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

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Summary

The T-cell repertoire is shaped by positive and negative selection of thymocytes through the interaction of α/β-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-β chains with a single rearranged TCR-β irrelevant to the selecting ligand, we show here that T cells selected to mature on a single MHC–peptide complex express highly restricted TCR-α chains in terms of Vα 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 β chain • T cell repertoire • transgenic knockout mice

Although the diversity of α/β-TCR theoretically reaches $10^{15}$ by random rearrangement of five gene segments (Vα, Jα, Vβ, Dβ, and Jβ) 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-CD8 immature thymocytes into CD4+CD8+ or CD4-CD8+ 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+ T cells using fetal thymic organ cultures derived from mutant mouse strains where a particular 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+ 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+ or CD4+ 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-α/β with no obvious structural features, this complex could not be a positively selecting ligand for T cells expressing a particular transgenic TCR-α/β 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-α/β in positive selection. However, experiments using TCR-α/β transgenic mice could not exclude the possibility that side chains of the peptides interfere with the interaction of the analyzed TCR-α/β 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-α/β. Sant’Angelo et al. (30) recently addressed this issue by analyzing a particular Vα-Jα segment in mice lacking H-2M (H-2M<sub>0/0</sub>) that catalyzes the dissociation of invariant chain-derived class II-associated peptide, CLIP, in the presence of a single rearranged TCR-β chain. Although this study has shown that alternation of self-peptide repertoire affects CDR3 length of the selected TCR-α 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<sub>0/0</sub> 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-α 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<sub>a</sub>-B6 (E<sub>a</sub>-B6 mice expressing 2B4 TCR-α and invariant chain, H-2M<sub>0/0</sub>-B6/2B4 transgenic for 2B4 TCR-α chain (31), introduction of the TCR-β chain irrelevant to I-A<sup>b</sup>-E<sub>a</sub>-B6-52 complex would give us a better chance to reveal structural features of the associated TCR-α chains selected by this ligand. Therefore, we have selected the 2B4 TCR-β chain derived from the TCR-A-β specific for moth cytochrome c peptide bound to I-E<sub>k</sub> or I-E<sub>k</sub> molecules (32). By comparing the TCR-α repertoire shaped by I-A<sup>b</sup>-E<sub>a</sub>-B6-52 complex with that by I-A<sup>b</sup> molecules with a normal array of self-peptides, including E<sub>a</sub>-B6-52, in the presence 2B4 TCR-β chain, we demonstrate here that expression of TCR-α chains with both particular Vα 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.

Materials and Methods

Mice. B2L mice that express the I-A<sup>b</sup> chain covalently bound to E<sub>a</sub>-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 β2-microglobulin-deficient mice carrying H<sup>-2</sup>-haplotype (β<sup>20/0</sup>; Jackson Laboratories, Bar Harbor, ME), and B2L mice lacking the endogenous I-A<sup>b</sup> chain, invariant chain, and β2-microglobulin were developed (B2L TKO). To develop B2L TKO/2B4<sub>b</sub> mice, we crossed B2L TKO with mice transgenic for 2B4 TCR-β chain that were maintained of C57BL/6 (B6) background (H-2<sup>d</sup>) in our facility (33). B2<sup>20/0</sup>-2B4<sub>b</sub> or E<sub>a</sub>-B6/2B4<sub>b</sub> mice were developed by crossing TKO/2B4<sub>b</sub> mice with β<sup>20/0</sup> or E<sub>a</sub> transgenic B6 (E<sub>a</sub>-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 (HS-A, J11D); FITC-anti-TCR Vα2 (B20.1); V<sub>α</sub>3.2 (R3-16); V<sub>α</sub>8 (B21.14). FITC-anti-TCR Vβ2 (B20.6); Vβ4 (KT4); Vβ5 (MR9-4); Vβ6 (R R4-7); Vβ7 (TR 310); Vβ8 (MR5-2); Vβ9 (MR 10-2); Vβ10 (B21.5); Vβ11 (R R3-15); Vβ12 (MR 11-1); Vβ13 (MR 12-3); Vβ14 (14-2); and biotinylated anti-TCR Vβ3 (K25). The mouse IgM mAb specific for CD8<sub>d</sub> used for the killing experiments were purchased from M eiji 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 Vβ3 mAb followed by streptavidin-Cy-Chrome (PharMingen). To assess the expression of TCR Vα or Vβ 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 Vα or Vβ mAb, with or without biotinylated anti-TCR Vβ3 followed by streptavidin-Cy-Chrome. Analyses were done on a FACSscan® (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-TCR Vα or Vβ and CD4<sup>-</sup>CD8<sup>-</sup> cells with or without biotinylated anti-TCR Vβ3 mAb followed by streptavidin-Cy-Chrome. CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>-</sup> Vβ3<sup>+</sup> thymocytes (1×10<sup>5</sup>) were sorted out using EPICS ELITE® (Coultier Corp., Hialeah, FL) or FACS® Vantage (Becton Dickinson) and were immediately placed in liquid nitrogen. All reagents were used at 10 μg/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-γR at IgG antibody and those coated with anti-mouse IgG antibody (both from DYNAL, Oslo, Norway). The lymph node CD4<sup>+</sup> T cells (1×10<sup>6</sup> cells/well) were cultured with irradiated spleen cells (1×10<sup>6</sup> cells/well) for 80 h and 1 μCi of [3H]thymidine was added during 16 h of the culture.

