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Immunology

Disruption of the CD4–major histocompatibility complex class II interaction blocks the development of CD4$^+$ T cells *in vivo*

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ABSTRACT **The experiments presented in this report were designed to specifically examine the role of CD4–major histocompatibility complex (MHC) class II interactions during T cell development *in vivo*. We have generated transgenic mice expressing class II molecules that cannot interact with CD4 but that are otherwise competent to present peptides to the T cell receptor. MHC class II expression was reconstituted in Aβ gene knock-out mice by injection of a transgenic construct encoding either the wild-type I-Aβ^b protein or a construct encoding a mutation designed to specifically disrupt binding to the CD4 molecule. We demonstrate that the mutation, EA137 and VA142 in the β2 domain of I-A^b, is sufficient to disrupt CD4–MHC class II interactions *in vivo*. Furthermore, we show that this interaction is critical for the efficient selection of a complete repertoire of mature CD4$^+$ T helper cells as evidenced by drastically reduced numbers of conventional CD4$^+$ T cells in animals expressing the EA137/VA142 mutant I-A^b and by the failure to positively select the transgenic AND T cell receptor on the mutated I-A^b. These results underscore the importance of the CD4–class II interaction in the development of mature peripheral CD4$^+$ T cells.**

The CD4 protein is expressed on a subset of T lymphocytes, primarily helper T cells, that recognize foreign antigen in the context of major histocompatibility complex (MHC) class II proteins. T cells recognize foreign antigen, presented as peptides bound to MHC molecules, by virtue of unique antigen-specific receptors, $\alpha\beta$ T cell receptors (TCRs), expressed on their surface. The CD4 and CD8 coreceptor molecules, also required for efficient T cell recognition of peptide–MHC complexes, interact with nonpolymorphic regions of the class II and class I MHC molecules, respectively. Collectively, these interactions dictate the cellular response to foreign antigen and result in the release of soluble mediators, antibody production, and immune cell recruitment to the area of injury or inflammation.

Animals deficient in CD4, CD8, or MHC class I and class II molecules have provided significant insight into the functions of these proteins during T cell development and in the generation of peripheral immune responses. Mice lacking MHC class II proteins, generated by targeted disruption of the I-Aβ gene in H-2b haplotype mice, display a profound lack of CD4$^+$ T cells in the periphery (1, 2); analogous results have been obtained in MHC class I-deficient (β_2-microglobulin gene-targeted) mice that fail to generate mature peripheral CD8$^+$ T cells (3, 4). Nevertheless, although these experiments emphasize the importance of MHC proteins in the selection of the T cell repertoire in the thymus, in the absence of MHC proteins, it is impossible to assess the relative contribution of TCR–MHC versus coreceptor–MHC interactions to T cell development and function.

It has been proposed that coreceptor–MHC interactions stabilize otherwise low-affinity TCR–MHC interactions and that it is the trimolecular complex of coreceptor, MHC, and TCR that provides the optimal signal for T cell activation and for "thymic education" of developing thymocytes (5). To test this hypothesis, a number of investigators generated transgenic (tg) mice expressing MHC class I molecules that were altered in the CD8 binding site in the α3 domain (6–9). These studies demonstrate that an intact CD8–class I interaction can affect the outcome of thymic selection either by inhibiting the positive selection of a unique TCR specificity or by allowing positive selection of T cells with higher TCR affinity for the MHC–peptide complex. This is consistent with recent kinetic analyses showing that the off rate of the TCR for its MHC–peptide ligand is substantially decreased in the presence of CD8 (10). Thus, there is substantive evidence that the CD8 coreceptor, via its interaction with MHC class I molecules, contributes to the overall avidity of the TCR–MHC complex.

Although it is assumed that the CD4 coreceptor acts similarly in its association with MHC class II proteins, models designed to explore its contribution to TCR–MHC class II interactions have been less informative (11–14). Animals lacking the CD4 coreceptor have been described (12–14); these animals displayed detectable, but reduced, levels of helper activity as measured by interleukin 2 production, antibody responses, and the ability to resist infection by *Leishmania* (15). Helper cell function was presumed to reside in an expanded population of CD4$^-$ CD8$^-$ $\alpha\beta$ TCR$^+$ cells, although the effector cell population was not clearly defined (16, 17). The simplest interpretation of these studies is that CD4-mediated interactions are not essential for the generation of an effective helper T cell response. However, recent studies have shown that CD4-deficient mice fail to mount an effective immune response when challenged with *Nippostrongylus brasiliensis* a potent inducer of T helper (Th) type 2-mediated immunity (18). In fact, CD4-deficient mice have been shown to have a general defect in Th2-mediated immune responses, thus implying that CD4 may play an important role in determining effector function (18, 19). A role for CD4–class II interactions in the regulation of memory T cell responses has also been described (20).

The experiments presented in this report were designed to specifically examine the role of the CD4–class II MHC interaction during T cell development *in vivo*. We have generated transgenic mice expressing class II molecules that cannot interact with CD4 but are otherwise competent to present peptides to the TCR. MHC class II expression was reconstituted in Aβ gene "knock-out" mice by injection of

Abbreviations: C2D, class II deficient; DP, double positive; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; SP, single positive; TCR, T cell receptor; Th, T helper; FITC, fluorescein isothiocyanate; PE, phycoerythrin; FACS, fluorescence-activated cell sorter.

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transgenic construct(s) encoding wild-type or mutant (EA37/VA142) I-Aβ^b proteins. The decision to mutate residues 137 and 142 was based on *in vitro* studies that had shown that this region in the β2 domain of the murine class II molecule is important for its interaction with murine CD4 (21).

