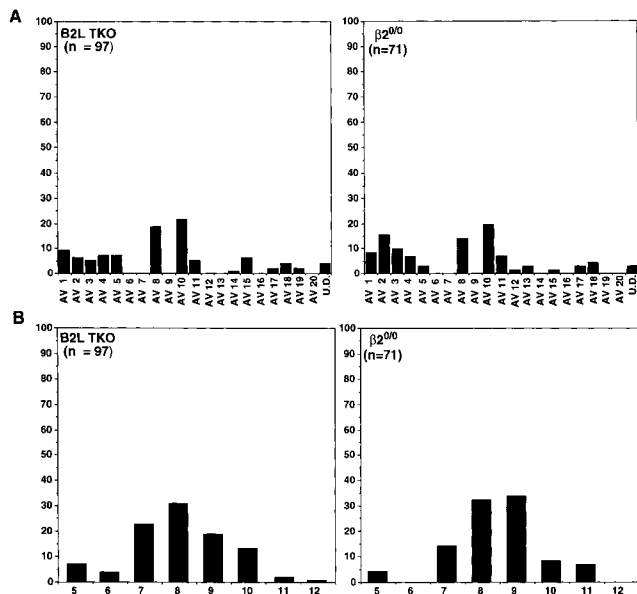
In contrast to the availability of the mAbs specific for TCR V $\beta$  segments, only limited numbers of mAbs are available for TCR V $\alpha$ s, some of which react with only the subfamily of a particular TCR V $\alpha$  family. To overcome this problem and thoroughly analyze the TCR- $\alpha$  repertoire, we sorted CD4<sup>+</sup>CD8<sup>-</sup> thymocytes and examined TCR- $\alpha$  mRNA using anchored PCR followed by sequencing of the cloned PCR products. From B2L TKO

CD4<sup>+</sup>CD8<sup>-</sup> thymocytes, we obtained 97 clones originating from different templates, where 13 different V $\alpha$  families and 27 different J $\alpha$  segments were encoded. On the other hand, we obtained from  $\beta$ 2<sup>0/0</sup> CD4<sup>+</sup>CD8<sup>-</sup> thymocytes 71 independent TCR- $\alpha$  sequences that encoded 13 different V $\alpha$  families and 22 different J $\alpha$  segments. When the V $\alpha$  and J $\alpha$  usages were compared, no remarkable difference was observed (Fig. 2 A and data not shown). The distribution of CDR3 length showed Gaussian-like patterns in both cases (Fig. 2 B). In addition, no obvious structural features in the CDR3 loops could be deduced, even when clones from B2L TKO were analyzed (data not shown). These results indicate that a single ligand, I-A<sup>b</sup>-E $\alpha$ 52-68 complex, selects a quite diverse T cell repertoire.