Acknowledgments. The following oligonucleotides were used: PCo of 1TTTGGAGCATCAAGTCGGTCAC; Ccoulout, AACAGGCGAGGTGGCTGTC; Cwinn, CTTGACCGGAGGACTTCTTTTAAAC; AP, GGGCAACGCCTGACTAGTACGGG1-3GIAGG111GJ3; AUAP, GGGCAACGCCGTGACTAGTAC.

Total cellular RNA was extracted from the sorted thymocytes using ISOGEN in the presence of 1 μl 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 Cα-specific antisense oligonucleotide, PCαF1, was carried out using SuperScript™ 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 PCαF1 and was subjected to homopolymeric tailing using terminal deoxynucleotidyl transferase and dCTP. With this dc-tailed cDNA, several PCR were carried out using C6-out and AP primers, under the following conditions: 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 Cαin 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 DH5α. Each colony was picked up and cultured in LB medium (100 μl) at 37°C for 5 h using 96-well U-bottom plates, then the supernatant was subjected to PCR using AUAP and Cαin2 (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 terminators and sequenced using Cαseq primer (AGACCGAGG ATCTTTTAACT) and were analyzed on an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer Corporation, Foster City, CA) according to standard protocols.

Results

CD4+ T cell differentiation directed by the I-Aβ-Eαα52-68 complex in mice lacking endogenous MHC class I and class II expression. Earlier, we had reported three lines of transgenic mice that had been developed by introducing the gene encoding I-Aβ chain covalently bound to Eαα52-68 into mice lacking both endogenous I-Aβ and invariant chains (DKO; reference 24). Evidence that the I-Aβ molecule covalently bound to Eαα52-68 is expressed in these transgenic lines as a single species was obtained by complete inhibition with the mAb specific for I-Aβ-Eαα52-68 complex of staining with an anti-I-Aβ reagent, the inability of transgenic spleen cells to present other I-Aβ-binding peptides, and the robust proliferative response of transgenic CD4+ T cells to wild-type I-Aβ 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+ CD8− thymocytes in these transgenic mice include NK1.1+ thymocytes that are selected by nonclassical MHC class I molecules such as CD1 (35, 36). To purify CD4+ CD8− thymocytes selected to mature on I-Aβ-Eαα52-68 complex from those selected by nonclassical and probably classical MHC class I molecules, we introduced null mutation of β2-microglobulin required for MHC class I expression into B2L DKO mice that expressed I-Aβ-Eαα52-68 complex in the thymus at an intermediate level and showed most effective CD4+ T cell differentiation among three lines (24). Finally, B2L transgenic mice or nontransgenic mice lacking endogenous I-Aβ, invariant chain, and β2-microglobulin (B2L TKO and TKO) were developed and used in the present study.

