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In a Transgenic Model of Spontaneous Autoimmune Diabetes, Expression of a Protective Class II MHC Molecule Results in Thymic Deletion of Diabetogenic CD8$^+$ T Cells

David J. Morgan,* C. Thomas Nugent,† Benjamin J. E. Raveney,* and Linda A. Sherman*‡

H-2$^d$ mice expressing both the influenza virus hemagglutinin (HA) as a transgene-encoded protein on pancreatic islet $\beta$ cells (InsHA), as well as the Clone 4 TCR specific for the dominant H-2K$^d$-restricted HA epitope, can be protected from the development of spontaneous autoimmune diabetes by expression of the H-2$^b$ haplotype. Protection occurs due to the deletion of K$^d$HA-specific CD8$^+$ T cells. This was unexpected as neither the presence of the InsHA transgene nor H-2$^b$, individually, resulted in thymic deletion. Further analyses revealed that thymic deletion required both a hybrid MHC class II molecule, E$^b$H$^b$ E$^d$, and the K$^d$ molecule presenting the HA epitope, which together synergize to effect deletion of CD4$^+$CD8$^+$ T cells. This surprising example of protection from autoimmunity that maps to a class II MHC molecule, yet effects an alteration in the CD8$^+$ T cell repertoire, suggests that selective events in the thymus represent the integrated strength of signal delivered to each cell through recognition of a variety of different MHC-peptide ligands. The Journal of Immunology, 2004, 172: 1000–1008.

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which the insulin-producing $\beta$ cells of the pancreatic islets are destroyed by an autoimmune process that requires participation of both CD4$^+$ and CD8$^+$ T lymphocytes (1, 2). As with many autoimmune diseases, a predisposition to IDDM is strongly associated with the expression of particular alleles of the MHC complex (3). In the nonobese diabetic (NOD) mouse model of spontaneous autoimmune diabetes, such a linkage has been studied extensively and has been correlated with the expression of a unique MHC class II molecule, I-A$^\beta$ (4–10). This is the only class II molecule expressed in this strain, and the introduction of additional class II molecules prevents disease (11–13). Linkage studies in humans have revealed similar susceptibility and suppressive genes among alleles at the DQB1 locus (3, 5, 14).

The mechanism that explains class II association with disease susceptibility remains unresolved, as does the basis for class II-mediated suppression of disease. At least two main hypotheses have been proposed to explain the latter. One is based on repertoire deletion and proposes that suppressive alleles encode MHC molecules that are responsible for thymic deletion of potentially autoreactive CD4$^+$ T cells that would otherwise respond to islet Ags and thereby lead to destruction of $\beta$ cells (12, 15, 16). This is supported by studies conducted by Schmidt and coworkers using a transgenic mouse line that expresses a $\beta$ cell-specific, I-A$^\beta$-restricted TCR, NY 4.1 TCR (13). NOD mice expressing this TCR develop early-onset diabetes. However, expression of a second class II molecule in these mice results in thymic deletion of the TCR-bearing CD4$^+$ T cells and protection from diabetes (17).

There is also good evidence to support an alternative hypothesis that proposes an immunoregulatory mechanism for suppression of diabetes by certain class II alleles (11, 18). Using another $\beta$ cell-specific, I-A$^\beta$-restricted TCR-transgenic murine line, the BDC-2.5 TCR (19), Mathis and coworkers reported that expression of I-A$^b$, although protective, did not result in deletion of T cells bearing this receptor. Rather, this led to enhanced expression of endogenous TCRs within the CD4$^+$ T cell repertoire. Such a result is consistent with immunoregulation, rather than deletion, of islet-specific T cells (20).

In this report, we present data that suggests a novel mechanism by which class II molecules may regulate potentially autoimmune T cells. Clone 4 TCR-transgenic mice express a CD8$^+$ T cell-derived TCR that is specific for the dominant H-2K$^d$-restricted epitope of the hemagglutinin (HA) molecule of the influenza virus A/PR/8. When Clone 4 TCR mice are mated with InsHA-transgenic mice that express the HA molecule on their pancreatic islet $\beta$ cells (21), the expression of the InsHA transgene in H-2$^d$ (InsHA × Clone 4 TCR)F1 progeny does not affect thymic development or function of the Clone 4 TCR-bearing thymocytes, and these mice develop spontaneous autoimmune diabetes and die by 2 wk of age (22). In contrast, the current study demonstrates that, in the H-2$^{bxd}$ (InsHA × Clone 4 TCR)F1 progeny, there is deletion of Clone 4 TCR-bearing thymocytes and the absence of HA-responsive Clone 4 CD8$^+$ T cells within the peripheral T cell pool. As a consequence of this deletion, mice do not develop spontaneous autoimmune-mediated diabetes. We show that thymic elimination requires recognition by the Clone 4 TCR bearing CD4$^+$CD8$^+$ double positive (DP) thymocytes of both K$^d$ HA and a hybrid class II molecule composed of E$^b$H$^b$ E$^d$. Moreover, these results illustrate a situation in which expression of a class II molecule leads to protection from IDDM via alteration in the CD8$^+$ T cell repertoire.
Materials and Methods

