Alterations in CD4-Binding Regions of the MHC Class II Molecule I-E\textsuperscript{k} Do Not Impede CD4\textsuperscript{+} T Cell Development

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The T cell coreceptors CD4 and CD8 enhance T cell responses to TCR signals by participating in complexes containing TCR, coreceptor, and MHC molecules. These ternary complexes are also hypothesized to play a seminal role during T cell development, although the precise timing, frequency, and consequences of TCR-coreceptor-MHC interactions during positive selection and lineage commitment remain unclear. To address these issues, we designed transgenic mice expressing mutant I-Eκ molecules with reduced CD4-binding capability. These transgenic mice were crossed to three different lines of I-Eκ-specific TCR transgenic mice, and the efficiency of production of CD4⁺ lineage cells in the doubly transgenic progeny was assessed. Surprisingly, replacing wild-type I-Eκ molecules with these mutant molecules did not affect the production of CD4⁺ CD8⁻ thymocytes or CD4⁺ peripheral T cells expressing any of the three TCRs examined. These data, when considered together with other experiments addressing the role of coreceptor during development, suggest that not all MHC class II-specific thymocytes require optimal and simultaneous TCR-CD4-MHC interactions to mature. Alternatively, it is possible that these particular alterations of I-Eκ do not disrupt the CD4-MHC interaction adequately, potentially indicating functional differences between I-A and I-E MHC class II molecules. The Journal of Immunology, 1999, 162: 1348–1358.

The cell-surface molecules CD4 and CD8 are well established as T cell coreceptors, capable of enhancing T cell responses to TCR signals (for review, see Ref. 1). CD4 and CD8 are expressed on mutually exclusive subsets of peripheral T cells: lymphokine-secreting “helper” T cells and cytotoxic “killer” T cells, respectively. CD4 has been shown to bind specifically to MHC class II molecules and not to class I molecules, correlating with the MHC specificity of the helper T cell subset (2). The converse is true for CD8, which binds to MHC class I molecules and not to class II molecules, correlating with the MHC specificity of the killer T cell subset (3). CD4 and CD8 can also transmit intracellular signals, in part via the association of their cytoplasmic tails with p56lck, a lymphocyte-specific protein tyrosine kinase (4–6). This kinase phosphorylates critical residues in cytoplasmic tails of the TCR/CD3 complex, facilitating the recruitment of other kinases, like ZAP-70, and thereby initiating a signaling cascade (for review, see Ref. 7). Coreceptor and TCR molecules aggregate when they both bind to MHC molecules during T cell activation (8–10). This colocalization enhances the degree of T cell stimulation (3, 11), presumably by stabilizing T cell-APC adhesion and recruiting p56lck molecules to the TCR/CD3 complex (12). Thus, the formation of a ternary complex including the TCR, MHC, and coreceptor molecules is a key event in the activation of mature T cells.

CD4 and CD8 are also coexpressed on immature thymocytes, and the formation of a similar ternary complex has been hypothesized to play a seminal role during T cell development. Several lines of evidence suggest that coreceptors play a role in positive selection (rescue from apoptosis), as well as in lineage commitment (the decision to become a CD4⁺ or a CD8⁺ T cell). Early experiments employing anti-CD4 or anti-CD8 Abs demonstrated that CD4 expression was critical for the development of helper T cells, and CD8 for the development of cytotoxic T cells (13, 14). More recently, mice lacking expression of CD4 or CD8α have been shown to be deficient in the helper T cell or cytotoxic T cell lineages, respectively (15–18). While there is general agreement that coreceptors can influence positive selection and lineage commitment, the details of the timing, frequency, and consequences of coreceptor-MHC interactions during these processes remain unclear. These details have been the subject of intense investigation, but the results are not clearly consistent with a simple model describing the influence of coreceptors on thymocyte cell fate (for review, see Ref. 19). Some experiments suggest an absolute requirement for coreceptor-MHC interactions during development (e.g., Ref. 18), while other data suggest that T cell maturation can sometimes proceed without coreceptor engagement (e.g., Ref. 20). In addition, recent studies have suggested that the CD4 lineage and CD8 lineage pathways may not have equivalent requirements for coreceptor (e.g., Refs. 21 and 22). Thus, our understanding of the role of coreceptor during T cell development requires further refinement.

One way to explore this issue is to disrupt coreceptor-MHC interactions in vivo by expressing mutant MHC molecules in transgenic mice (23–25). For example, Aldrich et al. (23) examined transgenic mice expressing a mutant version of the MHC class I molecule Ld that could not be bound by CD8. They assayed the peripheral T cells from these mice for cytotoxic T cell activity against targets presenting cytomegalovirus peptides bound to Ld and found no such activity, suggesting that the formation of the TCR-CD8-MHC class I ternary complex was required for maturation of CD8⁺ cells. In addition, Killeen et al. (24) performed a more definitive experiment with similar MHC class I transgenic mice bred to the MHC class I-specific H-Y TCR transgenic line. In these doubly transgenic animals, CD8⁺ T cells expressing the H-Y TCR did not develop efficiently, demonstrating that a simultaneous...
TCR-CD8-MHC class I interaction was necessary for their maturation. Is the formation of a simultaneous TCR-CD4-MHC class II complex similarly important for the development of MHC class II-specific thymocytes? The current ambiguity regarding the symmetry of the CD4 and CD8 lineage pathways enhances the interest in performing analogous experiments with mutant MHC class II molecules.