Although no definite CD4+ CD8− thymocytes were observed in TKO mice lacking MHC class I and class II expression, significant numbers of CD4+ CD8− thymocytes were selected to mature on a single I-Aβ−Eαα52-68 complex in B2L TKO, reaching a level of ~25% of that seen in mice that express wild-type I-Aβ molecules but lack β2-microglobulin expression (β20/0; Fig. 1A). Although the proportion of CD4+ CD8− thymocytes in B2L TKO was comparable to that in B2L DKO, CD4+ CD8− thymocytes markedly decreased in B2L TKO. Such a decrease, compared with DKO or C57BL/6 (B6) mice, was also found in TKO and β20/0 mice. These observations are consistent with the previous report (37), supporting the model that

![Figure 1. CD4+ T cell differentiation in B2L TKO and B2L DKO mice.](image)
CD4^+CD8^- thymocytes represents developing thymocytes to CD4^-CD8^- phenotype as well as those to CD4^+CD8^- phenotype (38). When NK1.1 expression was analyzed in B2L DKO, around 10% of CD4^+CD8^- thymocytes expressed this surface marker. However, as we expected, CD4^-CD8^-NK1.1^- T cells were scarcely observed in the thymus from B2L TKO (Fig. 1 B).

CD4^-CD8^- T thymocytes selected to mature on the I-A^b-E\x52-68 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^-CD8^- thymocytes from B2L TKO with those from \beta2^0/0 mice, using a panel of mAbs. Although the proportions of CD4^-CD8^- thymocytes expressing TCR V\beta4, 12, and 14 slightly increased in B2L TKO (V\beta4, 12.7 \pm 0.5% vs. 8.3 \pm 0.3%; V\beta12, 3.7 \pm 0.5% vs. 2.7 \pm 0.1%; V\beta14, 12.7 \pm 0.7% vs. 10.2 \pm 0.2%), other TCR \beta V\beta were similarly expressed on CD4^-CD8^- 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, 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^-CD8^- thymocytes and examined TCR-\alpha mRNA using anchored PCR followed by sequencing of the cloned PCR products. From B2L TKO and data not shown).