Mice

B10.D2, C57BL/6, B10.BR, B6.C-H-2\textsuperscript{b}H-2\textsuperscript{b}C/ByJ, B10.A (5R), and B10.GD mice were purchased from the breeding colony of The Scripps Research Institute, C57BL/6K(O)AP\textsuperscript{a} and B10.D2 (I-Tac-\textsuperscript{b}KO)Rag\textsuperscript{2} mice were purchased from Taconic (Germantown, NY), and B6.129.56-Cd4\textsuperscript{mko} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). InsHA-transgenic mice and Clone 4 TCR-transgenic mice were generated and characterized as previously described (21, 22). All mice were bred and maintained under specific pathogen-free conditions in The Scripps Research Institute vivarium. All experimental procedures were conducted in strict accordance with the guidelines laid out in the National Institutes of Health “Guide for the Care and Use of Laboratory Animals”.

Peptide

Influenza virus A/PR/8 (H1N1) HA peptide, sequence IYSTVASSL\textsubscript{115−122} (23), was synthesized by The Scripps Research Institute core facility using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The purity was found to be greater than 85%, as determined by mass spectrometry and reverse-phase HPLC analysis using a C\textsubscript{18} column (Vydac, Hesperia, CA).

Cytometry

Cloned 4 TCR-bearing T cells were detected by incubating with PE-conjugated anti-CD4 Ab (BD PharMingen, San Diego, CA) and FITC-conjugated anti-CD8 Ab (BD PharMingen, San Diego, CA). Cells were analyzed with a FACSscan and CellQuest software (BD Biosciences, Mountain View, CA).

Analysis of blood glucose levels

Blood samples from mice were obtained from the retro-orbital plexus. The level of blood glucose was determined using an Accu-Check III blood glucose monitor system (Boehringer Mannheim, La Jolla, CA). Mice were considered diabetic if their blood-glucose level was greater than 300 mg/dl on two consecutive readings.

Generation of radiation bone marrow chimeras

Bone marrow cells were prepared from Clone 4 TCR mice and incubated with anti Thy1.1 Abs and complement to remove mature T cells. B10.D2 or InsHA\textsuperscript{+/-} mice were lethally irradiated (1100 rad) and injected i.v. with 2\times10\textsuperscript{6} T cell-depleted bone marrow cells.

T cell proliferation assay

APC were prepared as follows: a single-cell suspension of mouse splenocytes was irradiated (3000 rad), and the cells were resuspended at 20\times10\textsuperscript{6} cells/well. Cells were then incubated at 37\textdegree\ C for 45 min with or without 5 \mu g/ml of the K\textsubscript{d}-restricted HA peptide (IYSTVASSL). Splenocytes were washed twice in complete RPMI 1640 and then seeded into 96-well plates at 5\times10\textsuperscript{4} cells/well. Responder cells were prepared from lymph node or thymus seeded into 96-well plates at the indicated cell number. Cells were incubated for 36 h followed by a further 12 h in the presence of 2\muCi of [\textit{methyl-\textsuperscript{3}H}]thymidine (New England Nuclear, Boston, MA). Cells were collected and radioactivity determined using a beta scintillation counter (Wallac, Wellesley, MA).

Nested RT-PCR analyses

Mouse tissue was processed for nucleic acid isolation by homogenization in TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s specifications. Nucleic acid was precipitated, washed, and subsequently resuspended in diethyl pyrocarbonate-treated water in a final volume of 150 \mu l. Eight microliters was then subjected to reverse transcription using SuperScript II reverse transcriptase (Life Technologies) according to the specifications of the manufacturer, except for the addition of 1 U of RNAsin (Life Technologies) to the reaction. Five microliters of each cDNA reaction was amplified using 250 ng of each primer specific for the InsHA transgene (see below) or using a 1-\mu l final concentration of primers for dihydrofolate reductase as a positive control (Stratagene, La Jolla, CA). An annealing temperature of 55\textdegree\ C was used in both rounds of nested PCR, and the second round of amplification was preceded by a chloroform extraction and wash. Nested-set PCR primers were designed using GenBank sequences for the rat insulin 2 gene promoter (accession no. M25583, Ref. 24) and the influenza virus type A mRNA sequence for HA (accession no. A19638, Ref. 25). The first primer pair, RIP-1 and HA-6

\text{RIP-1, 5'-CCCTAATGTACGATCGTACGT-3', HA-6, 5'-CAGCTCCAGGATCAGGTAAG-3'} was located externally to the second primer pair, RIP-2 (5'-CAGTCGGCCATCAGCTAGAAG-3') and HA-7 (5'-CCCTAATGTACGATCGTACGT-3'). PCR were analyzed by gel electrophoresis. Bands corresponding to the expected sizes of cDNA or gDNA were excised and cloned into pCR II using TA cloning (Invitrogen, Carlsbad, CA). Colonies containing plasmids with inserts were propagated and DNA was isolated using Wizard miniprep systems (Promega, Madison, WI). Purified DNA was subjected to fluorescent sequencing, which was conducted by The Scripps Research Institute core facility on an ABI 377XL automated sequencer (PerkinElmer, Foster City, CA). Sequencing results were analyzed using MacDNAsis (Hitachi Software) and the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST) (26).