In this report, we describe the phenotype of mice carrying the mutant Aβ (EA137/VA142) transgene. A feature of this model is that animals express only the mutated class II molecules. In this way, the repertoire of CD4 single-positive (SP) T lymphocytes selected *in vivo* could be analyzed. These animals are distinct from other systems that have been used previously to examine CD4 coreceptor function. They differ from mice lacking MHC class II proteins in that CD4–class II binding is the only interaction disrupted. Unlike CD4-deficient mice, the fate of CD4 T cells, selected in the absence of coreceptor interactions, can be monitored phenotypically. Finally, they are distinct from "tailless" CD4 transgenic mice in which CD4 signaling has been disrupted while leaving the CD4–class II adhesive interaction intact (22). Thus, we can specifically address the role of CD4–class II MHC interactions during T cell ontogeny and in the generation of peripheral immune responses.

MATERIALS AND METHODS

Constructs and Mutagenesis. The genomic construct encoding the wild-type sequence for I-Aβ^b was subcloned from the cosmid clone I$_\beta$-101 (23), a gift from Per Peterson, Scripps Clinic, San Diego, CA). For mutagenesis of residues Glu-137 and Val-142, the 2.1-kb *Bam*HI fragment containing the β2 exon was subcloned into pBluescript II KS$^+$ (Stratagene) for mutagenesis using the Chameleon mutagenesis kit (Stratagene). Nucleotides encoding Glu-137 and Val-142 were modified by using a single oligonucleotide (5′-AAGCTGTGTGGATGAGGCCCCCACCGTCTCCGCCGTGCCATTC-3′) and DNA sequences were confirmed by dideoxynucleotide sequence analysis.

Transgenic Animals. The genomic sequences encoding Aβ^b (wild type or mutant) were separated from plasmid sequences on 40 ml of 10–40% sucrose gradients centrifuged in an SW28 rotor for 24 h (26,000 rpm, 16°C). DNAs were microinjected into fertilized oocytes isolated from class II-deficient (C2D) mice (GenPharm International, Mountain View, CA); oocytes were transferred into pseudopregnant C57BL/6J $\times$ SJL F_1 female recipients (The Jackson Laboratory). Offspring were screened by standard Southern blot hybridization of genomic tail DNA (24). All animals used were housed in a specific pathogen-free barrier facility (Duke University, Durham, NC). Founder animals were bred with C2D animals to propagate the transgenic lines. Homozygous AND (H-2^b, B6 $\times$ SJL) TCR transgenics (provided by Stephen Hedrick, University of California, San Diego) were bred with C2D animals (C57BL/6 background) to generate AND$^{+/-}$, I-A$^{b/-}$ F_1 heterozygotes. The F_1 offspring were then mated to generate AND$^+$ C2D animals that were mated with EA137/VA142 transgenics.

Immunohistology. Immunohistology was performed by the Immunohistology Laboratory (Department of Pathology, Duke University Medical Center). Briefly, acetone-fixed thymus sections were incubated with biotinylated KH74 (anti-I-A^b, PharMingen) in PBS plus Fc block (50 μg/ml, PharMingen) followed by an avidin-horseradish peroxidase conjugate (Vectastain Elite ABC kit, Vector Laboratories). Staining was visualized by incubation with 3,3′-diaminobenzidine and counterstaining with Harris' hematoxylin.

Flow Cytometry. Single cell suspensions of spleen, lymph node, or thymus (1–2 $\times$ 10^6 cells) were incubated with Fc block (50 μg/ml, PharMingen) and stained alone or in the indicated combinations with antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin in a final volume of 50 μl. Anti-CD4 (H129.19), CD8 (53–6.7), and CD3ε (29B) were from GIBCO/BRL; anti-I-A^b (AF6–120.1), B220/CD45R (RA3–6B2), $\alpha\beta$ TCR (H57–597), and NK1.1 (PK136) were from PharMingen. Streptavidin-Cy-Chrome conjugate (PharMingen) was used to visualize samples stained with biotinylated antibodies. Analyses were performed on a FACScan (Becton Dickinson) using CELLQUEST software.

RESULTS

Expression of Wild-Type and Mutant (EA137/VA142) I-Aβ^b Proteins in MHC C2D Mice. The transgenic DNA construct used to reconstitute MHC C2D mice with wild-type I-Aβ^b is diagrammed in Fig. 1*A*. An additional construct, harboring a two-amino acid alteration, EA137/VA142, in the β2 domain was engineered by replacement of the wild-type 2.1-kb *Bam*HI fragment with the same fragment encoding the mutations. The constructs were microinjected directly into fertilized oocytes isolated from C2D mice. Integration of the Aβ transgene(s) was confirmed by Southern blot hybridization of tail DNA (Fig. 1*B*). Several transgenic founder lines were established for both wild-type (four lines) and EA137/VA142 mutant (two lines) Aβ constructs (Fig. 1*B*). Whereas initial studies were performed with animals harboring high copy number integrations, 6 copies for the wild-type reconstituted and 10 copies for the EA137/VA142 mutant (Fig. 1*B*, lanes labeled with an asterisk), a later comparison of high and low (1 or 2) copy-number lines (both wild-type reconstituted and EA137/