To perform these experiments, we chose to construct transgenic mice expressing mutant MHC class II molecules unable to interact with CD4. In previous work, our laboratory had identified two MHC class II I-E<sup>b</sup> regions involved in CD4 interactions. I-E<sup>b</sup> molecules with substitutions in these regions were able to present peptide and bind to the TCR normally but were defective in a CD4-dependent in vitro thymocyte stimulation assay (26). Accordingly, we designed transgenic mice expressing these mutant I-E<sup>b</sup> molecules to examine the role of CD4-MHC interactions during development. Based on the studies of other groups examining mice lacking expression of CD4 altogether (15, 16), we speculated that disrupting CD4-I-E<sup>b</sup> interactions specifically in vivo would further demonstrate the importance of these interactions to the positive selection and lineage commitment of CD4<sup>+</sup> T cells. We crossed our transgenic mice expressing the mutant I-E<sup>b</sup> molecules to 2B4, SJL, and AND TCR transgenic mice and analyzed the efficiency of CD4<sup>+</sup> T cells in the double transgenic mice. Surprisingly, we found that these suboptimal conditions for CD4-I-E<sup>b</sup> interaction did not impede the maturation of CD4<sup>+</sup> T cells expressing any of these three TCRs.

Materials and Methods

Mice

2B4 TCR transgenic (27), SJL TCR transgenic (28) (obtained from M. Davis, University of California San Diego, La Jolla, CA), and AND TCR transgenic (29) (obtained from S. Hedrick, Stanford University Medical School, Stanford, CA), I-E<sup>k</sup>km1 transgenic, I-E<sup>k</sup>km2 transgenic, and B10.BR and B10.D2 strains of mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in a specific pathogen-free facility. Mice were interbred to obtain the genotypes of interest. Progeny sacrificed for experiments were 6–11 wk old. All transgenic mice analyzed were on the fourth or greater backcross to the B10 background and no longer expressed superantigen (30). Genotypes of 2B4 TCR transgenic progeny were determined by PCR on genomic DNA to identify the presence of the 2B4 TCR a and b transgenes (31). Other genotypes were determined by Southern blot analysis or by FACS analysis of mAb-stained PBLs, as described below.

Analysis of mutant MHC molecules

The mutant MHC molecules described in Fig. 1 have been previously reported (26). The thymocyte dulling assay was performed as previously described (26).

Transgene construction and analysis of founder mice

A cosmid clone containing the I-E<sup>b</sup> locus was digested with EcoRI (NEB, Beverly, MA) and subcloned for simpler manipulation (for more information on the organization of this locus, see Ref. 32). The 2.0-kb EcoRI fragment containing exon 2 (encoding the β1 domain) was replaced with the 2.0-kb EcoRI fragment containing the corresponding exon of I-E<sup>b</sup>. To introduce mutations into the genomic clone, the 6.0-kb EcoRI fragment containing exon 3 and the 2.0-kb EcoRI fragment containing exon 2 were subjected to site-directed mutagenesis via the duentung method (33), using previously described oligonucleotides (26). After confirming the correct sequence of the mutated exons, the altered EcoRI fragments were reincorporated into full-length genomic clones in a Bluescript (Stratagene, La Jolla, CA) vector. To aid reconstruction of the genomic clone and facilitate screening for transgenic founders, additional restriction sites were introduced at two of the EcoRI sites.

Transgenic mice were made by standard methods (34). Fertilized eggs from (C57BL/6 × (C57BL/6 × He/J))F<sub>2</sub> mice were injected with the transgenes after free mixing of transgene sequences from vector sequences by digestion with SalI (NEB). To identify transgenic founders, genomic DNA was prepared from tail cuts of 10-day-old mice. Southern blots of DNA digested with BamHI (NEB) were probed with the PstI (NEB) fragment illustrated in Fig. 2.

2. DNA preparation, blotting, and hybridization techniques were all standard (35). These blots displayed an 8.0-kb fragment from endogenous I-E<sup>b</sup> genes, a 5.3-kb fragment from endogenous I-E<sup>k</sup> genes, and a 3.0-kb fragment from the transgene (Fig. 2).

Abs and FACS analysis

The following Abs and secondary reagents were used in these studies: H120.19-phycoerythrin (PE) (rat anti-mouse CD4; Life Technologies, Gaithersburg, MD), 53-6.7-RED 613 (rat anti-mouse CD8α; Life Technologies), A2B4-FITC (anti-2B4 TCRα; Ref. 36), RR8-1-FITC (rat anti-Vα11; PharMingen, San Diego, CA), 14.4.4-biotin (anti-I-E<sup>b</sup>; Ref. 37), 17.3.3-biotin (anti-I-E<sup>k</sup>; Ref. 37), 39-10-8-biotin (anti-I-A<sup>k</sup>; PharMingen), Y17-biotin (anti-I-E<sup>b</sup>; Ref. 38), IM7-biotin (anti-CD44; PharMingen), M1/69-biotin (anti-heat stable Ag (HSA); PharMingen), streptavidin-FITC (Southern Biotechnology Associates, Birmingham, Alabama), and streptavidin-PE (Southern Biotechnology Associates). For analyses of thymocytes, splenocytes, and PBLs, cells were prepared as previously described (39). Flow cytometric analysis was performed on a FACScan instrument (Becton Dickinson, Mountain View, CA). Ungated data (30,000–50,000 events per thymocyte sample; 10,000–50,000 events per PBL sample; 30,000–50,000 events per splenocyte sample) were collected and analyzed using CellQuest software (Becton Dickinson). Before analysis, samples were gated on live lymphocytes based on forward- and side-scatter parameters. Dot plots and histograms of live-gated samples shown in the figures are representative of many similar mice (see Table II). For statistical analyses of these data, the percentages of live cells falling in the appropriate gates from all individual mice of a particular genotype were pooled, and then the mean and SD for each data set were calculated. The means of sets of percentages were then compared using the two-tailed Student’s t test for independent samples (40). The resulting p values are provided throughout the text.