Results

The presence of H-2\textsuperscript{b} prevents diabetes in InsHA mice expressing the K\textsuperscript{b}HA-specific Clone 4 TCR

When InsHA mice were mated with the Clone 4 TCR mice, expressing a transgenic TCR (\textalpha\text{a}10,\textbeta\text{b}8.2), which recognizes the dominant K\textsuperscript{b} epitope of HA, the resulting double transgenic progeny demonstrated one of two very distinct phenotypes depending upon whether or not H-2\textsuperscript{b} was present. As reported previously, double transgenic mice that were homozygous for H-2\textsuperscript{d} rapidly developed hyperglycemia within the first few days of life and by day 14 all had died from diabetes (22). In contrast, double transgenic mice that expressed both H-2\textsuperscript{d} and H-2\textsuperscript{b} did not develop diabetes or thrive normally. To determine how the expression of H-2\textsuperscript{b} was affecting thymocyte development in the double transgenic mice, thymocytes and peripheral lymphocytes were taken from both H-2\textsuperscript{d} and H-2\textsuperscript{b/d} (InsHA \times Clone 4\textalpha}F\textsubscript{1} mice. The majority of thymocytes from the H-2\textsuperscript{d} double transgenic mice expressed high levels of \textit{V}\textsubscript{8}, whereas the majority of thymocytes from H-2\textsuperscript{b/d} mice were \textit{V}\textsubscript{8} negative (Fig. 1, A and B). K\textsuperscript{d}HA-responsive CD8\textsuperscript{+} T cells could not be detected in the thymus or the peripheral lymph nodes of the H-2\textsuperscript{b/d} (InsHA \times Clone 4\textalpha}F\textsubscript{1} mice (Table I, Experiment 1). In addition, cell recovery was ~70% less for thymic from the H-2\textsuperscript{b/d} double transgenics. Thus, the presence of the H-2\textsuperscript{b} haplotype resulted in the deletion of Clone 4 TCR-bearing single positive (SP) thymocytes in the H-2\textsuperscript{b/d} (InsHA \times Clone 4\textalpha}F\textsubscript{1} mice and protection from CD8\textsuperscript{+} T cell-mediated IDDM.

![FIGURE 1. Deletion of Clone 4 TCR-bearing thymocytes occurs in H-2\textsuperscript{b/d} (InsHA \times Clone 4\textalpha}F\textsubscript{1} mice. Thymocytes from H-2\textsuperscript{d} (InsHA \times Clone 4\textalpha}F\textsubscript{1} mice (A) and H-2\textsuperscript{b/d} (InsHA \times Clone 4\textalpha}F\textsubscript{1} (B) mice were analyzed for Clone 4 TCR expression by staining with PE-conjugated anti-V\textit{J}\textbeta\textsubscript{8} Abs. Total number of thymocytes was 58 \times 10\textsuperscript{6} and 21 \times 10\textsuperscript{6}, respectively. Data is representative of n = 4 H-2\textsuperscript{d} (Clone 4 \times InsHA\textalpha}F\textsubscript{1} mice. The mean and SE values of V\textit{J}\textbeta\textsubscript{8}\textsuperscript{low} were V\textit{J}\textbeta\textsubscript{8}\textsuperscript{low} = 4 + 0.48\%, V\textit{J}\textbeta\textsubscript{8}\textsuperscript{low} = 21 + 1.31\%, V\textit{J}\textbeta\textsubscript{8}\textsuperscript{high} = 75 + 1.78\%, n = 3, H-2\textsuperscript{b/d} (Clone 4 \times InsHAF\textalpha}F, mice mean and the SE values are V\textit{J}\textbeta\textsubscript{8}\textsuperscript{low} = 64 + 1.67\%, V\textit{J}\textbeta\textsubscript{8}\textsuperscript{low} = 21 + 0.88\%, V\textit{J}\textbeta\textsubscript{8}\textsuperscript{high} = 6 + 0.67\%.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.1001035)
The presence of H-2b alters thymic development in Clone 4 TCR mice

These data demonstrated that the presence of the H-2b haplotype had a profound effect upon the maturation of functional Clone 4 TCR-bearing thymocytes in (InsHA × Clone 4)F1 mice. This result was unexpected, as previous FACS analyses had revealed that there was no difference between the phenotype of peripheral T cells taken from the H-2d and H-2bxd Clone 4 TCR mice (Fig. 2A). Also, no difference was observed in the proliferative response of peripheral T cells from H-2d and H-2bxd Clone 4 mice in response to cognate Ag (Table I, Experiment 2). However, FACS analyses of thymocytes (Fig. 2B) demonstrated that there was a difference in the development of Clone 4 TCR-bearing thymocytes between these two strains. Although thymi were comparable in total cell numbers, the proportion of Vβ8high cells was less in the H-2bxd Clone 4 TCR mice than in the H-2d Clone 4 TCR mice (34 vs 50%) and the proportion of CD8 SP thymocytes (R4) was greater in the H-2d Clone 4 TCR mice than in the H-2bxd Clone 4 TCR mice (26 vs 9%). Also, there were more TCRlow DP (R2) cells in H-2bxd mice than in H-2d mice (43 as compared with 25%). Further examination of other H-2bxd and H-2d Clone 4 TCR mice indicated variation in the proportion of thymocytes in each subset; however, in all cases it was observed that the ratio of TCRlow: TCRhigh thymocytes was greater than 1 in mice expressing H-2bxd and less than 1 in mice homozygous for H-2d. Taken together, these results suggest that the transition from DP TCR low to CD8 SP is less efficient in the presence of H-2b.