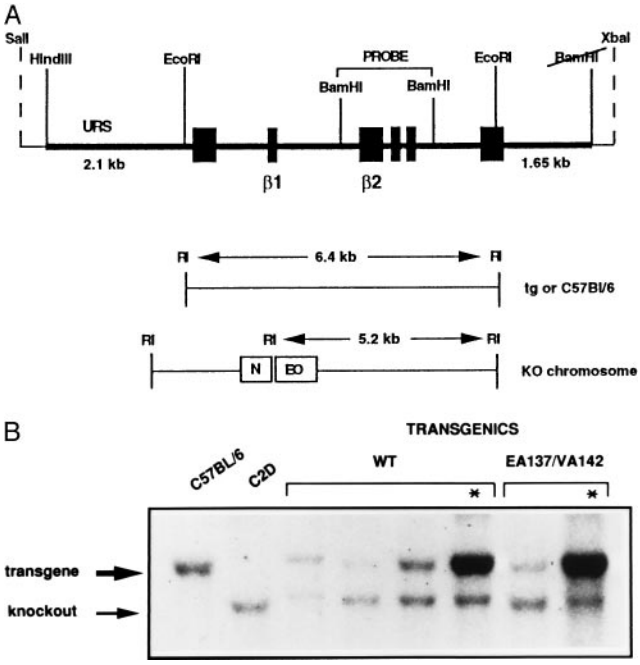

FIG. 1. (*A*) The 10.2-kb genomic construct, derived from the I$_\beta$-101 cosmid clone, contains 2.1 kb of upstream regulatory sequence (URS), the Aβ coding region within a 6.4-kb *Eco*RI fragment, and 1.65 kb of 3′ untranslated sequence. The Aβ tg construct was excised from plasmid sequences by digestion with *Sal*I and *Xba*I, purified by sucrose gradient density centrifugation, and microinjected into fertilized oocytes isolated from C2D mice. C2D mice were engineered by insertion of the gene encoding neomycin resistance within the β1 exon of I-A^b (1, 2). (*B*) Southern blot of 5 μg of genomic DNA isolated from proteinase K-treated tail samples from C57BL/6, C2D, wild-type (WT) tg, and EA137/VA142 tg founder pups. The DNA was digested with *Eco*RI, separated on 0.7% agarose gels, transferred to nylon membranes, and probed with a 2.1-kb *Bam*HI fragment containing the β2 exon (PROBE in *A*). A 6.4-kb fragment corresponds to the wild-type or tg allele (large arrow). A 5.2-kb fragment corresponds to the endogenous knock-out chromosome (small arrow) due to the addition of an *Eco*RI site within the neomycin-resistance gene introduced into the Aβ locus with the gene-targeting construct.

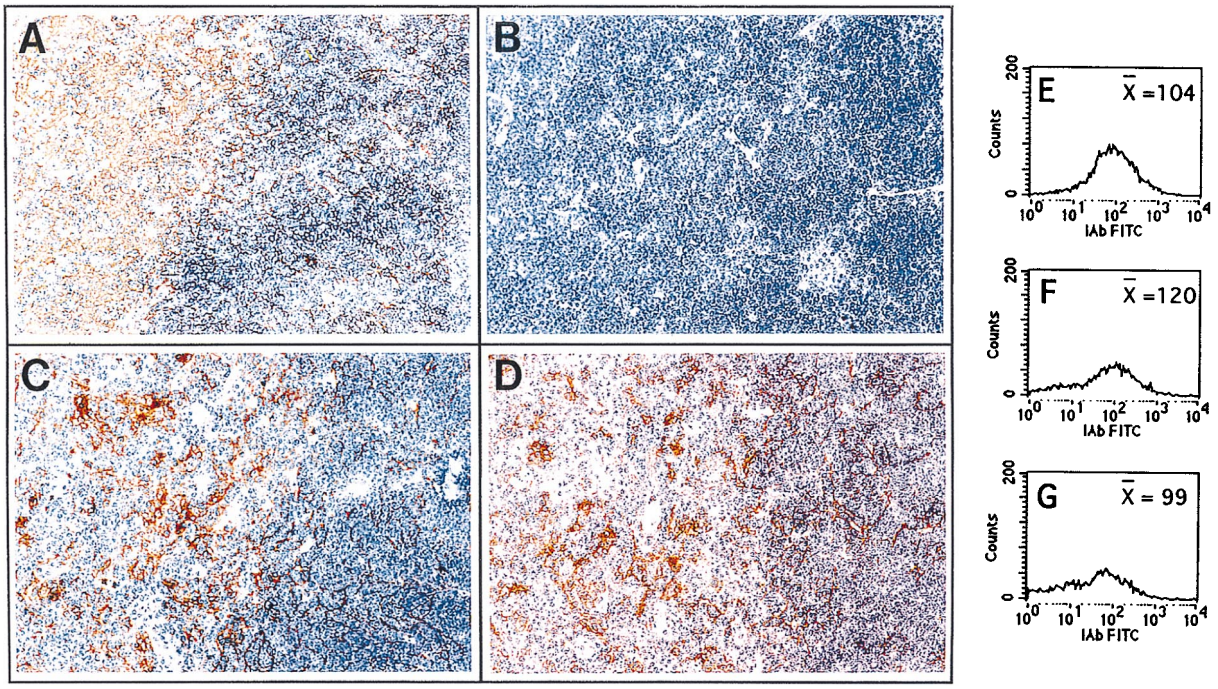

FIG. 2. Immunohistology of frozen thymic sections from C57BL/6 (*A*), C2D (*B*), wild-type reconstituted tg (*C*), and EA137/VA142 tg (*D*) mice. Sections are stained for I-A^b (immunoperoxidase staining in brown); counterstaining with hematoxylin reveals thymocytes (staining in blue). FACS profiles of gated $B220^+$ splenocytes stained for I-A^b (FITC): *E*, C57BL/6; *F*, wild-type reconstituted tg; *G*, EA137/VA142 mutant tg. The MFIs ($\bar{X}$ = mean) for $B220^+$ I-A^+ lymphocytes are given.