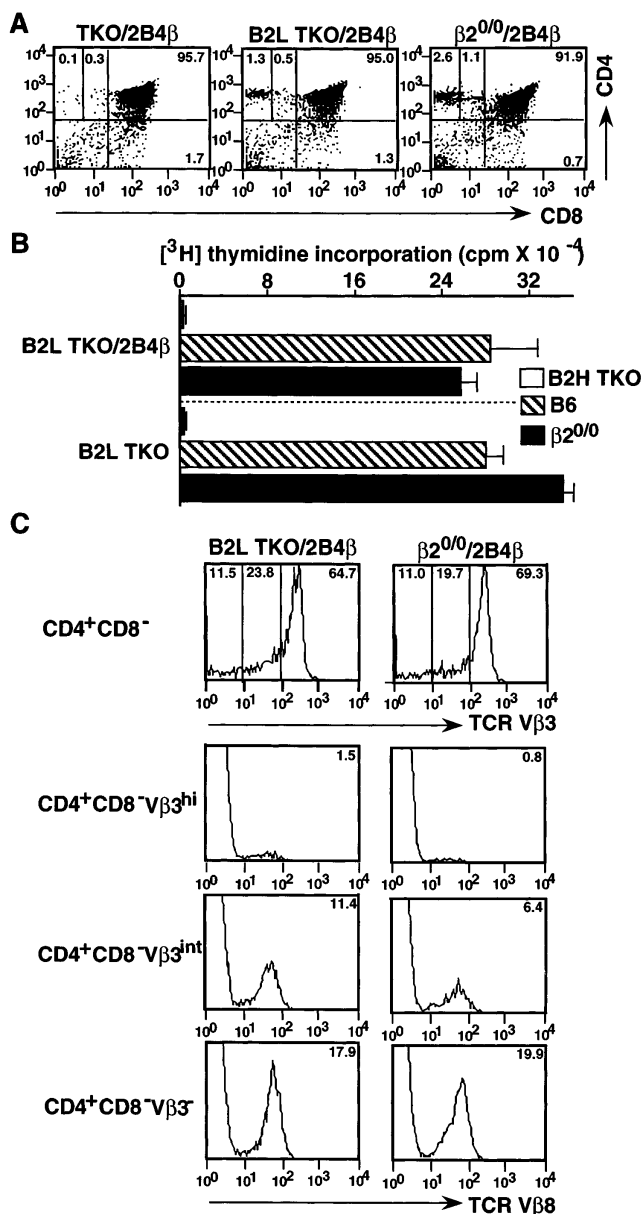
*CD4<sup>+</sup> T Cell Differentiation Directed by the I-A<sup>b</sup>-E $\alpha$ 52-68 Complex in the Presence of a Single Rearranged TCR- $\beta$  chain.* The specificity of TCR for MHC-peptide ligands is determined by both TCR- $\alpha$  and  $\beta$  chains. In light of the low affinity interaction of TCR- $\alpha/\beta$  with MHC-peptide ligands in positive selection (31), it would be difficult to visualize the imprint of selecting peptides on T cell repertoire when TCR- $\alpha$  or TCR- $\beta$  chains are separately analyzed, even though selecting peptide is a single species. However, some structural imprints might be revealed, if TCR- $\alpha$  or TCR- $\beta$  chains were to be analyzed for CD4<sup>+</sup>CD8<sup>-</sup> thymocytes expressing a particular TCR- $\beta$  or TCR- $\alpha$  with irrelevant specificity for the selecting ligand. To test this idea, we employed the transgenic mice expressing 2B4 TCR- $\beta$  (V $\beta$ 3-D $\beta$ 1.1-J $\beta$ 1.2) derived from the TCR- $\alpha/\beta$  specific for moth cytochrome c peptide bound to I-E<sup>k</sup> or I-E<sup>b</sup> molecules (7, 39), and, by crossing these mice with B2L TKO, developed TKO mice expressing both B2L transgene and the rearranged 2B4 TCR- $\beta$  (B2L TKO/2B4 $\beta$ ).

Although no CD4<sup>+</sup>CD8<sup>-</sup> thymocytes were observed in TKO/2B4 $\beta$  mice lacking MHC class I and class II expression, significant numbers of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes were selected to mature in B2L TKO/2B4 $\beta$  and  $\beta$ 2<sup>0/0</sup> mice expressing 2B4 TCR- $\beta$  chain ( $\beta$ 2<sup>0/0</sup>/2B4 $\beta$ ; Fig. 3 A). Lymph node CD4<sup>+</sup> T cells from B2L TKO/2B4 $\beta$  mice as well as B2L TKO responded well to spleen cells from  $\beta$ 2<sup>0/0</sup> or C57BL/6 (B6) mice expressing wild-type I-A<sup>b</sup> molecules with self-peptides other than E $\alpha$ 52-68, but did not show any response to B2H TKO spleen cells that express I-A<sup>b</sup>-E $\alpha$ 52-68 complex as a single species at higher level than those from B2L TKO (24; Fig. 3 B). Taken together, these observations suggest that positive and negative selection occurs normally in immature thymocytes expressing the essentially fixed 2B4 TCR- $\beta$  and randomly rearranged TCR- $\alpha$  in B2L TKO/2B4 $\beta$  mice.