A possible explanation for altered thymocyte development in the H-2bxd Clone 4 TCR-transgenic mice could be the reduced level of expression of the positively selecting Kd ligand (27). Cells from both H-2kxd and H-2bxd mice express half the amount of Kd as cells from H-2d homozygous mice (data not shown). However, the level of Vβ8 expression on thymocytes from H-2kxd Clone 4 TCR mice was identical with that of homozygous H-2d Clone 4 TCR mice (Fig. 2B). This suggests that a reduced level of Kd is not responsible for the altered thymic selection of Clone 4 TCR-bearing thymocytes observed in H-2bxd mice.

One complication in evaluating the amount of Clone 4 TCR expression in these mice arises from the fact that an anti-clonotypic Ab, which recognizes the Clone 4 TCR, is not available. Therefore, it is possible that many of the cells expressing Vβ8 may be expressing endogenous TCRα chains (28–30). To learn more about the way in which H-2b was affecting Clone 4 cells during their development in the thymus, both H-2d and H-2bxd Clone 4 TCR mice were back-crossed on to the Rag 2−/− /H11002 DUAL RECOGNITION OF CLASS I AND CLASS II BY CD8+ T CELLS

![FIGURE 2.](http://www.jimmunol.org/Downloadedfrom/fig2.jpg)
background to prevent the production of T cells expressing endogenous TCRs. FACS analyses revealed that the vast majority of thymocytes in the H-2d Clone 4 TCR Rag 2−/− mice were Vβ8<sup>high</sup> (Fig. 3A), whereas in H-2<sup>bxd</sup> Clone 4 TCR Rag 2−/− mice both Vβ8<sup>high</sup> and Vβ8<sup>low</sup> thymocytes were present (Fig. 3E). Despite these differences in thymic maturation of Clone 4 TCR-bearing thymocytes, the proportion of mature CD8 SP cells in the peripheral lymph nodes was the same in both groups of mice (compare Fig. 3, D with H). Furthermore, they expressed similar levels of both TCR and CD8. Again, these data suggest that H-2<sup>b</sup> is affecting thymic development of Clone 4 TCR cells as they transition from the DP to the CD8 SP stage of development. Once mature, the CD8 SP cells in H-2<sup>d</sup> and H-2<sup>bxd</sup> Clone 4 TCR are comparable in phenotype and function.

**CD8 SP T cells that express the Clone 4 TCR do not respond to H-2<sup>bxd</sup> APCs.**

Thus far, the data suggest that thymocytes, but not peripheral T cells, are affected by the presence of an alloantigen expressed on H-2<sup>bxd</sup> APCs. The availability of Rag 2−/− Clone 4 TCR mice provided an opportunity to directly assess the ability of CD8 SP T cells that express only the Clone 4 TCR (and no endogenous TCRs) to respond to H-2<sup>bxd</sup> APCs. To this end, lymph node cells from H-2<sup>d</sup> Rag 2−/− Clone 4 TCR mice were assessed for their ability to proliferate in response to H-2<sup>bxd</sup> APCs. As indicated by the data in Table II, no such alloresponse was observed. They were, however; fully competent in their response to H-2<sup>d</sup> cells pulsed with K<sup>d</sup>HA peptide.

**Identification of the H-2<sup>b</sup> molecule affecting Clone 4 TCR expression**

The previous results implied that Clone 4 TCR thymocytes recognized a molecule encoded by H-2<sup>b</sup>. As the Clone 4 TCR was obtained from a CD8<sup>+</sup> T cell that was class I restricted, it was considered most likely that the TCR was cross-reactive with a class I molecule of the H-2<sup>b</sup> haplotype. To determine whether K<sup>b</sup> was a likely candidate, H-2<sup>d</sup> B10.D2 Clone 4 TCR mice were mated with B6.C-H2<sup>bm1</sup>/CByJ, which are of the H-2<sup>b</sup> haplotype but express the mutant K<sup>bm1</sup> molecule. This particular mutation results in profound alteration of TCR recognition such that few T cells that recognize K<sup>b</sup> are able to recognize K<sup>bm1</sup> (31, 32). FACS analyses of the H-2<sup>d</sup> x H-2<sup>bm1</sup> F<sub>1</sub> progeny showed the same effect upon Clone 4 TCR<sup>high</sup> thymocytes as was observed in mice expressing a wild-type K<sup>b</sup> molecule (TCR<sup>low</sup>:TCR<sup>high</sup> > 1), suggesting that a role for K<sup>b</sup> was unlikely (Fig. 4). To assess the contribution of the D<sup>b</sup> molecule, B10.D2 Clone 4 TCR mice were crossed with B10.A(5R) mice that express K<sup>b</sup>, D<sup>b</sup>. Surprisingly, these offspring demonstrated the same type of profile of thymic maturation as H-2<sup>bxd</sup> mice, suggesting that D<sup>b</sup> was also not responsible for this effect on Clone 4 TCR expression (Fig. 4). Thus, it appeared that neither of the class I molecules expressed in the H-2<sup>b</sup> haplotype was affecting development of Clone 4 TCR-expressing thymocytes.