3 Abbreviations used in this paper: HSA, heat stable Ag. MCC moth cytochrome c.
Results

Generation of transgenic mice expressing mutant MHC class II molecules

First, it was necessary to identify substitutions of MHC class II I-E\(^b\) residues that would disrupt interactions with CD4 without affecting the capacity of the I-E\(^b\) molecule to bind peptides or the TCR. Based on the studies of others investigating CD4-class II interactions (41–45), we chose to mutate regions of the I-E \(\beta\)-chain in both the \(\beta1\) and \(\beta2\) domains. As a result of this analysis, we identified two regions of I-E\(^b\) important for CD4 interactions; a complete description of these studies including all the relevant controls has been reported previously (26). Fig. 1 shows one example of these data, demonstrating that APCs expressing the mutant I-E\(^b\) molecules are impaired in their ability to induce thymocyte dunning, an assay which is strongly dependent on CD4-MHC interactions (26). As shown in Fig. 1 and previously (26), substitution of two residues in the 3–4 (C-D) loop of the membrane-proximal \(\beta2\) domain of I-E\(^b\) (Glu\(^{137}\) and Glu\(^{138}\) replaced with leucines; “km1”) reduces thymocyte responsiveness; combining substitution of these two residues with a change in the membrane-distal \(\beta1\) domain (Glu\(^{137}\) and Glu\(^{138}\) replaced with leucines, Arg\(^{48}\) replaced by glu; “km2”) reduces thymocyte responsiveness even further. The \(\beta1\) domain substitution alone has the same effect as combining the \(\beta1\) and \(\beta2\) substitutions (26). Thus, as previously reported, these mutations disrupt thymocyte signaling; altogether, it is extremely likely that this disruption is the result of altered CD4-MHC interactions caused by the substitutions in residues 48 and 137 of I-E\(^b\) (26).

On the basis of these results, we chose to construct two types of transgenic mice: one type, expressing I-E\(^b\) molecules with only the \(\beta2\) mutations, was predicted to cause a less severe defect than the second type, with both the \(\beta1\) and \(\beta2\) mutations. We designed a transgene to drive expression of I-E\(^b\) in cell types that normally express MHC class II. Although the precise promoter and enhancer elements responsible for MHC class II expression are still under investigation, it seemed likely that using a large enough portion of genomic sequence would provide the bona fide class II promoter and upstream regulatory sequences, as has been the case for genomic clones of I-E\(^a\) and I-A\(^b\) (47–50). We therefore made use of an existing genomic clone that encompasses approximately 19 kb of the I-E\(^b\) locus, including approximately 3 kb of 5' and 5 kb of 3' flanking sequence (32). As a genomic clone of I-E\(^a\) was not available, the construct shown in Fig. 2A was generated from an I-E\(^b\) cosmid clone (kindly provided by Dr. Patricia Jones, Stanford University, Stanford, CA) by replacing the 2.0-kb EcoRI fragment containing exon 2 (encoding the \(\beta1\) domain) with the 2.0-kb EcoRI fragment containing the corresponding exon of I-E\(^a\) (kindly provided by Dr. Ronald Germain, National Institutes of Health, Bethesda, MD). All of the four amino acid differences between I-E\(^b\) and I-E\(^b\) reside in exon 2; all other exons are identical (32). To introduce mutations into the genomic clone, the 2.0-kb EcoRI fragment containing the \(\beta1\) domain and the 6.0-kb EcoRI fragment containing the \(\beta2\) domain were subjected to site-directed mutagenesis and then reincorporated into full-length transgenes. One of these transgenes, referred to as I-E\(^b\)\(^{km1}\), includes only the \(\beta2\) domain substitutions. The other transgene, referred to as I-E\(^b\)\(^{km2}\), includes the \(\beta1\) domain and the \(\beta2\) domain substitutions.

Transgenic mice were generated by standard procedures (34), and Southern blot analysis of tail DNA identified a total of twenty founders (Fig. 2B, for example). Transgenic founders were crossed to B10.D2 (H-2\(^d\)) mice to generate lines. B10.D2 mice express the I-A\(^d\) and I-E\(^d\) (but not the I-E\(^b\)) MHC class II molecules. We predicted that introduction of an I-E\(^b\) transgene would result in the surface expression of an I-E dimer formed by the endogenous I-E\(^d\) molecule and the transgene-encoded I-E\(^b\) molecule; this complex has been shown to be functionally indistinguishable from