VA142 mutant) yielded similar results, arguing against a gene dose effect. This is not unexpected because assembly of Aβ with endogenous Aα, as well as invariant chain proteins, is likely to be the limiting factor in regulating expression levels of class II molecules at the cell surface (25).

To analyze the tissue-specific expression of MHC class II molecules in transgenic animals, immunohistologic staining of frozen sections of thymus (Fig. 2) and spleen (data not shown) was performed. A confluent pattern of immunoperoxidase staining, corresponding to MHC class II-positive cells, is visible in medullary regions as opposed to a more reticular staining in cortical regions. This pattern of I-A^b expression on thymic epithelium in both wild-type (Fig. 2*C*) and mutant (Fig. 2*D*) transgenic animals is similar to that seen in C57BL/6 (Fig. 2*A*) control animals, indicating that authentic class II expression is restored in thymii of transgenic animals. Further evidence of accurate class II expression was shown by immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis of splenocytes that revealed that I-A^b proteins are expressed on $B220^+$ B lymphocytes in transgenic animals at levels comparable to C57BL/6 controls (Fig. 2 *E–G*), although there are more $B220^+$, I-A^- splenocytes in transgenic animals as compared with C57BL/6 controls. Importantly, all experiments presented in this report compare the transgenic wild-type mice with transgenic EA137/VA142 Aβ mutant animals. Thus, any phenotypic differences between wild-type and mutant transgenic animals must be attributed to the EA137/VA142 mutation rather than to differences in the level or tissue-specific expression of the I-A^b protein.

TCR and CD4 Are Expressed at Higher Levels on Double-Positive (DP) Thymocytes in EA137/VA142 Mutant Mice. It was important to demonstrate that the EA137/VA142 mutation actually disrupted the interaction between CD4 and I-A^b MHC class II molecules *in vivo*. It is known that the constitutive engagement of CD4 on DP thymocytes by MHC class II molecules expressed on thymic stromal cells maintains low-level expression of TCR on immature thymocytes (26, 27). Consequently, a substantial increase in TCR levels is detected when DP thymocytes are cultured in single-cell suspension at 37°C in the absence of MHC class II-expressing epithelial cells. Increased levels of TCR are also seen in MHC class II-deficient mice (2, 28). To determine whether the EA137/VA142 mutation was sufficient to disrupt CD4–class II interactions in the thymus, we compared levels of TCR expression on DP thymocytes in our wild-type reconstituted and EA137/VA142 mutant mice with C57BL/6 and C2D animals. Thymocytes were triple labeled with anti-CD4 (FITC), anti-CD8 (PE), and anti-TCRβ (biotinylated followed by Cy-Chrome-labeled streptavidin) and the levels of TCR expression on gated $CD4^+$ $CD8^+$ DP thymocytes were plotted as histograms, comparing wild-type reconstituted (Fig. 3*A Upper*) and EA137/VA142 Aβ mutant (Fig. 3*A Lower*) mice with C57BL/6 and C2D animals. Whereas DP thymocytes from wild-type reconstituted animals displayed TCR levels that were comparable to C57BL/6 DP thymocytes [wild-type mean

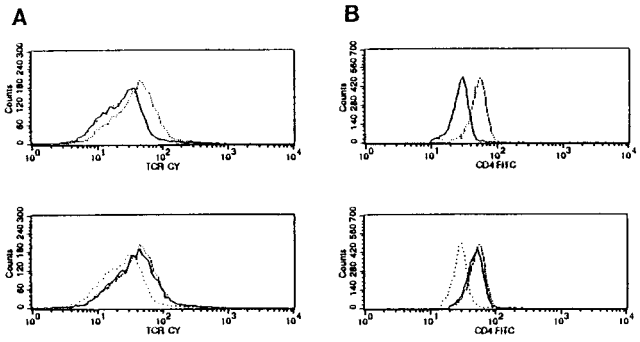

FIG. 3. (*A*) Histograms of TCR (CY-Chrome) expression on gated $CD4^+$ $CD8^+$ thymocytes from wild-type reconstituted (*Upper*) and EA137/VA142 (*Lower*) tg thymocytes (solid lines) overlaid on profiles of DP thymocytes from C2D (closely spaced dots) and C57BL/6 (widely spaced dots). (*B*) Histograms of CD4 (FITC) expression on gated $CD4^+$ $CD8^+$ thymocytes from wild-type reconstituted (*Upper*) and EA137/VA142 (*Lower*) tg thymocytes (solid lines) overlaid on profiles of DP thymocytes from C2D (closely spaced dots) and C57BL/6 (widely spaced dots).

fluorescence intensity, $MFI_{(WT)} = 35.0$; $MFI_{(C57BL/6)} = 32.07$], TCR levels on DP thymocytes from EA137/VA142 Aβ mutant animals were indistinguishable from TCR levels on DP thymocytes from class II-deficient animals [$MFI_{(EA137/VA142\ mutant)} = 49.52$; $MFI_{(C2D)} = 49.62$].