These data led us to consider the possibility that a class II MHC molecule contributed by the H-2<sup>b</sup> haplotype was affecting thymic development of the Clone 4 TCR-bearing cells. To determine whether this was the case, B10.GD Clone 4 TCR mice (E<sup>Bb</sup> apo<sup>−</sup>) (33) were generated and mated with the class II-negative C57BL/6 class II Ab<sup>−</sup>/K<sup>d</sup> F<sub>1</sub> mice (E<sup>Bb</sup> apo<sup>−</sup>/K<sup>d</sup>). FACS analyses of the resulting progeny revealed that the thymic profile of Vβ expression was similar to that of the H-2<sup>d</sup> Clone 4 TCR mice (TCR<sup>low</sup>:TCR<sup>high</sup> < 1) indicating that a class II MHC molecule was indeed responsible for altered thymic expression of the Clone 4 TCR (Fig. 4). To determine which class II molecule was responsible, the B10.GD Clone 4 TCR mice were crossed with C57BL/6 mice to generate mice expressing I-A<sup>b</sup>. The presence of A<sup>b</sup> did not alter thymic development of Clone 4 TCR cells (Fig. 4). This led to the conclusion that the effect of H-2<sup>b</sup> upon Clone 4 TCR expression must be mediated by an I-E molecule, and, by the process of elimination, this must be the hybrid molecule comprised of Eβ<sup>b</sup> Eα<sup>d</sup>.

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**FIGURE 3.** Lack of endogenous TCR<sub>a</sub> does not modify the effect of H-2<sup>bxd</sup> on Clone 4 TCR thymocytes. H-2<sup>d</sup> (A-D) and H-2<sup>bxd</sup> (E-H) Clone 4 TCR Rag2 KO mice were analyzed for expression of the clone TCR. Thymocytes were stained with CyChrome-conjugated anti-Vβ8 (A and E) and FITC-conjugated anti-CD8 plus PE-conjugated anti-CD4 (B and F). Total thymocyte numbers were comparable (H-2<sup>d</sup>, 42 ± 10<sup>6</sup>; H-2<sup>bxd</sup>, 47 ± 10<sup>6</sup>). Data is representative of two H-2<sup>d</sup> Clone 4 TCR Rag2 KO mice that were analyzed with values of Vβ8<sup>low</sup> = 1%, Vβ8<sup>high</sup> = 5%, Vβ8<sup>high</sup> = 94% for the other mouse; and two H-2<sup>b</sup> Clone 4 TCR Rag2 KO mice with Vβ8<sup>low</sup> = 10%, Vβ8<sup>low</sup> = 46%, Vβ8<sup>high</sup> = 44% for the other mouse. Peripheral lymphocytes were stained with CyChrome-conjugated anti-Vβ8 (C and G) and FITC-conjugated anti-CD8 plus PE-conjugated anti-CD4 (D and H). Peak mean fluorescence of anti-Vβ8 is: H-2<sup>d</sup>, 196; H-2<sup>b</sup>, 148; H-2<sup>bxd</sup>, 170. Total numbers of peripheral lymphocytes were: H-2<sup>d</sup>, 23 ± 10<sup>6</sup>; H-2<sup>bxd</sup>, 20 ± 10<sup>6</sup>.

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**Table II.** CD8<sup>+</sup> Clone 4 T cells do not respond to H-2<sup>bxd</sup> APCs<sup>a</sup>