![FIGURE 2. The design of the I-E\(^b\) transgenes. A, The organization of the I-E\(^b\) genomic clone used for microinjection into fertilized mouse eggs is shown, indicating the domains encoded by each exon. Boxes represent exons, and the line connecting the exons represents intron sequences. The exons are labeled as follows: “L” denotes the exon encoding leader sequences, “\(\beta1\)” encodes the \(\beta1\) domain, “\(\beta2\)” encodes the \(\beta2\) domain, and the exons marked “TM/CYT/3’UT” encode the transmembrane, cytoplasmic, and 3’ untranslated regions. The 2.0-kb EcoRI fragment including the \(\beta1\) exon (gray regions) indicates the region where I-E\(^b\) sequences have replaced I-E\(^a\) sequences. Mutations were introduced in the codon for residue 48 in the \(\beta1\) domain (labeled “48”) and in the codons for residues 137 and 138 (labeled “137”) in the \(\beta2\) domain. The restriction sites for EcoRI (E) and BamHI (B) and the location of the Psrl fragment used as a probe for Southern blotting are indicated. B\(_1\) and B\(_2\) are BamHI sites found only in the endogenous I-E\(^b\) locus or in the transgene, respectively. B, Genomic Southern blot of tail DNA from six pups derived from embryos injected with the I-E\(^b\) transgene. DNA was digested with BamHI, and the blot was probed with the Psrl fragment shown in Fig. 1A. Note the distribution of endogenous I-E\(^b\) (k) and I-E\(^b\) (b) genes in the (C57Bl/6J × C3H/He)F\(_1\)F\(_2\) pups, as well as the high number of copies of the transgene (tg) incorporated into the DNA of pup number 3.

Table I. MHC alleles expressed in mouse strains used in these studies*

<table>
<thead>
<tr>
<th>MHC allele</th>
<th>I-E(^a)</th>
<th>I-E(^b)</th>
<th>I-A</th>
<th>Class 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2(^a)</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td></td>
</tr>
<tr>
<td>I-E(^b)(^{km1})</td>
<td>d + tg k (137,138)</td>
<td>d</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>I-E(^b)(^{km2})</td>
<td>d + tg k (48,137,138)</td>
<td>d</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>H-2(^b)</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
</tbody>
</table>

*Each row describes the alleles of each MHC gene present in a particular mouse strain. For example, “k” indicates homozygosity of the k alleles of a specific endogenous gene. In the transgenic strains I-E\(^b\)\(^{km1}\) and I-E\(^b\)\(^{km2}\), “tg k” indicates the introduction of an I-E\(^b\) transgene that includes substitutions in the residues indicated in parentheses.
an I-E<sup>k</sup>/I-E<sup>b</sup> dimer (51, 52). At least four generations of crossing to B10.D2 were performed to establish homozygosity at the H-2<sup>k</sup> or H-2<sup>d</sup> background as “I-E<sup>k</sup>km1” or “I-E<sup>b</sup>km2” transgenic mice (Table I).

We chose to express our mutant I-E<sup>b</sup> molecules on the H-2<sup>d</sup> background rather than on a MHC class II-deficient background (53, 54) for two reasons. First, mice lacking all MHC class II molecules, or having low class II expression due to a mutation in the invariant chain gene (55, 56), have abnormally high levels of CD4 on their CD4<sup>+</sup> thymocytes. Thus, an overall deficiency in MHC class II expression, potentially altering signaling through CD4, therefore, we elected to perform our experiments on a background of wild-type MHC class II expression to preserve normal CD4 expression. Second, we wanted to alter only the specific ternary complex involving in positive selection of the TCRs being examined, all of which are positively selected by I-E<sup>k</sup> (see below). This is most directly accomplished by disrupting only the interactions between CD4 and I-E<sup>k</sup>; in I-E<sup>k</sup>km1 and I-E<sup>b</sup>km2 mice, only I-E<sup>k</sup> is defective, while I-A<sup>k</sup> and I-E<sup>b</sup> retain their normal interactions with CD4.

The expression of transgenic I-E<sup>b</sup> molecules is similar to the expression of endogenous MHC class II molecules

We examined MHC class II expression in eight I-E<sup>b</sup>km1 transgenic lines and one I-E<sup>b</sup>km2 line. Transgenic expression on an H-2<sup>d</sup> background can be monitored using the I-E<sup>b</sup> specific mAb, 17.3.3 (37). This Ab does not recognize I-E<sup>k</sup> and, therefore, detects only the I-E molecules containing the transgenic I-E<sup>b</sup> and not the endogenous I-E<sup>k</sup> molecules. Six of eight I-E<sup>b</sup>km1 lines express transgenic I-E<sup>b</sup> on splenic B cells (BB20<sup>+</sup>) at levels comparable to the amount of I-E<sup>b</sup> in an H-2<sup>b</sup> (B10.BR) mouse (Fig. 3A and data not shown). Two I-E<sup>b</sup>km1 lines express lower levels of I-E<sup>b</sup> and are described elsewhere (57). No transgenic lines express more I-E<sup>b</sup> than in an H-2<sup>b</sup> mouse; presumably, the number of cell-surface I-E<sup>b</sup>...
dimers is limited by the endogenous levels of I-E<sub>k</sub>. Additionally, these data demonstrate that the transgenic I-E<sub>b</sub><sup>km1</sup> molecule can out-compete the endogenous I-E<sub>b</sub><sup>km2</sup> for pairing with I-E<sub>k</sub>, as suggested by previous studies (58). Staining splenic B cells with the I-E<sub>k</sub> specific mAb 14.4.4 (37) and the I-A<sub>d</sub>-specific mAb 39-10-8 (PharMingen) demonstrates that transgene expression does not alter the total amount of I-E or I-A on the cell surface (Fig. 3, A and C).