In addition to increased levels of TCR, increased levels of CD4 are also detected on DP thymocytes from MHC class II-deficient animals (2, 28). Therefore, we compared CD4 levels on DP thymocytes from wild-type reconstituted (Fig. 3*B Upper*) and EA137/VA142 Aβ mutant (Fig. 3*B Lower*) mice with C57BL/6 and C2D controls. Again, wild-type reconstituted animals were similar to C57BL/6 controls [$MFI_{(WT)} = 28.07$; $MFI_{(C57BL/6)} = 29.57$], whereas EA137/VA142 Aβ mutant mice were indistinguishable from C2D animals [$MFI_{(EA137/VA142\ mutant)} = 49.16$; $MFI_{(C2D)} = 53.85$]. Thus, as measured by increased TCR and CD4 levels on DP thymocytes, the EA137/VA142 mutation in I-A^b is sufficient to disrupt CD4–class II interactions *in vivo*.

Expression of the EA137/VA142 Mutant Aβ Protein Results in the Inefficient Maturation of CD4$^+$ T cells. To determine whether disruption of the CD4–MHC class II interaction *in vivo* had altered the process of T cell selection in the thymus, thymocytes were double-stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry (Fig. 4*A* and Table 1). Of note, there was no overall effect on cellularity; the total number of thymocytes in transgenic animals was the same as C57BL/6 controls. The number of CD4 SP thymocytes in animals reconstituted with the wild-type Aβ transgene (mean = 12.6%, SD = 2.8) was equivalent to C57BL/6 controls (mean = 12.7%, SD = 2.6). By contrast, the number of CD4 SP thymocytes in EA137/VA142 transgenics (mean = 5.7%, SD = 1.9) was significantly lower than wild-type controls (Student's *t* test, $P < 0.05$). This was often accompanied by an increase in the number of CD8$^+$ SP thymocytes in EA137/VA142 transgenics [mean = 8.1% (SD = 2.2) versus mean = 4.3% (SD = 1.6) in wild-type reconstituted animals]; a similar phenomenon has been reported for MHC C2D mice (1, 2). Finally, although fewer CD4$^+$ SP thymocytes are found in animals expressing the mutated class II protein, these cells are phenotypically similar to those found in control animals; i.e., CD4 SP thymocytes are TCR^{hi}, CD5^{hi}, and dexamethasone-resistant. This is in marked contrast with the small population of CD4$^+$ SP thymocytes found in MHC C2D animals (1, 29).

Few CD4$^+$ T Cells Are Found in Peripheral Lymphoid Compartments in EA137/VA142 Aβ Mutant Mice. To determine whether the inefficient selection of CD4$^+$ T cells in animals expressing the mutated class II proteins resulted in fewer numbers of CD4$^+$ T cells in peripheral lymphoid organs, splenocytes and lymph node cells were double-stained with anti-CD4 and anti-CD8 mAbs. The frequency of CD4$^+$ splenocytes in EA137/VA142 Aβ mutant transgenics (mean = 8.8%, SD = 2.8) was significantly lower ($P < 0.005$) than wild-type reconstituted (mean = 24.4%, SD = 8.1) or C57BL/6 (mean = 24.1%, SD = 7.2) animals (Fig. 4*B* and Table 1). This difference was also apparent in the mesenteric lymph nodes: mean = 14.5% (SD = 2.1) in EA137/VA142 Aβ mutant, mean = 51% (SD = 6.0) in wild-type reconstituted; mean = 47% (SD = 7.1) in C57BL/6 mice. The total cellularity in peripheral lymphoid organs was unchanged and thus reflects a true reduction in the number of CD4$^+$ T cells found in animals expressing the mutant Aβ protein. No age-related differences were detected; neonates, mice 6–8 weeks old, and animals 12–18 months old were analyzed. Moreover, for all experiments presented in this report, a careful comparison of low- and high-copy-number lines (wild type and mutant) yielded identical results. Thus, the paucity of CD4 SP cells seen in EA137/VA142 transgenic animals is not the consequence of gene dosage, age, or site of transgene integration; rather, it is the direct result of mutation of the Aβ polypeptide in the CD4 binding region.

Table 1. Subpopulations in thymus and spleen of C57BL/6, C2D, wild-type reconstituted, and EA137/VA142 Aβ mutant tg mice

	C57BL/6	C2D	WT tg	EA137/VA142 tg
Thymus				
CD4 SP	12.7 (2.6)	2.0 (0.8)	12.6 (2.8)	5.7 (1.9)
CD8 SP	4.0 (1.6)	5.9 (1.8)	4.3 (1.6)	8.1 (2.2)
Spleen				
$CD3^+CD4^+$	24.1 (7.2)	2.4 (0.6)	24.4 (8.1)	8.8 (2.8)
$CD3^+CD8^+$	11.8 (2.0)	18.5 (3.4)	15.9 (3.4)	17.9 (4.6)

Mean percent (of total lymphocytes) and SD (in parentheses) of indicated subpopulations in thymus and spleen of C57Bl/6 ($n = 8$). C2D ($n = 10$), wild-type (WT) reconstituted ($n = 22$), and EA137/VA142 Aβ mutant ($n = 22$) tg animals. Comparing wild-type reconstituted and EA137/VA142 Aβ mutant transgenics, the differences in the number of CD4 SP cells in the thymus and spleen are significant, $P < 0.05$ and $P < 0.005$, respectively (Student's *t* test). No other differences are significant.

The reduction in the number of CD4 SP T cells selected in EA137/VA142 Aβ mutant mice was further emphasized by staining of CD3$^+$ CD4$^+$ thymocytes and peripheral T cells with an antibody specific for the NK1.1 surface marker (30). It has been shown previously that the majority of CD4 SP T cells present in C2D mice belong to the NK1.1$^+$ subset, selected by CD1 molecules in the thymus (29). Our results reveal that a significant proportion (34–39%) of the CD3$^+$ CD4$^+$ peripheral T cells in animals expressing the EA137/VA142 Aβ mutant transgene belong to the NK1.1$^+$ subset (Table 2).