<table>
<thead>
<tr>
<th>APC (splenocytes)</th>
<th>Lymph Node Cells from H-2&lt;sup&gt;d&lt;/sup&gt;</th>
<th>[H]Thymidine Response by 47,380</th>
<th>188</th>
<th>155</th>
<th>47,380</th>
<th>31,682</th>
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<tbody>
<tr>
<td>H-2&lt;sup&gt;d&lt;/sup&gt;bxd</td>
<td>188</td>
<td>188</td>
<td>155</td>
<td>47,380</td>
<td>31,682</td>
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<tr>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;bxd + K&lt;sup&gt;d&lt;/sup&gt;HA peptide</td>
<td>47,380</td>
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<tr>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;bxd + K&lt;sup&gt;d&lt;/sup&gt;HA peptide</td>
<td>31,682</td>
<td>31,682</td>
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<sup>a</sup>A total of 5 × 10<sup>6</sup> lymph node cells were incubated with 5 × 10<sup>6</sup> of the indicated stimulator spleen cells with or without K<sup>d</sup>HA peptide, as described under Materials and Methods.
To determine the significance of our results, we performed a statistical analysis to determine whether the profiles of Vβ expression were significantly similar between individual mice within each congenic group studied in Fig. 4. These results, provided in the legend to Fig. 4, strongly indicated that the various individuals within a group exhibited a high degree of similarity. Furthermore, it appeared that the groups of mice exhibited one of two distinct phenotypes based on their distribution of Vβ as gated for high and low expression. To determine whether this was indeed the case, we performed a statistical analysis comparing the mean values of Vβlow cells between each of the various groups of mice analyzed in Fig. 4. With respect to this parameter, there was a greater than 95% confidence that the following types of Clone 4 mice were identical: B10.D2, (B10.D2 × B10.BR)F1, and (B10.GD × B6)F1. There was also a greater than 95% confidence that the (B10.D2 × B6)F1 and (B10.D2 × B10.A(5)R)F1 mice were identical. Furthermore, the difference between these two sets of groups is highly statistically significant. Although the sample size for the other remaining congenic strains was too small to perform statistical analyses, the Vβlow values for the two individuals representing the (B10.GD × B6 class II Aβb KO)F1 mice were very similar to each other and to the sets represented by the (B10.D2 × B6)F1 mice. Furthermore, the Vβlow values for the two individuals representing the (B10.GD × B6 class II Aβb KO)F1 mice were very similar to each other and to the sets represented by B10.D2. Accordingly, we conclude that the expression pattern of Vβ is determined on the basis of the presence or absence of the Eβb Eαd molecule.

**Thymic expression of CD4 is required in order for Eβb Eαd to affect thymic expression of the Clone 4 TCR**

It has been estimated that expression of the CD4 coreceptor can augment the response to class II by as much as 100-fold (34). Considering that DP thymocytes, but not mature T cells, in Clone 4 TCR mice express CD4, a requirement for CD4 could explain why evidence for recognition of the Eβb Eαd molecule was only observed during thymic development. If this were the case, we would predict that in the absence of CD4, thymic expression of the Clone 4 TCR should be identical in mice that express H-2b and H-2d. To test this hypothesis, we examined thymic expression of the Clone 4 TCR in mice that are genetically deficient in CD4 expression. Indeed, as exhibited by the results in Fig. 5, elimination of CD4 in the thymus of H-2b and H-2d Clone 4 mice restored the
respectively. Of CD4 expression in both groups of mice, whereas 70% and of thymocytes were negative for transgene transcripts. This was not due to degradation of RNA in the sample, as dihydrofolate reductase amplification products were readily detected. Sequencing analysis of gel-purified PCR products confirmed that the 470-bp product corresponded to transgene gDNA and the 350-bp product was amplified from transgene cDNA (data not shown).

Although these results indicated that the InsHA transgene was expressed, albeit at low levels, in the thymus, we wished to determine whether Clone 4 TCR-bearing cells could detect its presence. Previous studies had demonstrated that homozygous expression of the InsHA transgene resulted in higher levels of HA epitopes in the pancreas than was found in the heterozygous mice (36). If this increased β cell expression of HA was accompanied by an increase in expression of HA in the thymus, then the amount of HA may achieve a concentration sufficient to alter the thymic development of Clone 4 TCR-bearing T cells. However, it is not possible to generate (InsHA × Clone 4 TCR) mice homozygous for InsHA, because (InsHA × Clone 4 TCR) mice heterozygous for the InsHA transgene die within the first 2 wk of life and are, therefore, unavailable for further breeding. To determine whether thymic development of Clone 4 TCR-expressing cells was affected by homozygous expression of the InsHA transgene, radiation chimeras were produced by reconstituting lethally irradiated InsHA+/− mice with bone marrow from Clone 4 TCR mice. Analyses of these chimeras revealed that, as compared with the control B10.D2 recipients, there was a 4-fold diminution in the total number of thymocytes recovered from InsHA+/− recipients. This was accompanied by a reduction in the percentage of Clone 4 TCRhigh thymocytes and CD8+ SP cells (Fig. 7).

Neonatal H-2b/d (InsHA × Clone 4 TCR)F1 mice develop diabetes in the absence of Eβb Eae4

To demonstrate directly that Eβb Eae4 was responsible for deletion of Vβ8-expressing cells in the thymus of the H-2b/d (InsHA × Clone 4 TCR)F1 mice, we utilized the chimeric approach. Neonatal H-2b/d (InsHA × Clone 4 TCR)F1 mice were reconstituted with bone marrow from either Clone 4 TCR mice (TCR-) or wild-type C3H/HeJ mice (TCR+). The TCR− chimera revealed that, as compared with the control B10.D2 recipients, there was a 4-fold diminution in the total number of thymocytes recovered from InsHA+/− recipients. This was accompanied by a reduction in the percentage of Clone 4 TCRhigh thymocytes and CD8+ SP cells (Fig. 7).