Figure 2. V\alpha usage (A) and CDR3 length distribution (B) of TCR-\alpha repertoire shaped by the I-A^b-E\x52-68 complex. CD4^-CD8^- thymocytes were sorted from B2L TKO or \beta2^0/0 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. When the expression of TCR V\beta3 was analyzed, 65-75% of CD4^-CD8^- thymocytes from both B2L TKO/2B4\beta and \beta2^0/0/2B4\beta mice expressing 2B4 TCR-\beta chain (\beta2^0/0) were detected in B2L TKO responded well to spleen cells from B2L TKO/2B4\beta mice expressing both B2L transgene and the rearranged 2B4 TCR-\beta (B2L TKO/2B4\beta). Although no CD4^-CD8^- thymocytes were observed in TKO/2B4\beta mice lacking MHC class I and class II expression, significant numbers of CD4^-CD8^- thymocytes were selected to mature in B2L TKO/2B4\beta and \beta2^0/0 mice expressing 2B4 TCR-\beta chain (\beta2^0/0; Fig. 3 A). Lymph node CD4^- T cells from B2L TKO/2B4\beta mice as well as B2L TKO responded well to spleen cells from \beta2^0/0 or C57BL/6 (B6) mice expressing wild-type I-A^b molecules with self-peptides other than E\x52-68, but did not show any response to B2H TKO spleen cells that express I-A^b, E\x52-68 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\beta3 was analyzed, 65-75% of CD4^-CD8^- thymocytes from both B2L TKO/2B4\beta and \beta2^0/0/2B4\beta mice expressing on their cell surface TCR V\beta3 at a high level (Fig. 3 C). Considerable numbers of CD4^-CD8^- V\beta3^- and CD4^-CD8^- V\beta8^- thymocytes expressed TCR V\beta3 that is most frequently observed in CD4^-CD8^- thymocytes from both B2L TKO and \beta2^0/0 mice (B2L TKO, 18.0 \pm 0.4%; \beta2^0/0, 18.2 \pm 0.5%), whereas no definite expression of TCR V\beta8 was detected.
Figure 3. Phenotypic and functional analysis for CD4+ T cell differentiation directed by the I-A\(^{-}\)E\(\alpha\)52-68 complex in the presence of the B24 TCR-\(\beta\) chain. (A) Thymocytes were prepared from TKO/2B4, B2L TKO/2B4, and \(\beta^{20/0}/2B4\) mice at 6–7 wk old and analyzed for CD4 and CD8 expression. The percentages of CD4+CD8+, CD4+CD8+, CD4+CD8+, and CD4+CD8- thymocytes are indicated. For each line, at least three mice were analyzed. The each value for the percentage of CD4+CD8- thymocytes was as follows: TKO/2B4, 0.1, 0.1, 0.1, B2L TKO/2B4, 1.3, 0.9, 0.7, 0.7, \(\beta^{20/0}/2B4\), 2.6, 2.3, 2.7. (B) Lymph node CD4+ T cells from B2L TKO/2B4 or B2L TKO mice were cultured with irradiated spleen cells from B2H TKO, B6, and \(\beta^{20/0}\) mice, and [\(\text{H}\)]thymidine incorporation was measured. The data indicate the mean plus one SD of triplicate cultures. (C) Thymocytes were prepared from B2L TKO/2B4 or \(\beta^{20/0}/2B4\) mice at 6–7 wk old, and the expression of TCR V\(\beta\)3 was analyzed for gated CD4+CD8- thymocytes (top). The percentages of CD4+CD8- V\(\beta\)3, CD4+CD8- V\(\beta\)3, CD4+CD8- V\(\beta\)3, and CD4+CD8- V\(\beta\)3 thymocytes are indicated. For each line, four or three mice were analyzed. The each value for the percentage of CD4+CD8- V\(\beta\)3 thymocytes was as follows: B2L TKO/2B4, 64.7, 71.0, 65.2, 75.0, \(\beta^{20/0}/2B4\), 69.3, 75.3, 70.8. The expression of TCR V\(\beta\)8 on CD4+CD8- V\(\beta\)3, CD4+CD8- V\(\beta\)3, CD4+CD8- V\(\beta\)3, and CD4+CD8- V\(\beta\)3 thymocytes and the percentages of positive population are shown below.

on CD4+CD8- thymocytes expressing TCR V\(\beta\)3 at a high level from both B2L TKO/2B4 and \(\beta^{20/0}/2B4\) (Fig. 3 C). These observations indicate that allelic exclusion by the transgenic B24 TCR-\(\beta\) is almost completed in CD4+CD8- V\(\beta\)3 thymocytes and suggest that these thymocytes exclusively express the transgene.

Highly Restricted Expression of TCR-\(\alpha\) Chains on CD4+CD8- T Cells. T Cells Selected to Maturate on the I-A\(^{-}\)E\(\alpha\)52-68 Complex in the Presence of T Ransgenic B24 TCR-\(\beta\). With the strategy described above, we then compared TCR-\(\alpha\) chains expressed on CD4+CD8- V\(\beta\)3 thymocytes from B2L TKO/2B4 with those from \(\beta^{20/0}/2B4\) or B6 mice expressing both E\(\alpha\) and B24 TCR-\(\beta\) chains (E\(\alpha\)-B6/2B4). In two independent experiments using different B2L TKO/2B4 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 17A2) that was not observed in samples from \(\beta^{20/0}/2B4\) 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 mice, such restricted TCR V\(\alpha\) usage was not observed with clones obtained from E\(\alpha\)-B6/2B4 or \(\beta^{20/0}/2B4\) mice expressing wild-type I-A\(^{\beta}\) molecules with or without I-A\(^{-}\)-E\(\alpha\)52-68 complex, respectively, though the TCR V\(\alpha\) repertoire differed between these strains, probably because of the presence of I-E\(^{\beta}\) molecules in E\(\alpha\)-B6/2B4 mice. When the length of their CDR3 loops was compared, the distribution pattern in B2L TKO/2B4 mice differed from those in \(\beta^{20/0}/2B4\) and E\(\alpha\)-B6/2B4 (Fig. 4 B).