Since our mutation in the β1 domain of I-E<sub>b</sub><sup>km1</sup> eliminates the epitope of the 17.3.3 mAb, expression of the I-E<sub>b</sub><sup>km2</sup> transgene was monitored with the mAb Y17 (38), which recognizes I-E<sub>k</sub> but not I-E<sub>b</sub> dimers containing I-E<sub>b</sub><sup>km2</sup>. As was the case for the I-E<sub>b</sub><sup>km1</sup> transgenic lines, the I-E<sub>b</sub><sup>km2</sup> line expresses as much I-E<sub>b</sub> on splenic B cells as H-2<sup>k</sup> mice express (Fig. 3D). As expected, this expression cannot be detected with 17.3.3 (Fig. 3E). Expression of the I-E<sub>b</sub><sup>km2</sup> transgene, as is the case for the I-E<sub>b</sub><sup>km1</sup> transgene, does not affect the total cell-surface I-E or I-A expression (data not shown).

In addition to the splenic B cells shown above, lymph node and peripheral blood B cells, splenic dendritic cells, and splenic macrophages from the transgenic mice also expressed I-E<sub>b</sub> at levels comparable to those seen in H-2<sup>k</sup> mice (data not shown). Transgenic I-E<sub>b</sub> molecules are expressed in the thymic cortex and medulla in patterns indistinguishable from those seen in H-2<sup>k</sup> thymi (data not shown). Furthermore, the transgenic MHC molecules function appropriately. Transgenic B cells process cytochrome<sub>c</sub> protein and present its I-E<sub>b</sub>-binding epitope as well as H-2<sup>k</sup> B cells do (data not shown), indicating that the transgenic I-E<sub>b</sub> molecules proceed normally through the intracellular trafficking patterns required for peptide loading. Therefore, all evidence suggests that the thymic stroma in I-E<sub>b</sub><sup>km1</sup> and I-E<sub>b</sub><sup>km2</sup> transgenic mice contain normal numbers of I-E<sub>b</sub> molecules that should be fully accessible to the normal array of thymic peptides, indicating that the comparison of I-E<sub>b</sub><sup>km1</sup> and I-E<sub>b</sub><sup>km2</sup> transgenic mice with H-2<sup>k</sup> mice is appropriate.

TCR transgenic mice used to assess positive selection

We crossed the I-E<sub>b</sub><sup>km1</sup> and I-E<sub>b</sub><sup>km2</sup> transgenic mice to three lines of TCR transgenic mice. All three TCRs used, 2B4, 5C.C7, and AND, recognize an identical cytochrome<sub>c</sub> peptide bound to I-E<sub>k</sub> (29, 46, 59). In addition to their similar specificity, these three TCRs are structurally similar. All three incorporate V<sub>α1</sub>L and V<sub>β3</sub> gene segments. In fact, the β-chains of the 5C.C7 and AND TCRs are completely identical (29, 60). Thymocytes expressing any of these three similar TCRs are positively selected by I-E<sub>k</sub> but not by I-E<sub>d</sub> (27–29, 39, 57, 61). Although these three TCRs share general structural features, each has a unique fine specificity for recognition of cytochrome<sub>c</sub> peptide variants and allo-MHC Ags (39, 61–64). These unique specificities lead to functional consequences. Thymocytes are not selected with equal efficiency in 2B4/H-2<sup>k</sup>, 5C.C7/H-2<sup>k</sup>, and AND/H-2<sup>d</sup> mice, and 2B4, 5C.C7 and AND T cells differ in their dependence on CD4 for maximal responsiveness (57). By using three related TCRs with some distinct characteristics, we hoped to elucidate whether the effects of our I-E<sub>k</sub> mutations would apply generally to multiple TCRs.
For each type of TCR transgenic, we compared the thymocytes and PBLs of TCR/H-2k, TCR/H-2d, TCR/I-Eβ km1, and TCR/I-Eβ km2 mice (Table I). All of the TCR/I-Eβ km1 and TCR/I-Eβ km2 mice were homozygous for the endogenous H-2d loci. In TCR/H-2k mice, formation of the ternary complex (TCR-CD4-MHC) is unhindered, and thymocytes are positively selected by endogenous H-2k molecules. In contrast, no MHC class II molecules are recognized by the transgenic TCRs in TCR/H-2d mice, so efficient positive selection cannot occur. Finally, in TCR/I-Eβ km1 or TCR/I-Eβ km2 mice, the TCRs can recognize the mutant I-Eβ3 molecules, although CD4-I-Eβ interactions are inhibited.

Positive selection proceeds normally in thymic TCR transgenic mice expressing mutant I-Eβ km1 molecules