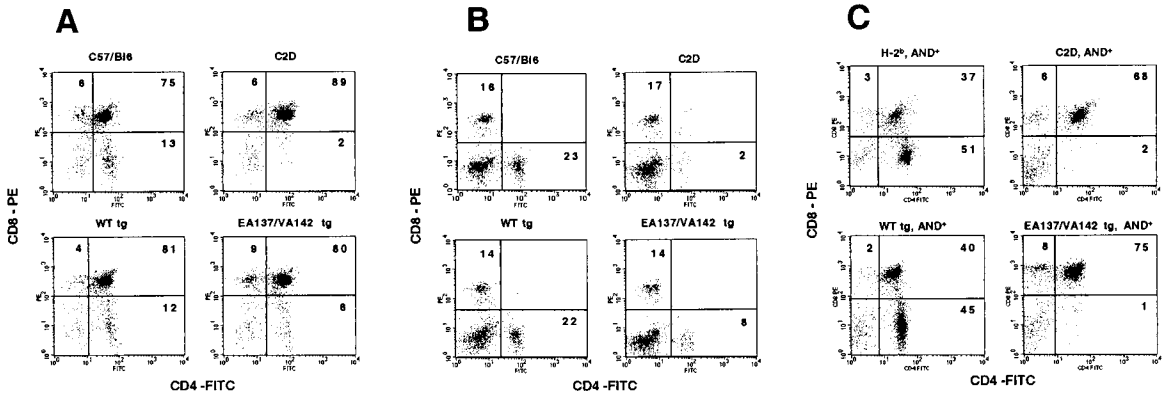

FIG. 4. (*A*) FACS profiles of C57BL/6, C2D, wild-type reconstituted, and EA137/VA142 tg thymocytes stained for CD4 (FITC) and CD8 (PE). (*B*) FACS profiles of C57BL/6, C2D, wild-type reconstituted, and EA137/VA142 tg splenocytes stained for CD4 (FITC) and CD8 (PE). (*C*) FACS profiles of thymocytes isolated from H-2^b/AND$^+$, C2D/AND$^+$, wild-type reconstituted WT tg/AND$^+$, and EA137,VA142/AND$^+$ tg thymocytes stained for CD4 (FITC) and CD8 (PE).

Table 2. Subpopulations of $CD4^+$, $NK1.1^+$ lymphocytes in thymus and spleen of C57BL/6, C2D, wild-type reconstituted, and EA137/VA142 Aβ mutant tg mice

	C57BL/6	C2D	WT tg	EA137/VA142 tg
Thymus				
CD4 SP, $NK1.1^-$	10.0 (0.5)	2.5 (1.2)	9.3 (1.3)	4.9 (0.4)
CD4 SP, $NK1.1^+$	1.5 (0.4)	0.5 (0.1)	1.2 (0.5)	0.7 (0.3)
Spleen				
$CD3^+CD4^+NK1.1^-$	20.9 (0.7)	2.4 (0.6)	27.0 (6.9)	7.0 (0.8)
$CD3^+CD4^+NK1.1^+$	2.5 (1.1)	3.0 (0.7)	3.0 (1.2)	2.4 (0.3)

Mean percent (or total lymphocytes) and SD (in parentheses) of indicated subpopulations in thymus and spleen of C57Bl/6, C2D, wild-type (WT) reconstituted and EA137/VA142 Aβ mutant transgenic animals. In each case, values presented are from three to eight animals (ages 6–8 weeks), stained in independent experiments.

Importantly, this does not reflect an expansion of the peripheral NK1.1 subset in EA137/VA142 Aβ mutant mice, but rather a loss of conventional CD4 SP T cells, because the total number of $CD3^+$ $CD4^+$ $NK1.1^+$ cells is similar to wild-type controls. Thus, there are actually only one-fourth as many conventional $CD4^+$ $NK1.1^-$ T cells in the periphery of EA137/VA142 Aβ mutant mice, even though the proportion of $NK1.1^+$ CD4 SP thymocytes in EA137/VA142 mice (11.5%) is similar to wild-type (12.4%) controls. The increased proportion of $CD4^+$ $NK1.1^+$ T cells found in the periphery of Aβ mutant mice (26% in Aβ mutant versus 10% in wild type) might indicate that fewer $CD4^+$ $NK1.1^-$ thymocytes reach peripheral lymphoid compartments in these animals. Alternatively, conventional CD4 T cells may not persist in the periphery in EA137/VA142 Aβ mutant mice. The latter interpretation is consistent with a recent report showing that class II expression in the periphery may be required for the long-term survival of $CD4^+$ T cells (31).

Failure to Positively Select AND Transgenic TCR Thymocytes in EA137/VA142 Mutant Mice. We have shown that the EA137/VA142 mutation is sufficient to disrupt the CD4 binding site on the I-A^b molecule. The simplest interpretation of the results presented thus far is that only those class II-restricted T cells that express TCRs of sufficient affinity for MHC plus peptide to be selected in the absence of CD4 coreceptor function are positively selected in EA137/VA142 Aβ mutant mice. To assess the consequences of this mutation in a uniform population of T cells expressing a unique TCR specificity, mice expressing the EA137/VA142 mutant I-A^b protein were bred with mice expressing the AND TCR transgenes (32, 33). These mice express productively rearranged Vα11, Vβ3 TCR genes that were originally isolated from a hybridoma recognizing an immunodominant peptide derived from pigeon cytochrome *c* in the context of I-E^k, and the transgenic TCR is expressed on >90% of $CD4^+$ peripheral T cells in I-E^k-expressing mice. Fortuitously, the AND receptor is also positively selected in mice that express I-A^b in the thymus (34). Hence, the AND transgenic line was bred with the EA137/VA142 Aβ mutant transgenic described in this study to ask whether this mutation in the β2 domain of I-A^b interferes with the positive selection of the CD4-dependent AND TCR.