Having found that CD4+CD8- thymocytes selected to mature on I-A\(^{-}\)-E\(\alpha\)52-68 complex preferentially expressed V\(\alpha\)18 in the presence of the B24 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 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 \(\beta^{20/0}/2B4\) 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: valine, threonine, glycine, and leucine (Table 1). AV18S1 encodes threonine at this position, whereas AV18S2 encodes lysine (41). Although the subfamily was not determined in the present study, the high level expression of V\(\alpha\)18 was found in clones encoding this V\(\alpha\) family and analyzed amino acid sequences of their CDR3 loops (\(\alpha\)36 clones) encoded the V\(\alpha\)36 segment (67%) originating from different templates were found to encode the V\(\alpha\)18 family. The expression of TCR V\(\beta\)8 on CD4+CD8- V\(\beta\)3, CD4+CD8- V\(\beta\)3, CD4+CD8- V\(\beta\)3, and CD4+CD8- V\(\beta\)3 thymocytes and the percentages of positive population are shown below.
Discussion

In this study, we compared the TCR-α repertoire in CD4⁺CD8⁺ thymocytes selected to mature on a single I-A<sup>b</sup>-Exα52-68 ligand with that 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-β 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<sub>α</sub> 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-α chains. Although the frequencies of V<sub>α</sub>3 or V<sub>α</sub>8 usage in B2L TKO and β<sup>20/0</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<sub>α</sub>2 usage estimated by this approach in several mouse lines including B2L TKO, β<sup>20/0</sup>, and B2L TKO/2B4 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-α repertoire in CD4⁺CD8⁺ thymocytes.

By introducing the 2B4 TCR-β chain with irrelevant specificity for the I-A<sup>b</sup>-Exα52-68 complex, CD4⁺CD8⁺ thymocytes selected to mature on this single ligand expressed highly restricted TCR-α chains encoding V<sub>α</sub>18 or its related sequence and negatively charged amino acid residues at α93 in their CDR3 loops. Taking into consideration that multiple TCR-α rearrangements cease only after positive selection (48, 49), a small number of clones encoding TCR-α chains without such structural features might represent nonselectable in-frame rearrangements (50). Alternatively, these clones might be derived from a trace of CD4⁺CD8⁻V<sub>β3</sub>thymocytes where allelic exclusion by 2B4 TCR-β is not accomplished. Since such structural features of TCR-α chain were not observed with CD4⁺CD8⁻V<sub>β3</sub>thymocytes selected to mature in β<sup>20/0</sup>/2B4β mice expressing wild-type I-A<sup>b</sup> molecules with a normal array of self-peptides, it is clear that highly restricted V<sub>α</sub> usage and amino acid residues at α93 in B2L TKO/2B4β mice does not result from efficient pairing of particular TCR-α chains with 2B4 TCR-β as was previously reported (51, 52), but rather from thymic selection.
### Table 1. Comparison of Vα18-encoding TCR-αC chains between B2L TKO / 2B4β and β2β/2B4β Mice