Evidence for expression of HA in the thymus

As reported previously and shown again in Fig. 1A, thymocytes in H-2d (InsHA × Clone 4 TCR) mice were unaffected by the presence of the InsHA transgene (22). This result suggested that HA epitopes were not expressed in the thymus of mice heterozygous for the InsHA transgene. This was consistent with our earlier data that showed that, using the 3SR method for amplifying RNA transcripts, thymic expression of the InsHA transgene was not detectable in the heterozygous InsHA mice (21). However, this is inconsistent with the current observation that in the presence of Eβb Eae4, expression of InsHA prevents development of functional Clone 4 TCR CD8+ T cells (Fig. 1B). This paradox led us to investigate further the possibility that HA epitopes were expressed in the thymus. This was examined at both the molecular and functional level as described below.

To determine whether or not transcription of the InsHA transgene was occurring in the thymus, the highly sensitive technique of nested RT-PCR was performed (35). Total nucleic acid was isolated from the thymus of InsHA mice. Nucleic acid was also extracted from the pancreas and liver to serve as positive and negative controls, respectively. RT-PCR analysis was performed on total nucleic acid. The transgene contains an intron of 120 bp within the rat insulin promoter portion of the transgene, which is spliced out and therefore absent in bona fide transgene mRNAs. Therefore, PCR products amplified from cDNA can be distinguished from PCR products amplified from genomic DNA (gDNA) based upon their smaller size as determined by agarose gel electrophoresis. As depicted in Fig. 6, both PCR products were detected in samples isolated from the pancreas, as expected. In addition, a PCR product corresponding to InsHA cDNA was also detected in samples isolated from the thymus of InsHA mice, suggesting that transcription of the transgene was indeed occurring in this organ. Only the higher molecular mass product was detected in samples originating from the liver, indicating that liver samples were negative for transgene transcripts. This was not due to degradation of RNA in the sample, as dihydrofolate reductase amplification products were readily detected. Sequencing analysis of gel-purified PCR products confirmed that the 470-bp product corresponded to transgene gDNA and the 350-bp product was amplified from transgene cDNA (data not shown).
Clone 4 TCR;F₁ double transgenic mice, H-2b InsHA mice (Eβb Eαb), were mated with B10.GD Clone 4 TCR mice (Eβb Eαβ) to produce double transgenic progeny that were negative for I-E. As anticipated, the I-E-negative offspring did not demonstrate the deleted thymic profile found in H-2b,xd InsHA × Clone 4 TCR;F₁ mice that express Eβb Eαd (compare Fig. 8 with Fig. 1B). Furthermore, the I-E-negative (InsHA × Clone 4 TCR;F₁) neonates died from spontaneous diabetes 7–10 days after birth. Blood glucose levels greater than 300 mg/dl were achieved within the first 4 days after birth. Critically, these results demonstrate that in this transgenic model of autoimmune diabetes, the presence of the Eβb Eαd molecule confers protection from CD8+ T cell-mediated disease.

FIGURE 7. FACS analyses of thymocytes from bone marrow chimeras. Clone 4 TCR bone marrow was injected into lethally irradiated B10.D2 (A and C) and InsHA;H-2b (B and D) mice. After 6 wk, thymus was removed and stained with the CyChrome-conjugated anti-Vβ8 Ab (A and B) or a mixture of PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 Abs (C and D). Total numbers of thymocytes were: B10.D2, 96 × 10⁶; InsHA;H-2b, 25 × 10⁶.

Clonal deletion of CD8 SP thymocytes expressing the Clone 4 TCR is reduced through negative selection (46). Even in the absence of the HY Ag, expression of CD4+ T cells expressing the HY TCR is reduced through negative selection (46). Here we present data that suggest that dual recognition of both class I and class II ligands by a single TCR may result in a overall decrease in the avidity of interaction of DP thymocytes such that they become subject to negative selection. Individually, neither the presence of the Eβb Eαd molecule nor the K'HA complex results in deletion of CD8 SP thymocytes expressing the Clone 4 TCR. However, expression of both of these molecules results in extensive deletion of DP thymocytes. Indeed, the presence of the InsHA;H-2b transgene has no effect upon Clone 4 TCR-bearing thymocytes unless the class II Eβb Eαd molecule is also present. This report represents the first description of negative selection that requires co-expression of two different ligands. Moreover, these results raise the interesting possibility that the fate of a T cell is determined on the basis of positive and negative selection by many different ligands, which may act synergistically in determining the fate of a developing thymocyte.

There is strong evidence that selection of a highly diverse TCR repertoire requires the presence of a diverse set of peptide ligands. This has been interpreted as evidence that each TCR is extremely specific and requires a unique peptide ligand to achieve positive selection 

Discussion

In this report, evidence is presented that demonstrates recognition of a class II molecule by DP thymocytes expressing a class I-restricted TCR. Such recognition was revealed in Clone 4 TCR mice that express both the H-2b and H-2xd haplotypes not as deletion, but rather as an increase in the proportion of immature DP TCRlow thymocytes and a reduction in the proportion of SP CD8 T cells in the thymus. Although the numbers of thymocytes was not significantly different in the H-2b vs H-2xd mice that express the Clone 4 TCR, we cannot rule out the possibility that some low level of deletion of Clone 4 TCR-bearing thymocytes does occur in the H-2b,xd thymus. For example, some of the DP thymocytes may transition to CD4 SP T cells that are subsequently deleted on the basis of their high affinity for class II. When the Clone 4 TCR is expressed on SP CD8 T cells, no evidence of class II recognition was observed. This most likely reflects the importance of CD4 expression in enhancing signal transduction during TCR recognition of class II (34). Indeed, when CD4 expression is extinguished in DP thymocytes through the use of CD4-deficient mice, we find the thymic influence of the Eβb Eαd molecule on thymic expression of the Clone 4 TCR is eliminated.