For each of the three types of TCR transgenic mice, there are no significant differences between the total number of thymocytes found in age-matched TCR/H-2k and TCR/I-Eβ km1 or TCR/I-Eβ km2 mice, indicating that neither mutation has a gross effect on thymocyte production (Table II). Using FACS analysis, we examined thymocyte subpopulations defined by CD4, CD8, and TCR expression (Figs. 4 and 5, Table II). Consistent with the MHC class II specificity of these TCRs, the mature thymocytes produced in TCR/H-2k mice are overwhelmingly CD4+CD8+ cells and not CD4+CD8− cells. In TCR/I-Eβ km1 and TCR/I-Eβ km2 mice, the percentage of CD4+CD8− cells per thymus is similar to the percentage observed in TCR/H-2d mice. For instance, Fig. 4A depicts representative thymi from 2B4/H-2k and 2B4/I-Eβ km1 mice that have 10.0% and 11.5% CD4+CD8− cells, respectively. Furthermore, the 2B4α profiles for these CD4+CD8− cells show high homogeneous levels of the transgenic TCR α-chain. Expression of Vβ3 is also comparable (data not shown), demonstrating that the CD4+CD8− cells are expressing the transgenic TCR. Additionally, the production of 2B4α+CD4+CD8− cells in 2B4/I-Eβ km1 mice is obviously distinct from the lack of production in 2B4/H-2d mice (Fig. 4A, Table II). Inspection of 5C.C7 (Fig. 5, Table II) and AND (Table II) TCR transgenic mice yielded similar results. In all TCR/I-Eβ km1 and TCR/I-Eβ km2 mice, the percentage of TCRα+CD4+CD8− cells per thymus is statistically indistinguishable (p < 0.01, comparing data sets using the two-tailed Student’s t test; see Materials and Methods) from the percentage observed in TCR/H-2k mice (Fig. 4, Fig. 5, Table II). Thus, replacement of I-Eβ with mutant I-Eβ km1 or I-Eβ km2 molecules does not interfere with positive selection in these cases.

The production of peripheral CD4+ T cells is unaffected in I-Eβ km1 and I-Eβ km2 mice

We confirmed the efficiency of positive selection in TCR/I-Eβ km1 and TCR/I-Eβ km2 mice by examining peripheral CD4+ T cells. Figs. 6 and 7 show representative FACS profiles for pairs of 2B4 and 5C.C7 mice. In these cases, as well as in the AND TCR transgenic mice, TCR/I-Eβ km1 and TCR/I-Eβ km2 mice have no statistically significant defect (p < 0.01) in the production of TCRα+CD4+ T cells (Table II). Additionally, production of CD4+ cells in the presence of I-Eβ, whether wild-type or mutant, far surpasses production of CD4+ cells in TCR/H-2d mice (Table II).
II). Analyses of HSA, CD44, and CD69 expression on these peripheral CD4+ T cells suggest that, in all TCR/I-Eβkm1 and TCR/I-Eβkm2 mice, these cells are phenotypically normal, naive peripheral T cells (data not shown). Consistent with this conclusion, preliminary functional data assessing cytochrome c-specific responses indicate that the CD4+ T cells in TCR/I-Eβkm1 and TCR/I-Eβkm2 mice proliferate and secrete IL-2 as well as CD4+ T cells from TCR/H-2k mice do (data not shown). Thus, it appears that CD4+ cells expressing any of these three TCRs can mature in the thymus and emigrate to the periphery normally, even in the presence of mutant I-Eβ molecules.

CD44 surface expression is increased on 2B4/I-Eβkm1 and 2B4/I-Eβkm2 thymocytes

Because we were surprised by the generally normal production of CD4+ T cells in TCR/I-Eβkm1 and TCR/I-Eβkm2 mice, we looked closely at the expression of the differentiation markers HSA, CD44, and CD69 on thymocytes from mice of all genotypes. Modulation of the levels of these cell-surface markers accompanies the process of positive selection: HSA levels decrease as maturation proceeds (65), CD44 levels increase during this process (66, 67), and CD69 levels increase and then decrease again before mature cells exit the thymus (68). Given the normal expression of these markers on CD4+ peripheral T cells from all of the mice analyzed, we hypothesized that, if development were truly unaffected, we would observe comparable expression patterns on thymocytes from TCR/H-2k, TCR/I-Eβkm1, and TCR/I-Eβkm2 mice. This was indeed the case for the 5C.C7 and AND thymocytes. No differences in differentiation marker expression are obvious when comparing cells from H-2k, I-Eβkm1, and I-Eβkm2 mice (data not shown). However, in contrast to the thymocytes in the 5C.C7 and AND thymocytes, thymocytes from 2B4/I-Eβkm1 and 2B4/I-Eβkm2 mice express higher levels of CD44 than thymocytes from 2B4/H-2k mice (Table III, Fig. 8). The enhanced levels are more striking on 2B4/I-Eβkm2 thymocytes than on 2B4/I-Eβkm1 thymocytes (Table III). Furthermore, heightened expression is most evident on the mature CD4+CD8+ thymocytes but could also be noted on immature thymocytes (Table III). In contrast, HSA and CD69 expression patterns on 2B4/I-Eβkm1 and 2B4/I-Eβkm2 thymocytes were normal, relative to 2B4/H-2k thymocytes (Table III and data not shown).

FIGURE 6. Normal populations of CD4+ T cells are found in the peripheral blood of 2B4/I-Eβkm1 and 2B4/I-Eβkm2 mice. Peripheral blood cells from age-matched 2B4/H-2k, 2B4/I-Eβkm1, and 2B4/I-Eβkm2 mice were stained with Abs to CD4, CD8, and 2B4α. Live-gated dot plots and histograms are as indicated in Fig. 3. In A, PBLs from 2B4/H-2k and 2B4/I-Eβkm1 mice are compared. In B, PBLs from 2B4/H-2k and 2B4/I-Eβkm2 mice are compared. In all cases examined, the populations of CD4+ T cells found in the lymph nodes and spleens of 2B4 transgenic mice expressing mutant I-Eβ molecules are also normal in comparison to 2B4/H-2k mice (data not shown).