| CDR 3 loops sequence | length | Jα | S1 | S2 | ND* | total (n = 73) | CDR 3 loops sequence | length | Jα | S1 | S2 | ND* | total (n = 20) |
|----------------------|-------|----|----|----|-----|----------------|----------------------|-------|----|----|----|-----|----------------|
| 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  | 1  | 0   | 1              |
| DDNAGYKL             | 8     | 8  | 0  | 1  | 0   | 1             | EKTGGYKV            | 8     | 10 | 0  | 0  | 1   | 1              |
| DAVNAGYKL            | 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  | 1  | 0   | 1             | ASSGSWQL            | 8     | 17 | 0  | 0  | 1   | 1              |
| DAEGYKV              | 7     | 10 | 0  | 1  | 0   | 1             | ETNAYKV             | 8     | 15 | 0  | 0  | 0   | 1              |
| DVGGYKV              | 7     | 10 | 0  | 1  | 0   | 1             | TNTGKL              | 6     | 21 | 0  | 0  | 1   | 1              |
| EAGGYKV              | 7     | 10 | 0  | 1  | 0   | 1             | VATNAYKV            | 8     | 23 | 0  | 0  | 1   | 1              |
| ETGGYKV              | 7     | 10 | 0  | 1  | 0   | 1             | EGNAYKV             | 8     | 23 | 0  | 0  | 0   | 1              |
| DQGGRAL              | 7     | 12 | 0  | 2  | 1   | 3             | DGNMNRI             | 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             | PTGSNK              | 8     | 30 | 0  | 0  | 1   | 1              |
| YRGSTGLR             | 9     | 15 | 0  | 1  | 0   | 1             | ASNNAGAKL           | 9     | 32 | 0  | 1  | 0   | 1              |
| DNYNGKGL             | 8     | 18 | 0  | 0  | 1   | 1             | DASGSNKL            | 10    | 34 | 0  | 1  | 0   | 1              |
| DEYNQGK              | 8     | 18 | 0  | 0  | 1   | 1             | EDDNAP              | 6     | 35 | 0  | 1  | 0   | 1              |
| DSNYNQKL             | 9     | 18 | 0  | 1  | 0   | 1             | DGNF                | 8     | 41 | 0  | 1  | 0   | 1              |
| PTASLGL              | 9     | 19 | 0  | 0  | 1   | 1             | RASSSFSKL           | 9     | 42 | 0  | 0  | 1   | 1              |
| DGYAGQGL             | 7     | 20 | 0  | 1  | 0   | 1             | TEASSSFSKL          | 10    | 42 | 0  | 0  | 1   | 1              |
| DNYAQGQ              | 7     | 20 | 0  | 0  | 4   | 6             | 10                 |                   |      |    |    |    |     |                |
| DQGAQGQ              | 7     | 20 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DADYAGQ              | 7     | 20 | 0  | 0  | 1   | 1             |                    |                   |      |    |    |    |     |                |
| YQYAQG              | 7     | 20 | 0  | 1  | 1   | 2             |                    |                   |      |    |    |    |     |                |
| DNNAQGQ              | 8     | 20 | 0  | 0  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DANYAQG              | 8     | 20 | 0  | 0  | 3   | 4             |                    |                   |      |    |    |    |     |                |
| DHNAQGQ              | 8     | 20 | 0  | 0  | 3   | 3             |                    |                   |      |    |    |    |     |                |
| DGNAQGQ              | 8     | 20 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DDNAQGQ              | 8     | 20 | 0  | 6  | 0   | 6             |                    |                   |      |    |    |    |     |                |
| DQNAQGQ              | 8     | 20 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DADYAGQ              | 8     | 20 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DGNNYAQGQ            | 9     | 20 | 0  | 0  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DDNTNQGKL            | 9     | 21 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DTNAQKV              | 7     | 23 | 0  | 1  | 1   | 2             |                    |                   |      |    |    |    |     |                |
| DDTNAQKV             | 8     | 23 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DGSQGNKL             | 8     | 25 | 0  | 0  | 1   | 1             |                    |                   |      |    |    |    |     |                |
| DGNQGKL              | 8     | 30 | 0  | 1  | 0   | 1             |                    |                   |      |    |    |    |     |                |
| DDPQNTQKL            | 9     | 30 | 0  | 2  | 0   | 2             |                    |                   |      |    |    |    |     |                |
| DAYRTGNTQKL          | 11    | 30 | 0  | 1  | 1   | 2             |                    |                   |      |    |    |    |     |                |
| DNGAQKL              | 7     | 32 | 0  | 0  | 3   | 3             |                    |                   |      |    |    |    |     |                |
| DPNNNAP              | 8     | 35 | 0  | 0  | 1   | 1             |                    |                   |      |    |    |    |     |                |
| DVNNNAP              | 8     | 35 | 0  | 0  | 1   | 1             |                    |                   |      |    |    |    |     |                |