When the expression of TCR V $\beta$ 3 was analyzed, 65–75% of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from both B2L TKO/2B4 $\beta$  and  $\beta$ 2<sup>0/0</sup>/2B4 $\beta$  mice expressed on their cell surface TCR V $\beta$ 3 at a high level (Fig. 3 C). Considerable numbers of CD4<sup>+</sup>CD8<sup>-</sup>V $\beta$ 3<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup>V $\beta$ 3<sup>int</sup> thymocytes expressed TCR V $\beta$ 8 that is most frequently observed in CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from both B2L TKO and  $\beta$ 2<sup>0/0</sup> mice (B2L TKO, 18.0  $\pm$  0.4%;  $\beta$ 2<sup>0/0</sup>, 18.2  $\pm$  0.5%), whereas no definite expression of TCR V $\beta$ 8 was detected



**Figure 2.** V $\alpha$  usage (A) and CDR3 length distribution (B) of TCR- $\alpha$  repertoire shaped by the I-A<sup>b</sup>-E $\alpha$ 52-68 complex. CD4<sup>+</sup>CD8<sup>-</sup> thymocytes were sorted from B2L TKO or  $\beta$ 2<sup>0/0</sup> mice, and TCR- $\alpha$  transcripts were analyzed using anchored PCR followed by sequencing of the cloned PCR products. The results are shown as the percentages of clones originating from different templates. The clones encoding undefined TCR V $\alpha$  are indicated as U.D. in A. CDR3 length in (B) is indicated as the number of amino acid residues between two amino acids downstream from the conserved cysteine at position 90 in the V region and two amino acids upstream from the conserved GXG motif (where G is glycine and X is any amino acid) in the J region (64).