FIGURE 7. Normal populations of CD4+ T cells are found in the peripheral blood of 5C.C7/I-Eβkm1 and 5C.C7/I-Eβkm2 mice. Peripheral blood cells from age-matched 5C.C7/H-2k, 5C.C7/I-Eβkm1, and 5C.C7/I-Eβkm2 mice were stained with Abs against CD4, CD8, and Vα11. Live-gated dot plots and histograms are as indicated in Figs. 3 and 4. In A, PBLs from 5C.C7/H-2k and 5C.C7/I-Eβkm1 mice are compared. In B, PBLs from 5C.C7/H-2k and 5C.C7/I-Eβkm2 mice are compared. In all cases examined, the populations of CD4+ T cells found in the lymph nodes and spleens of 5C.C7 transgenic mice expressing mutant I-Eβ molecules are also normal in comparison to 5C.C7/H-2k mice (data not shown).
mocytes. Thymocytes from age-matched 2B4/H-2 k and 2B4/I-E b mice were stained with Abs against CD4, CD8, and CD44. "Mature" and "immature" CD4 T cells were defined as shown in Fig. 7A. A mean channel value (on a scale of 0–1024) for the fluorescence indicating CD44 or HSA surface expression was calculated for each sample. The values shown here are the average of at least three mean channel values for each genotype. SDs are given in parentheses.

### Discussion

A clear conclusion emerges from these studies: the I-E b km1 and I-E b km2 mutant molecules do not hinder the maturation of CD4 + T cells expressing the 2B4, 5C.C7, or AND TCRs. In all cases, TCR/I-E b km1 and TCR/I-E b km2 mice produce as many CD4 + CD8 + thymocytes and peripheral T cells as TCR/H-2 k mice. Our initial expectation was that if these mutations impeded CD4-I-E k interactions, we would observe striking defects in the development of CD4 + T cells. However, since this expectation was not fulfilled, we must consider several possible interpretations of these data.

First, it is formally possible that the particular mutations made in I-E b do not adequately disrupt the CD4-I-E k interaction. Given the modest defects seen with these mutant molecules in our in vitro assays (Fig. 1, Ref. 26), we cannot be absolutely certain that these mutations have significant consequences for the formation of the TCR-CD4-MHC ternary complex in vivo. Unfortunately, it is not possible to demonstrate CD4 interacting directly with these sites, due to the low affinity of murine CD4 for murine MHC class II molecules (69). However, several other lines of evidence support the conclusion that these sites are engaged by CD4. For instance, both functional and biochemical studies by other groups have identified the analogous loop of the β2 domain of other MHC class II molecules as a CD4 binding site. Specifically, König et al. (42) have demonstrated that altering residue 137 of the MHC class II molecule I-A b eliminates responsiveness almost entirely in a CD4-dependent functional assay. Additionally, a peptide spanning the analogous β2 region (residues 134–148) of the human MHC class II molecule DR4 has been shown to bind directly to human CD4 (41). The structural similarities between DR and I-E make this particularly compelling evidence in favor of residues 137 and 138 in I-E being involved in CD4 binding (70). Finally, the possibility that residue 48 also binds directly to CD4 is supported by several experiments demonstrating the ability of MHC class II-derived peptides spanning this region to inhibit CD4-dependent T cell activation (43–45). It is also interesting to note that the recent crystal structure of a CD8-MHC class I complex vividly demonstrates the interaction of CD8 with residues 223–229 of the MHC class I α3 domain (the loop analogous to the proposed MHC class II β2 CD4-binding site), as well as with residues in the MHC class I α2 domain (the region analogous to the proposed MHC class II β1 CD4-binding site) (71). Taken together, these results make it extremely likely that our substitutions in residues 48 and 137 of I-E b genuinely disrupt CD4-MHC interactions.

Second, the ineffectiveness of our mutations may suggest the more interesting possibility that CD4-I-E b interactions are not essential for the development of CD4 + T cells expressing the 2B4, 5C.C7, and AND TCRs. This interpretation is in apparent conflict with the severe defect in CD4 + T cell development observed in mice lacking CD4 (15, 16). There are at least two possible explanations for this apparent discrepancy. First, a direct comparison of our mice to mice lacking CD4 may not be appropriate, as it is possible that eliminating CD4 entirely creates a situation distinct from inhibiting CD4-I-E b interactions specifically. For instance, coreceptor interactions with the positively selecting MHC molecule (i.e., I-E b) may not be essential if coreceptor interactions with noncognate MHC molecules (i.e., I-A b and I-E b) are also available. Second, it is possible that CD4 + T cells expressing the 2B4, 5C.C7, and AND TCRs belong to a special subset of T cells that do not require a CD4-MHC class II interaction during development. This idea is supported by studies of several TCR transgenic lines, including the AND line, crossed to the CD4 knockout (72).

In these studies, no decrease in the number of mature thymocytes expressing the AND TCR was observed in the absence of CD4. This result directly supports our own observations that some TCRs selected on I-E class II molecules do not require a CD4-class II interaction for maturation. This idea is further supported by evidence from a close examination of the peripheral T cells in CD4 knockout mice. Specifically, these studies revealed the presence of MHC class II-specific helper T cells responsive to Leishmania infection and also cells capable of mediating Ab class switching (20, 73). These cells become members of the CD4 + T cell lineage, by
functional criteria, without ever expressing CD4. Thus, it is probable that a subset of thymocytes expressing MHC class II-restricted TCRs are exempt from the requirement for CD4 during development. Furthermore, the 2B4, 5C.C7, and AND thymocytes may be members of this group.