The high frequency of alloreactive T cells detected in the pre- and postthymic repertoire attests to the degeneracy with which TCRs can recognize MHC molecules (37–40). However, the frequency of TCRs that can recognize both class I and class II MHC molecules has been more difficult to assess. This is due to a requirement that CD8 and CD4 coreceptor molecules be expressed to generate a response to class I and class II MHC molecules, respectively (41). Thus, unless provided with an appropriate coreceptor molecule, the inherent specificity of a TCR for either a class I or a class II MHC molecule is insufficient to effect T cell activation. Ecotopic expression of a CD8 transgene has revealed class I alloseactivity by CD4+ T cells (42, 43), and the expression of a CD4 transgene has demonstrated the inherent class II specificity among TCRs expressed by CD8+ T cells (44, 45). These findings imply that during development some DP thymocytes bear TCRs that can recognize and are susceptible to positive and negative selection by both class I and class II MHC molecules. Recent studies have provided evidence to support this possibility. When expressed on CD4+ T cells, the H-2Dd-restricted TCR that recognizes the HY "male" Ag was found to be cross-reactive on Aβ. Even in the absence of the HY Ag, expression of CD4+ T cells expressing the HY TCR is reduced through negative selection (46).

Here we present data that suggest that dual recognition of both class I and class II ligands by a single TCR may result in an overall increase in the avidity of interaction of DP thymocytes such that they become subject to negative selection. Individually, neither the presence of the Eβb Eαd molecule nor the K'HA complex results in deletion of CD8 SP thymocytes expressing the Clone 4 TCR. However, expression of both of these molecules results in extensive deletion of DP thymocytes. Indeed, the presence of the InsHA;H-2b transgene has no effect upon Clone 4 TCR-bearing thymocytes unless the class II Eβb Eαd molecule is also present. This report represents the first description of negative selection that requires co-expression of two different ligands. Moreover, these results raise the interesting possibility that the fate of a T cell is determined on the basis of positive and negative selection by many different ligands, which may act synergistically in determining the fate of a developing thymocyte.
selection (47–54). However, an alternative interpretation of these findings would be that due to the low concentration at which some ligands may be expressed, in some instances, simultaneous recognition of multiple ligands may be required to achieve positive or (as in the current situation) negative selection of a single TCR (34).

Initial characterization of the InsHA mice revealed high levels of the HA protein were expressed by the pancreatic β cells; however, HA expression was not detected in the thymus (21). Further evidence for lack of InsHA transgene expression in the thymus of the InsHA mice was gained by the fact that the presence of the InsHA transgene had no effect upon expression of the transgenic, K^b HA-specific, Clone 4 TCR in the thymus of H-2^d (InsHA × Clone 4 TCR)/F1 mice (22). Nevertheless, the ability to demonstrate functional gene expression within a particular tissue will, invariably, be determined by the sensitivity of the assay used for detection, and, through the use of a more sensitive nested RT-PCR technique, evidence for HA expression in the thymus was detected. Previous studies from a number of laboratories have demonstrated thymic expression of transgenes under the control of the insulin promoter (55–58). Also, we have demonstrated partial thymic deletion of Clone 4 TCR-bearing thymocytes can occur in radiation chimeras – 58). Also, we have demonstrated partial thymic deletion of expression of transgenes under the control of the insulin promoter.

Evidence for HA expression in the thymus was detected. Previous

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Clone 4 TCR-bearing thymocytes can occur in radiation chimeras
generated by reconstituting InsHA × Clone 4 mice with bone marrow
from Clone 4 TCR mice. Therefore, although the Clone 4 TCR is
unable to detect the level of HA expressed in the thymus of the
(InsHA × Clone 4 TCR)/F1 mice, the simultaneous recognition of
the class II ligand seen by DP thymocytes expressing Clone 4 TCR
(Eβ/Eα) has the effect of lowering the concentration threshold
for recognition of K^b HA epitope that must be present to achieve
deletion of the Clone 4 TCR cells. These results pose the interesting
possibility that the affinity for any one ligand may not dictate
the fate of a particular thymocyte as much as the combined avidity
for multiple ligands. Therefore, expecting the fate of all T cells to
reflect the consequences of the recognition of a single self-ligand
may be an oversimplification.

In conclusion, our results underscore the subtlety of thymic
selection events and their dependence on many factors including
coreceptor expression, TCR affinity, and, as indicated in these
results, the overall level of expression of multiple ligands presented
by both class I and class II molecules. Moreover, these results
suggest the possibility that, in some instances, disease susceptibil-
ity, or resistance, which maps to MHC class II may, be due to
effects on both CD4 and CD8 T lymphocytes.

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References


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