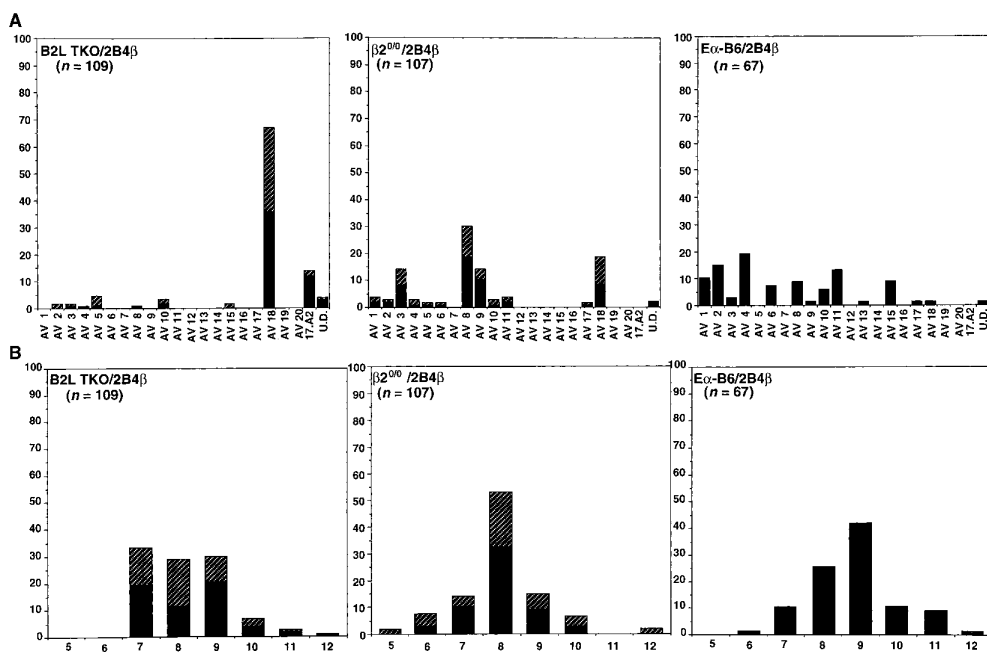


**Figure 3.** Phenotypic and functional analysis for CD4<sup>+</sup> T cell differentiation directed by the I-A<sup>b</sup>-E $\alpha$ 52-68 complex in the presence of the 2B4 TCR- $\beta$  chain. (A) Thymocytes were prepared from TKO/2B4 $\beta$ , B2L TKO/2B4 $\beta$ , and  $\beta 2^{0/0}$ /2B4 $\beta$  mice at 6–7 wk old and analyzed for CD4 and CD8 expression. The percentages of CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>int</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>+</sup> thymocytes are indicated. For each line, at least three mice were analyzed. The each value for the percentage of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes was as follows: TKO/2B4 $\beta$ , 0.1, 0.1, 0.1; B2L TKO/2B4 $\beta$ , 1.3, 0.9, 0.7, 0.7;  $\beta 2^{0/0}$ /2B4 $\beta$ , 2.6, 2.3, 2.7. (B) Lymph node CD4<sup>+</sup> T cells from B2L TKO/2B4 $\beta$  or B2L TKO mice were cultured with irradiated spleen cells from B2H TKO, B6, and  $\beta 2^{0/0}$  mice, and [<sup>3</sup>H]thymidine incorporation was measured. The data indicate the mean plus one SD of triplicate cultures. (C) Thymocytes were prepared from B2L TKO/2B4 $\beta$  or  $\beta 2^{0/0}$ /2B4 $\beta$  mice at 6–7 wk old, and the expression of TCR V $\beta 3$  was analyzed for gated CD4<sup>+</sup>CD8<sup>-</sup> thymocytes (*top*). The percentages of CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{-}$ , CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{int}$ , CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{hi}$  thymocytes are indicated. For each line, four or three mice were analyzed. The each value for the percentage of CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{hi}$  thymocytes was as follows: B2L TKO/2B4 $\beta$ , 64.7, 71.0, 65.2, 75.0;  $\beta 2^{0/0}$ /2B4 $\beta$ , 69.3, 75.3, 70.8. The expression of TCR V $\beta 8$  on CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{hi}$ , CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{int}$ , CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{-}$  thymocytes and the percentages of positive population are shown below.

on CD4<sup>+</sup>CD8<sup>-</sup> thymocytes expressing TCR V $\beta 3$  at a high level from both B2L TKO/2B4 $\beta$  and  $\beta 2^{0/0}$ /2B4 $\beta$  (Fig. 3 C). These observations indicate that allelic exclusion by the transgenic 2B4 TCR- $\beta$  is almost completed in CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{hi}$  thymocytes and suggest that these thymocytes exclusively express the transgene.

*Highly Restricted Expression of TCR- $\alpha$  Chains on CD4<sup>+</sup>CD8<sup>-</sup> Thymocytes Selected to Mature on the I-A<sup>b</sup>-E $\alpha$ 52-68 Complex in the Presence of Transgenic 2B4 TCR- $\beta$ .* With the strategy described above, we then compared TCR- $\alpha$  chains expressed on CD4<sup>+</sup>CD8<sup>-</sup>V $\beta 3^{hi}$  thymocytes from B2L TKO/2B4 $\beta$  with those from  $\beta 2^{0/0}$ /2B4 $\beta$  or B6 mice expressing both E $\alpha$  and 2B4 TCR- $\beta$  chains (E $\alpha$ -B6/2B4 $\beta$ ). In two independent experiments using different B2L TKO/2B4 $\beta$  mice, 73 out of 109 clones (67%) originating from different templates were found to encode the V $\alpha$ 18 family, and 42% of the remaining (15 of 36 clones) encoded the V $\alpha$  segment (denoted as 17.A2) that was not observed in samples from  $\beta 2^{0/0}$ /2B4 $\beta$  mice but identical to that on a T cell clone reported by Mohapatra et al. (40; Fig. 4 A). In contrast to the results on B2L TKO/2B4 $\beta$  mice, such restricted TCR V $\alpha$  usage was not observed with clones obtained from E $\alpha$ -B6/2B4 $\beta$  or  $\beta 2^{0/0}$ /2B4 $\beta$  mice expressing wild-type I-A<sup>b</sup> molecules with or without I-A<sup>b</sup>-E $\alpha$ 52-68 complex, respectively, though the TCR V $\alpha$  repertoire differed between these strains, probably because of the presence of I-E<sup>b</sup> molecules in E $\alpha$ -B6/2B4 $\beta$  mice. When the length of their CDR3 loops was compared, the distribution pattern in B2L TKO/2B4 $\beta$  mice differed from those in  $\beta 2^{0/0}$ /2B4 $\beta$  and E $\alpha$ -B6/2B4 $\beta$  (Fig. 4 B).