To assess AND thymocyte development, thymocytes derived from H-$2^{b/-}$ AND^+, C2D (I-$A^{-/-}$) AND^+, wild-type transgenic AND^+, and EA137/VA142 transgenic AND^+ mice were stained with anti-CD4 (FITC) and anti-CD8 (PE) and analyzed by FACS (Fig. 4*C*). As expected, the majority (mean = 51.3%, SD = 4.8) of thymocytes in wild-type I-A^b-expressing animals are mature CD4 SP thymocytes with fewer than 3% (mean = 2.5%, SD = 1.4) $CD8^+$ thymocytes (Fig. 4*C Upper Left* and *Lower Left*). By contrast, there are few CD4 SP (mean = 2.0%, SD = 0.8) or CD8 SP (mean = 5.7%, SD = 3.6) thymocytes present in C2D AND^+ animals (Fig. 4*C Upper Right*). In the absence of an appropriate MHC ligand in the thymus, virtually all of the transgenic thymocytes in C2D animals fail to progress beyond the $CD4^+$ $CD8^+$ DP stage of thymocyte development. Strikingly, few CD4 SP (mean = 2.1%, SD = 0.7) thymocytes (Fig. 4*C Lower Right*) or peripheral T cells (mean = 2.8%, SD = 1.0) were seen in doubly transgenic EA137 VA142/AND^+ mice ($P < 0.005$, $n = 13$). Of note, there was a significant increase ($P < 0.025$) in the number (mean = 15.4%, SD = 5.8) of CD8 SP thymocytes present in these animals as compared with H-2^b-expressing animals (mean = 2.5%, SD = 1.5). Selection of AND TCR transgenic T cells into the CD8 compartment was also seen when AND transgenic animals were bred onto the CD4 knock-out background (35), suggesting that in the absence of a productive CD4–class II interaction, the mismatched coreceptor may provide signals required for positive selection.

DISCUSSION

In summary, we have shown that a two-amino acid mutation in the β2 domain of the I-A^b protein is sufficient to disrupt the interaction of MHC class II molecules with CD4 proteins in the thymus *in vivo*. When compared with wild-type controls, animals expressing the mutated MHC class II protein displayed significantly reduced numbers of $CD4^+$ cells in the thymus and in peripheral lymphoid organs. Inasmuch as it is thought that coreceptor–MHC interactions stabilize otherwise low-avidity TCR–MHC interactions, the simplest interpretation of our results is that immature thymocytes that commit to the CD4 lineage in EA137/VA142 Aβ mutant mice express TCRs of sufficient affinity to be positively selected in the absence of CD4–class II interactions, although positive selection may also be facilitated by the increased levels of TCR present on immature DP thymocytes in EA137/VA142 Aβ mutant mice. Although an analysis of Vβ gene usage did not reveal an obvious lack of heterogeneity or skewing in the overall T cell repertoire (data not shown), the inability to select $CD4^+$ AND^+ T cells in animals expressing the mutated Aβ transgene demonstrates that the CD4–class II interaction is essential for the selection of at least some TCRs and may be essential for the generation of a complete and expansive repertoire of helper T cells.

Despite the reduction in the number of mature $CD4^+$ T cells, the CD4 SP cells that do appear in the periphery of EA137/VA142 Aβ mutant mice function as mature helper T cells. These animals can mount a T cell-dependent antibody response to a complex antigen (keyhole limpet hemocyanin) that is normal in both magnitude and isotype distribution (unpublished results). However, experiments using defined antigenic peptide epitopes are necessary to determine whether functional holes in the repertoire may exist. These studies are in progress. In addition, the animals described in this report provide an ideal system to analyze the role of CD4–MHC class II interactions in signaling events that occur during the development of peripheral T cell responses. This is particularly relevant as CD4–class II interactions have been implicated in the regulation of memory T cell responses (20) and Th1/Th2 differentiation (18, 19).

The results presented in this report provide insight into the role of CD4–class II MHC interactions during thymic ontogeny. Unlike CD4-deficient mice, the fate of $CD4^+$ T cells selected in EA137/VA142 Aβ mutant animals can be monitored phenotypically. The animals generated in our studies are also distinct from tailless CD4 transgenic mice, which were designed to investigate the role of CD4 signaling during thymic selection. Furthermore, the interpretation of the CD4 tailless experiments are complicated by the possible contribution of CD4–class II adhesive interactions. In our system, we can specifically examine the outcome of disrupting the CD4–class

II MHC interaction while leaving other interactions intact, i.e., TCR–MHC, CD4–p56^{lck}, and TCR–CD4. Moreover, this demonstrates *in vivo* that the CD4–class II interaction *per se* is critical for the development of an expansive repertoire of CD4$^+$ T cells and that this region of the β2 domain of class II is the major site of CD4 interaction.

Finally, these studies go further than analogous experiments in the CD8–class I system in which selection of only a few unique TCR specificities has been examined (6–9). In our system, all of the mature CD4 SP T cells have been selected on MHC class II molecules that cannot interact with the CD4 coreceptor. This allows us to study the complete repertoire of class II-restricted T cells selected in the absence of CD4–class II interactions. Analyses of antigen-specific T cell responses, including repertoire and functional analyses, in EA137/VA145 Aβ mutant mice is the focus of current studies in our laboratory.