It is possible that the T cells expressing the 2B4, 5C.C7, and AND TCRs may not require CD4-MHC class II interactions for maturation because the positively selecting ligand for these TCRs is exceptionally abundant. Other groups have demonstrated that altering the peptide ligands on thymic stromal cells can lead to the production of MHC class I-specific CD8** lineage cells from thymocytes deficient in CD8a expression (74, 75). The authors propose that coreceptor-MHC interactions may be important for development only when maturation is relatively inefficient, due to limiting amounts of thymic peptide ligands (74, 75), and furthermore, that this may be the case more often for MHC class I-specific thymocytes than for MHC class II-specific thymocytes (74). This model could explain our data: if the thymic ligands of the 2B4, 5C.C7, and AND TCRs were plentiful, then these thymocytes would be unaffected by the mutant I-E** molecules. However, the precise thymic ligands of these three TCRs remain unknown. Further elucidation of these ligands may ultimately demonstrate whether ligand density correlates with a requirement for TCR-C4-MHC class II ternary complex formation.

Considering this model, it is intriguing to note that the only observed effect of substituting wild-type I-E** molecules with mutant I-E** molecules was the up-regulation of CD44 expression by the 2B4/I-E** and 2B4/I-E** thymocytes. Of the three types of TCR transgenic thymocytes examined, those expressing the 2B4 TCR are the least efficiently selected on an H-2** background and the most dependent upon adequate expression of MHC class II for positive selection (57). Therefore, it is possible that this TCR recognizes a less abundant thymic peptide ligand than the 5C.C7 and AND TCRs. Consistent with this possibility, there may be a subtle effect of the I-E** transgene in the 2B4 background. There is a decrease in the percentage of CD4** thymocytes in the 2B4/I-E** mice compared with 2B4/I-E* mice (from 11.5% to 7.5%), although statistical analysis with this number of mice indicates that this decrease is not significant.

We cannot explain precisely how expression of the I-E** or I-E** molecules influences the expression of CD44 on 2B4 thymocytes. The degree of heightened expression of CD44 correlates with the increased severity of I-E** mutations, suggesting that a decrease in CD4-I-E** interactions somehow alters the kinetics of CD4** T cell development. However, this result is paradoxical because CD44 up-regulation normally indicates successful signal transduction through the TCR (66). Thus, the I-E** mutations would be expected, if any effect was seen, to reduce the expression of CD44. No other signs of heightened signal transduction (i.e., increased TCR levels on CD4** CD8** or CD4** CD8** thymocytes) were apparent in these mice. Our understanding of this result awaits more thorough comprehension of the events that lead to CD44 up-regulation during normal thymic ontogeny.

As a final note, it is interesting to compare our studies with other recent studies of the MHC class II molecule I-A**. Gillfillan et al., in collaboration with König and Germain, have designed transgenic mice expressing an I-A** molecule with substitutions in the 137 and 142 residues of the β2 domain (these data graciously provided by S.G., D.M., C.B., R.K., and R.G., unpublished data). This transgene was introduced onto an MHC class II-deficient background (53). The transgenic I-A** molecule dimerizes with endogenous I-A** to form the only MHC class II molecule expressed. The efficiency of production of total heterogeneous CD4** T cells in mice expressing the mutant I-A** transgene was then compared with that of mice expressing a wild-type I-A** transgene, and it was found that the mutant transgenic mice have significantly fewer (<50%) CD4** T cells than the wild-type transgenics. The contrast between the effectiveness of these I-A mutations and the ineffectiveness of our I-E mutations is striking, although differences in our experimental designs (i.e., MHC knockout background vs H-2d background) make direct comparisons difficult. Nevertheless, we speculate that this β2 domain CD4-binding site is less critical for CD4-I-E interactions than for CD4-I-A interactions, thereby accounting for the functional differences between the I-E and the I-A mutants in this region. This idea is also supported by the results of prior studies that have suggested an inherent difference in the functional capacities of I-A and I-E, with CD4-I-E interactions being weaker than CD4-I-A interactions (76, 77). Thus, the repertoire of TCRs positively selected on I-A molecules may be intrinsically more CD4-dependent than those selected on I-E molecules.

To further examine the effectiveness of the I-E** and I-E** molecules on CD4** T cell development, we plan to compare the CD4-dependence of I-E**-restricted T cells in non-TCR transgenic I-E** and I-E** mice to the CD4-dependence of I-E**-restricted T cells in wild-type H-2** mice. This analysis would help discriminate between the models described above. For instance, if Ag-specific I-E**-restricted T cells isolated from the mutant mice were significantly more CD4-independent than those isolated from wild-type mice, this would suggest that the 2B4, AND, and 5C.C7 TCRs do belong to a special subset of MHC class II-restricted T cells. Alternatively, if no differences in the CD4-dependence of the TCR repertoires between the mutant and wild-type I-E**-expressing mice could be discerned, this would suggest that, in general, I-E**-restricted TCRs are not dependent on maximal CD4-I-E** interactions during thymic selection. In this latter case, the three TCRs analyzed here, 2B4, AND, and 5C.C7, would be representative of all I-E**-restricted TCRs, indicating an important functional distinction between I-E and I-A.

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