Having found that CD4<sup>+</sup>CD8<sup>-</sup> thymocytes selected to mature on I-A<sup>b</sup>-E $\alpha$ 52-68 complex preferentially expressed V $\alpha$ 18 in the presence of the 2B4 TCR- $\beta$ , we focused on clones encoding this V $\alpha$  family and analyzed amino acid sequences of their CDR3 loops. Surprisingly, 70 of 73 clones bearing V $\alpha$ 18 from B2L TKO/2B4 $\beta$  mice encoded aspartic or glutamic acid at the first position of their CDR3 loops ( $\alpha 93$ ; Table 1). In contrast, only 9 of 20 clones bearing V $\alpha$ 18 from  $\beta 2^{0/0}$ /2B4 $\beta$  encoded aspartic or glutamic acid at  $\alpha 93$ , and 11 other clones encoded several amino acid residues including tryptophan, alanine, arginine, proline, valine, threonine, glycine, and leucine (Table 1). The V $\alpha$ 18 family includes two subfamilies, AV18S1 and AV18S2, that only differ by the amino acid residue at position 25: AV18S1 encodes threonine at this position, whereas AV18S2 encodes lysine (41). Although the subfamily was not determined for 23 clones from B2L TKO/2B4 $\beta$  mice due to shortness of the PCR products, 50 other clones encoded AV18S2 and 48 clones of these encoded aspartic or glutamic acid at  $\alpha 93$ . Because of the lack of complete information on the genomic sequence of AV18S2, we cannot determine at this stage whether aspartic or glutamic acid at this position is encoded by a germ line sequence or created by a random nucleotide (N-region) addition. Nonetheless, since the AV18S2-bearing TCR- $\alpha$  chains with various amino acid residues at  $\alpha 93$  were found in  $\beta 2^{0/0}$ /2B4 $\beta$ , it is suggested that, in B2L TKO/2B4 $\beta$  mice, there is a strong



**Figure 4.**  $V\alpha$  usage (A) and CDR3 length distribution (B) of TCR- $\alpha$  repertoire shaped by the I-A<sup>b</sup>-E $\alpha$ 52-68 complex in the presence of 2B4 TCR- $\beta$  chain. CD4<sup>+</sup>CD8<sup>-</sup>V $\beta$ 3<sup>hi</sup> thymocytes were sorted from B2L TKO/2B4 $\beta$ ,  $\beta$ 2<sup>0/0</sup>/2B4 $\beta$ , or E $\alpha$ -B6/2B4 $\beta$  mice, and TCR- $\alpha$  transcripts were analyzed, as described for Fig. 2. The results are shown as percentages of clones originating from different templates. The closed and hatched boxes represent clones obtained from independent experiments using different mice. The clones encoding undefined  $V\alpha$  are indicated as U.D. in A.

selection to conserve or introduce negatively charged amino acid residues at this position to survive the thymic selection directed by a single I-A<sup>b</sup>-E $\alpha$ 52-68 ligand.

Since AV18S2 and  $V\alpha$ 17.A2 were the major  $V\alpha$  gene segments obtained from B2L TKO/2B4 $\beta$ , we compared the NH<sub>2</sub>-terminal structure of these segments from the cysteine at position 90. As shown in Fig. 5 A, the predicted amino acid sequences are highly conserved between these  $V\alpha$  gene segments (80.7%). The CDR1 and CDR2 loops of TCR- $\alpha$  chains have been suggested to play an important role in interactions with MHC class II/peptide ligand (42-44). Although three amino acid substitutions are observed in their CDR1 loops, the property of these amino acid residues is relatively conserved and the CDR2 loops show no differences between AV18S2 and  $V\alpha$ 17.A2. Thus, it is concluded that  $V\alpha$ 17.A2 encodes the  $V\alpha$  chain that would be structurally related to that encoded by AV18S2. When CDR3 loops were analyzed for 15 clones encoding  $V\alpha$ 17.A2 from B2L TKO/2B4 $\beta$  mice, we again found that 11 clones encoded aspartic or glutamic acid at  $\alpha$ 93 (Fig. 5 B).