*The clone, of which Vα18 subfamily was not determined because of its shortness is indicated as ND.*
expressing TCR-α chains. This might raise the possibility that developing thymocytes from C57 BL/6 mice where I-A<sup>b</sup>-E<sub>α</sub>52-68 complexes are expressed in the thymus, probably at a higher level than that in B2L TKO/2B4<sub>β</sub> mice (34). Therefore, it is suggested that the structural features of TCR-α chains observed in B2L TKO/2B4<sub>β</sub> mice are imprinted under the process of positive selection directed by I-A<sup>b</sup>-E<sub>α</sub>52-68 complex.

Several groups recently reported that antigen-specific T cells selected by a given MHC-peptide complex express somewhat different TCR from those in normal mice, suggesting that selecting self-peptides influence mature T cell repertoire (53–55). However, it is difficult from these experiments to precisely determine whether the altered T cell repertoire mainly results from positive selection directed by a particular MHC-peptide ligand or antigen-driven expansion of some T cells that would be deleted in normal mice expressing the same MHC class II molecules at high level. In the thymus with a normal array of self-peptides, because negative selection to wild-type MHC molecules was lacking in some experiments (54) or was ineffective (53, 55). This issue was clarified in this study where CD4<sup>+</sup> CD8<sup>+</sup> thymocytes positively selected by a single I-A<sup>b</sup>-E<sub>α</sub>52-68 ligand were directly analyzed for TCR-α chains expressed in association with 2B4 TCR-β. Our findings clearly indicate that self-peptides involved in positive selection influence structure of the variable gene segment and CDR3 loops of the selected TCR repertoire. In some antigen-specific TCR recognition, the amino acid residue at α93 has been functionally or structurally shown to be involved in peptide contact (56, 57). Thus, the highly restricted amino acid residue at this position of TCR-α chains in B2L TKO/2B4<sub>β</sub> mice strongly suggests that specific TCR-peptide contacts are also involved in positive selection directed by I-A<sup>b</sup>-E<sub>α</sub>52-68 complex. Since expression of the I-A<sup>b</sup>-E<sub>α</sub>52-68 complex was readily detected in the thymus using a mAb specific for this complex (24), it is unlikely that this contribution of E<sub>α</sub>52-68 to positive selection in B2L TKO/2B4<sub>β</sub> mice is due to an extremely low expression of I-A<sup>b</sup> molecules, which might cause a more stringent requirement of specific selecting peptides than that under physiological conditions. In addition, CD4<sup>+</sup> CD8<sup>+</sup> thymocytes in B2L TKO/2B4<sub>β</sub> mice, different from studies using TCR-αβ transgenic mice, are selected from the semi-diverse T cell repertoire with randomly rearranged TCR-α chains and the essentially fixed 2B4 TCR-β chain. Although further analysis needs to address whether self-peptides with amino acid residues bearing bulky or charged side chains at positions facing TCR would mediate efficient positive selection, our findings on B2L TKO/2B4<sub>β</sub> mice provide definitive evidence that specific recognition of self-peptides by TCR is involved in positive selection of thymocytes and support the recent observation that the amino acid composition of CDR3 loops analyzed for particular Vβ<sub>j</sub>β segments in B2L DKO mice is slightly different from those in B6 mice (58).

**Figure 5.** Predicted amino acid sequence of V<sub>α</sub>17.A2-encoding TCR-α chains. (A) The NH<sub>2</sub>-terminal structure of V<sub>α</sub>17.A2 from cysteine at position 90 is compared with that of AV18S2. CDR1 and CDR2 are boxed. (B) The clones originating from different templates were analyzed for CDR3 loops and θ<sub>α</sub> gene segments.