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1. Grusby, M. J., Johnson, R. S., Papaioannou, V. E. & Glimcher, L. H. (1991) *Science* **253,** 1417–1420.
2. Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C. & Mathis, D. (1991) *Cell* **66,** 1051–1066.
3. Zijlstra, M., Bix, M., Simister, N. E., Loring, J. M., Raulet, D. H. & Jaenisch, R. (1990) *Nature (London)* **344,** 742–746.
4. Koller, B. H., Marrack, P., Kappler, J. W. & Smithies, O. (1990) *Science* **248,** 1227–1230.
5. Janeway, C. A., Jr. (1991) *Semin. Immunol.* **3,** 153–160.
6. Aldrich, C. J., Hammer, R. E., Jones-Youngblood, S., Koszinowski, U., Hood, L., Stroynowski, I. & Forman, J. (1991) *Nature (London)* **352,** 718–720.
7. Ingold, A. L., Landel, C., Knall, C., Evans, G. A. & Potter, T. A. (1991) *Nature (London)* **352,** 721–723.
8. Killeen, N., Moriarity, A., Teh, H.-S. & Littman, D. R. (1992) *J. Exp. Med.* **176,** 89–97.
9. Sherman, L. A., Hesse, S. V., Irwin, M. J., La Face, D. & Peterson, P. (1992) *Science* **258,** 815–818.
10. Garcia, K. C., Scott, C. A., Brunmark, A., Carbone, F. R., Peterson, P. A., Wilson, I. A. & Teyton, L. (1996) *Nature (London)* **384,** 577–581.
11. Yelon, D., Spain, L. M., Lim, K. & Berg, L. J. (1996) *Int. Immunol.* **8(7),** 1077–1090.
12. Rahemtulla, A., Kündig, T. M., Narendran, A., Bachmann, M. F., Julius, M., Paige, C. J., Ohashi, P. S., Zinkernagel, R. M. & Mak, T. W. (1994) *Eur. J. Immunol.* **24,** 2213–2218.
13. Killeen, N., Sawada, S. & Littman, D. R. (1993) *EMBO J.* **12,** 1547–1553.
14. Penninger, J. M., Schilham, M. W., Timms, E., Wallace, V. A. & Mak, T. W. (1995) *Eur. J. Immunol.* **25,** 2115–2118.
15. Locksley, R. M., Reiner, S. L., Hatam, F., Littman, D. R. & Killeen, N. (1993) *Science* **261,** 1448–1451.
16. Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W., Kündig, T. M., Sambhara, S. R., Narendran, A., Arabian, A., Wakeham, A., Paige, C. J., Zinkernagel, R. M. *et al.* (1991) *Nature (London)* **353,** 180–184.
17. Koh, D.-R., Ho, A., Rahemtulla, A., Penninger, J. & Mak, T. W. (1994) *Eur. J. Immunol.* **24,** 2250–2253.
18. Fowell, D. J., Magram, J., Turck, C. W., Killeen, N. & Locksley, R. M. (1997) *Immunity* **6,** 559–569.
19. Brown, D. R., Moskowitz, N. H., Killeen, N. & Reiner, S. L. (1997) *J. Exp. Med.* **186,** 101–107.
20. Farber, D. L., Luqman, M., Acuto, O. & Bottomly, K. (1995) *Immunity* **2,** 249–259.
21. König, R., Huang, L.-Y. & Germain, R. N. (1992) *Nature (London)* **356,** 796–798.
22. Killeen, N. & Littman, D. R. (1993) *Nature (London)* **364,** 729–732.
23. Larhammar, D., Hammerling, U., Denaro, M., Lung, T., Flavell, R. A., Rask, L. & Peterson, P. A. (1983) *Cell* **34,** 179–188.
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
25. Cresswell, P. (1996) *Cell* **84,** 505–507.
26. McCarthy, S. A., Kruisbeek, A. M., Uppenkamp, I. K., Sharrow, S. O. & Singer, A. (1988) *Nature (London)* **336,** 76–79.
27. Nakayama, T., June, C. H., Munitz, T. I., Sheard, M., McCarthy, S. A., Sharrow, S. O., Samelson, L. E. & Singer, A. (1990) *Science* **249,** 1558–1561.
28. Wiest, D. L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R. D., Glimcher, L. H., Samelson, L. E. & Singer, A. (1993) *J. Exp. Med.* **178,** 1701–1712.
29. Cardell, D., Tangri, S., Chan, S., Kronenberg, M., Benoist, C. & Mathis, D. (1995) *J. Exp. Med.* **182,** 993–1004.
30. Bendelac, A., Lantz, O., Quimby, M. E., Yewdell, J. W., Bennink, J. R. & Brutkiewicz, R. B. (1995) *Science* **268,** 863–865.
31. Takeda, S., Rodewald, H.-R., Arakawa, H., Bluethmann, H. & Shimuzu, T. (1996) *Immunity* **5,** 217–228.
32. Kaye, J., Hsu, M.-L., Sauron, M.-E., Jameson, S. C., Gascoigne, N. R. J. & Hedrick, S. M. (1989) *Nature (London)* **341,** 746–749.
33. Vasquez, N. J., Kaye, J. & Hedrick, S. M. (1992) *J. Exp. Med.* **175,** 1307–1316.
34. Kaye, J., Vasquez, N. J. & Hedrick, S. M. (1992) *J. Immunol.* **148,** 3342–3353.
35. Matechak, E. O., Killeen, N., Hedrick, S. M. & Fowlkes, B. J. (1996) *Immunity* **4,** 337–347.