## Discussion

In this study, we compared the TCR- $\alpha$  repertoire in CD4<sup>+</sup>CD8<sup>-</sup> thymocytes selected to mature on a single I-A<sup>b</sup>-E $\alpha$ 52-68 ligand with those selected by wild-type I-A<sup>b</sup> molecules with a normal array of self-peptides, in the presence or absence of a single rearranged TCR- $\beta$  chain irrelevant to this selecting ligand, using anchored PCR followed by sequencing of the PCR products. This method is based on competitive PCR without using specific primers for particular  $V\alpha$  segments, so that PCR bias is minimized. In addition, clones originating from different templates can be distinguished by differences in the anchored position, even when they encode the same TCR- $\alpha$  chains. Although the

frequencies of  $V\alpha$ 3 or  $V\alpha$ 8 usage in B2L TKO and  $\beta$ 2<sup>0/0</sup> mice estimated using this approach were much higher than frequencies assessed using the mAbs, RR3-16 or B21.14 (data not shown), this is likely the result of specificity of these mAbs that react with only some of these subfamilies (45, 46). On the other hand, the frequencies of  $V\alpha$ 2 usage estimated by this approach in several mouse lines including B2L TKO,  $\beta$ 2<sup>0/0</sup>, and B2L TKO/2B4 $\beta$  largely agreed with frequencies observed with the flow cytometric analysis using the mAb, B20.1, that reacts with almost all subfamilies (47; data not shown). Therefore, we conclude that heterogeneity of the anchored PCR products would reflect, to some extent, the real diversity of TCR- $\alpha$  repertoire in CD4<sup>+</sup>CD8<sup>-</sup> thymocytes.

By introducing the 2B4 TCR- $\beta$  chain with irrelevant specificity for the I-A<sup>b</sup>-E $\alpha$ 52-68 complex, CD4<sup>+</sup>CD8<sup>-</sup> thymocytes selected to mature on this single ligand expressed highly restricted TCR- $\alpha$  chains encoding  $V\alpha$ 18 or its related sequence and negatively charged amino acid residues at  $\alpha$ 93 in their CDR3 loops. Taking into consideration that multiple TCR- $\alpha$  rearrangements cease only after positive selection (48, 49), a small number of clones encoding TCR- $\alpha$  chains without such structural features might represent nonselectable in-frame rearrangements (50). Alternatively, these clones might be derived from a trace of CD4<sup>+</sup>CD8<sup>-</sup>V $\beta$ 3<sup>int</sup> thymocytes where allelic exclusion by 2B4 TCR- $\beta$  is not accomplished. Since such structural features of TCR- $\alpha$  chain were not observed with CD4<sup>+</sup>CD8<sup>-</sup>V $\beta$ 3<sup>hi</sup> thymocytes selected to mature in  $\beta$ 2<sup>0/0</sup>/2B4 $\beta$  mice expressing wild-type I-A<sup>b</sup> molecules with a normal array of self-peptides, it is clear that highly restricted  $V\alpha$  usage and amino acid residues at  $\alpha$ 93 in B2L TKO/2B4 $\beta$  mice does not result from efficient pairing of particular TCR- $\alpha$  chains with 2B4 TCR- $\beta$  as was previously reported (51, 52), but rather from thymic selection

**Table 1.** Comparison of V $\alpha$ 18-encoding TCR- $\alpha$  Chains between B2L TKO/2B4 $\beta$  and  $\beta$ 2<sup>0/0</sup>/2B4 $\beta$  Mice