directed by I-A<sup>b</sup>-E<sub>α</sub>52-68 complex. We cannot determine at this stage whether aspartic or glutamic acid at α93 is determined by the germ line sequence or created by the N-region. However, it is suggested that both structural features in the V<sub>α</sub> gene segment and amino acid residue at α93 are independently required to survive thymic selection by this single ligand, because other V<sub>α</sub> gene segments, including AV10S6 that encodes aspartic acid at this position, and negative selection directed by I-Ab molecules, which might cause a more stringent requirement of specific selecting peptides than that under physiological conditions. In addition, CD4<sup>+</sup> CD8<sup>+</sup> thymocytes in B2L TKO/2B4<sub>β</sub> mice, different from studies using TCR-αβ transgenic mice, are selected from the semi-diverse T cell repertoire with randomly rearranged TCR-α chains and the essentially fixed 2B4 TCR-β chain. Although further analysis needs to address whether self-peptides with amino acid residues bearing bulky or charged side chains at positions facing TCR would mediate efficient positive selection, our findings on B2L TKO/2B4<sub>β</sub> mice provide definitive evidence that specific recognition of self-peptides by TCR is involved in positive selection of thymocytes and support the recent observation that the amino acid composition of CDR3 loops analyzed for particular Vβ<sub>j</sub>β segments in B2L DKO mice is slightly different from those in B6 mice (58).
Studies on crystallography of two MHC class I–peptide complexes with their associated TCR-α/β have revealed that five CDs both from TCR-α and TCR-β chains, except for TCR-β CDR 2, 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-α/β 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 action also occurs in this case (43, 44). Since CDR1 and CDR2 are determined by a variable segment itself, our action also occurs in this case (43, 44). Since CDR1 and CDR2 are determined by a variable segment itself, our action also occurs in this case (43, 44). Since CDR1 and CDR2 are determined by a variable segment itself, our action also occurs in this case (43, 44).

When analyzing the affinity of TCR-α/β 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-β chain has CDR 1 or CDR 3 that fits with a selecting ligand, CDR of the associated TCR-α 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-α chains with good fits. Together with the degeneracy in recognition of peptides by TCR-α/β (60), this could explain why B2L TKO/2B4β mice but not B2L TKO show structural features in their mature TCR-α 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-α/β 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-2M0/0 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-Aβ molecules and contribute to the positive selection of CD4+ thymocytes (26). By comparing a particular Vα-Jα segment in H-2M0/0 mice with that in H-2M0/+ in the presence of a single rearranged TCR-β chain, Sant’Angelo et al. (30) recently reported that alteration of self-peptides bound to I-Aβ molecules affects CDR 3 length of the selected TCR-α repertoire. The somewhat biased distribution of CDR 3 length observed with the TCR-α repertoire in B2L TKO/2B4β mice largely supports their findings. However, it should be noted that homogeneity in the CDR 3 length in B2L TKO/2B4β mice depends on the analyzed Vα-Jα segments: 12 out of 12 clones encoding Vα17.A2-Jα15 have the same CDR 3 length, whereas this is not the case with the Vα18-Jα20 segment where 16 clones have a CDR 3 loop of 7 amino acids, 18 clones have a CDR 3 loop of 8 amino acids, and 1 clone has a CDR 3 loop of 9 amino acids. Therefore, different from the case with antigen-driven T cell expansion (62, 63), our findings in B2L TKO/2B4β mice suggest that homogeneity in the CDR 3 length does not serve as a hallmark of specific TCR–peptide interaction in the positive selection of thymocytes. In addition, the TCR-α repertoire in B2L TKO/2B4β mice, as compared with that in H-2M0/0 mice expressing the transgenic TCR-β chain, showed a stronger restriction to amino acid residue in the CDR 3 loops. This would result from differences in the expression level of I-Aβ molecules in the thymus and/or the diversity of selecting peptides, that is single or heterogeneous. Furthermore, the difference in the introduced TCR-β chain might affect the outcome, because D10 TCR-β chain used in their experiment is derived from the I-Aβ-reactive TCR-α/β (42).

In conclusion, we have shown that, by expression of a single rearranged TCR-β chain with irrelevant specificity for the selecting ligand, CD4+CD8− thymocytes selected to mature on a single MHC class II–peptide ligand express highly restricted TCR-α chains in terms of Vα usage and amino acid residue of their CDR 3 loops, thereby providing evidence for specific recognition of self-peptides by TCR-α/β 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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