B2L TKO/2B4 $\beta$							$\beta$ 2 <sup>0/0</sup> /2B4 $\beta$								
CDR3 loops		J $\alpha$	observed number				total (n = 73)	CDR3 loops		J $\alpha$	observed number				total (n = 20)
sequence	length		subfamily			ND*		sequence	length		subfamily				
DSTQVVGQL	9	5	0	1	0		1	DSGYNKY	7	9	0	0	1	1	
DWTQVVGQL	9	5	0	1	0	1	WTGGYKV	7	10	0	0	1	1		
DDNMGYKL	8	8	0	1	0	1	EKTGGYKV	8	10	0	0	1	1		
DAVNMGYKL	9	8	0	1	0	1	GAGNKL	6	14	0	2	0	2		
DLGGYKV	7	10	0	1	0	1	EERGSALGRL	10	15	0	1	0	1		
DEGGYKV	7	10	0	0	1	1	ASSGSWQL	8	17	0	0	1	1		
DAGGYKV	7	10	0	1	0	1	TNTGKL	6	21	0	0	1	1		
DVGGYKV	7	10	0	1	0	1	ETNAYKV	7	23	0	1	0	1		
EAGGYKV	7	10	0	1	0	1	VATNAYKV	8	23	0	1	0	1		
ETGGYKV	7	10	0	2	1	3	LDTNAYKV	8	23	0	1	0	1		
DQGGAL	7	12	0	2	1	3	DAGNNRI	8	24	0	0	1	1		
DGGAGNKL	8	14	0	1	0	1	DGPSSNTNKV	10	27	0	1	0	1		
DRGSALGRL	9	15	0	0	2	2	PTGNTGKL	8	30	0	0	1	1		
YRGSALGRL	9	15	0	1	0	1	ASNNAGAKL	9	32	0	1	0	1		
DNYNQGKL	8	18	0	0	1	1	DASGGSNAKL	10	34	0	1	0	1		
DEYNQGKL	8	18	0	1	0	1	EDNNAP	6	35	0	1	0	1		
DSWYNQGKL	9	18	0	1	0	1	DAQNF	5	41	0	1	0	1		
PTTASLGKL	9	19	0	0	1	1	RASSSFSKL	9	42	0	0	1	1		
DGYAQGL	7	20	0	1	0	1	TEASSSFSKL	10	42	0	0	1	1		
DNYAQGL	7	20	0	4	6	10									
DGQAQGL	7	20	0	1	0	1									
DDYAQGL	7	20	0	0	1	1									
DFYAQGL	7	20	0	1	1	2									
YQYAQGL	7	20	0	1	0	1									
DNNYAQGL	8	20	0	0	1	1									
DANYAQGL	8	20	0	3	1	4									
DHNYAQGL	8	20	0	3	0	3									
DGNYAQGL	8	20	0	1	0	1									
DDNYAQGL	8	20	0	6	0	6									
DQNYAQGL	8	20	0	1	0	1									
DADYAQGL	8	20	0	1	1	2									
DGNNYAQGL	9	20	0	0	1	1									
DDNTNTGKL	9	21	0	1	0	1									
DTNAYKV	7	23	0	1	1	2									
DDTNAYKV	8	23	0	1	0	1									
DGSSGNKL	8	25	0	0	1	1									
DAGNTGKL	8	30	0	1	0	1									
DDPGNTGKL	9	30	0	2	0	2									
DAYRTGNTGKL	11	30	0	1	1	2									
DNAGAKL	7	32	0	3	0	3									
DPYNNNAP	8	35	0	1	0	1									
DVNNNNAP	8	35	0	0	1	1									

\*The clone, of which V $\alpha$ 18 subfamily was not determined because of its shortness is indicated as ND.





Studies on crystallography of two MHC class I-peptide complexes with their associated TCR- $\alpha/\beta$  have revealed that five CDRs both from TCR- $\alpha$  and TCR- $\beta$  chains, except for TCR- $\beta$  CDR2, directly interact with MHC class I-peptide complex in a diagonal orientation (57, 59). Although no structural data are available for the interaction of TCR- $\alpha/\beta$  with MHC class II-peptide complex, functional studies have suggested that a similar but not identical interaction also occurs in this case (43, 44). Since CDR1 and CDR2 are determined by a variable segment itself, our finding on B2L TKO/2B4 $\beta$  mice that developing thymocytes expressing TCR- $\alpha$  chains with particular V $\alpha$  segments and CDR3 loops are allowed to mature on a single MHC-peptide ligand suggests that at least two good fits are required for the interaction of TCR- $\alpha/\beta$  with a selecting ligand to survive positive selection. Despite this requirement, however, TCR- $\alpha$  chains expressed on CD4<sup>+</sup>CD8<sup>-</sup> thymocytes from B2L TKO mice showed no obvious bias in V $\alpha$  usage and amino acid composition of their CDR3 loops. By analyzing the affinity of TCR- $\alpha/\beta$  for positively or negatively selecting MHC-peptide ligands in a cell-free system, Alam et al. (31) have suggested that the affinity window to survive both positive and negative selection is quite narrow. Therefore, provided that a TCR- $\beta$  chain has CDR1 or CDR3 that fits with a selecting ligand, CDR of the associated TCR- $\alpha$  chain on mature thymocytes would be less characteristic, because not only of low affinity interaction sufficient for surviving positive selection but also of negative selection of developing thymocytes expressing TCR- $\alpha$  chains with good fits. Together with the degeneracy in recognition of peptides by TCR- $\alpha/\beta$  (60), this could explain why B2L TKO/2B4 $\beta$  mice but not B2L TKO show structural features in their mature TCR- $\alpha$  repertoire. Although the degree of this structural fitness for a selecting MHC-peptide ligand would be affected by its cell surface density in thymic cortex and medulla (17–19, 24, 61), the partial fitness of TCR- $\alpha/\beta$  for their selecting peptide and/or MHC molecule might be a general feature of the mature T cell repertoire shaped by both positive and negative selection.

Although H-2M<sup>0/0</sup> mice had been used to define the role of particular self-peptides in positive selection (21–23, 25, 27), it has been shown that peptides other than CLIP are bound to I-A<sup>b</sup> molecules and contribute to the positive

selection of CD4<sup>+</sup> thymocytes (26). By comparing a particular V $\alpha$ -J $\alpha$  segment in H-2M<sup>0/0</sup> mice with that in H-2M<sup>0/+</sup> in the presence of a single rearranged TCR- $\beta$  chain, Sant'Angelo et al. (30) recently reported that alteration of self-peptides bound to I-A<sup>b</sup> molecules affects CDR3 length of the selected TCR- $\alpha$  repertoire. The somewhat biased distribution of CDR3 length observed with the TCR- $\alpha$  repertoire in B2L TKO/2B4 $\beta$  mice largely supports their findings. However, it should be noted that homogeneity in the CDR3 length in B2L TKO/2B4 $\beta$  mice depends on the analyzed V $\alpha$ -J $\alpha$  segments: 12 out of 12 clones encoding V $\alpha$ 17.A2-J $\alpha$ 15 have the same CDR3 length, whereas this is not the case with the V $\alpha$ 18-J $\alpha$ 20 segment where 16 clones have a CDR3 loop of 7 amino acids, 18 clones have a CDR3 loop of 8 amino acids, and 1 clone has a CDR3 loop of 9 amino acids. Therefore, different from the case with antigen-driven T cell expansion (62, 63), our findings in B2L TKO/2B4 $\beta$  mice suggest that homogeneity in the CDR3 length does not serve as a hallmark of specific TCR-peptide interaction in the positive selection of thymocytes. In addition, the TCR- $\alpha$  repertoire in B2L TKO/2B4 $\beta$  mice, as compared with that in H-2M<sup>0/0</sup> mice expressing the transgenic TCR- $\beta$  chain, showed a stronger restriction to amino acid residue in the CDR3 loops. This would result from differences in the expression level of I-A<sup>b</sup> molecules in the thymus and/or the diversity of selecting peptides, that is single or heterogeneous. Furthermore, the difference in the introduced TCR- $\beta$  chain might affect the outcome, because D10 TCR- $\beta$  chain used in their experiment is derived from the I-A<sup>b</sup>-reactive TCR- $\alpha/\beta$  (42).

In conclusion, we have shown that, by expression of a single rearranged TCR- $\beta$  chain with irrelevant specificity for the selecting ligand, CD4<sup>+</sup>CD8<sup>-</sup> thymocytes selected to mature on a single MHC class II-peptide ligand express highly restricted TCR- $\alpha$  chains in terms of V $\alpha$  usage and amino acid residue of their CDR3 loops, thereby providing evidence for specific recognition of self-peptides by TCR- $\alpha/\beta$  in positive selection. Thus, our experimental system made it feasible to visualize TCR structure required for surviving positive selection directed by a single MHC-peptide ligand. This approach would lead to the relevant application for elucidation of topology of TCR-MHC-peptide interaction in positive selection.

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