MHC Class I Allele Dosage Alters CD8 Expression by Intestinal Intraepithelial Lymphocytes

Bradley S. Podd, Caroline Åberg, Kimberly L. Kudla, Lataya Keene, Erin Tobias and Victoria Camerini

*J Immunol* 2001; 167:2561-2568; doi: 10.4049/jimmunol.167.5.2561
http://www.jimmunol.org/content/167/5/2561

---

**References**  
This article cites 34 articles, 21 of which you can access for free at:  
http://www.jimmunol.org/content/167/5/2561.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
MHC Class I Allele Dosage Alters CD8 Expression by Intestinal Intraepithelial Lymphocytes

Bradley S. Podd, Caroline Åberg, Kimberly L. Kudla, Lataya Keene, Erin Tobias, and Victoria Camerini

The development of TCR αβ+, CD8αβ+ intestinal intraepithelial lymphocytes (IEL) is dependent on MHC class I molecules expressed in the thymus, while some CD8αα+ IEL may arise independently of MHC class I. We examined the influence of MHC I allele dosage on the development of CD8+ T cells in RAG 2−/− mice expressing the H-2Db-restricted transgenic TCR specific for the male, Smcy-derived H-Y Ag (H-Y TCR). IEL in male mice heterozygous for the restricting (H-2Db) and nonrestricting (H-2Dd) MHC class I alleles (MHC F1) were composed of a mixture of CD8αβ+ and CD8αα+ T cells, while T cells in the spleen were mostly CD8αβ+. This was unlike IEL in male mice homozygous for H-2Dd, which had predominantly CD8αα+ IEL and few mostly CD8+ T cells in the spleen. Our results demonstrate that deletion of CD8αα+ cells in H-Y TCR male mice is dependent on two copies of H-2Dd, whereas the generation of CD8αα+ IEL requires only one copy. The existence of CD8αβ+ and CD8αα+ IEL in MHC F1 mice suggests that their generation is not mutually exclusive in cells with identical TCR. Furthermore, our data imply that the level of the restricting MHC class I allele determines a threshold for conventional CD8αβ+ T cell selection in the thymus of H-Y TCR-transgenic mice, whereas the development of CD8αα+ IEL is dependent on, but less sensitive to, this MHC class I allele. The Journal of Immunology, 2001, 167: 2561–2568.

The majority of CD8+ T cells found in the peripheral circulation and secondary lymphoid organs express the CD8αβ heterodimer, whereas CD8+ intestinal intraepithelial lymphocytes (IEL) express CD8 as an αβ heterodimer or as an α-β homodimer (1, 2). CD8αα+ IEL, like their CD8+ T cell counterparts in the spleen and peripheral circulation, express the TCR αβ and arise following positive selection by self-MHC class I molecules expressed in thymus (1, 3). By contrast CD8αα, a form of CD8 that is almost never found on T cells outside the intestine, is expressed on TCR γδ+ and TCR αβ+ IEL (1, 2). The development and selection of these IEL, however, are less defined and are probably distinct from those of CD8αβ+ IEL. For example, while CD8αβ+ IEL are dependent on MHC class I for their development, CD8αα+ IEL persist despite targeted deletion of the MHC class I K and D locus genes (4–6). Interestingly, while CD8αα+ IEL expressing the TCR γδ are present in β2-microglobulin knockout mice (β2m−/−), those expressing the TCR αβ are markedly reduced. These results suggest that TCR αβ+, CD8αα+ IEL require a β2-m-dependent, but nonclassical, MHC I molecule (MHC class Ib) for some aspect of their development or differentiation in the thymus, gut, or some other site in the periphery (7–9). As the enrichment of CD8αα+ IEL in certain mouse strains correlates with the level of Qa-2 expression, this β2m-dependent MHC class Ib molecule, or other related MHC molecules may be required for the development and differentiation of CD8αα+ IEL (10–12).

Although numerous mouse models have demonstrated that some CD8αα+ IEL develop independently of conventional MHC class I molecules, the IEL generated in these mice may encompass different TCR repertoires subject to varying selective pressures during development and differentiation in the thymus, gut, or periphery. TCR-transgenic mice, however, have been widely used to more precisely define the development of IEL. The H-2Db MHC class I-restricted αβ TCR-transgenic mouse, whose TCR is reactive to a male peptide derived from the Y-linked gene, Smcy (H-Y TCR), has been used widely to define the selection of CD8+ T cells in the thymus and gut. In this model, positive selection of CD8αβ+ T cells occurs in the thymus of female mice, which lack the agonist ligand, and negative selection of these T cells occurs in male mice, which express the agonist ligand (13, 14). Therefore, female mice have CD8αβ+ T cells in the lymph node and spleen, whereas male mice lack these T cells due to deletion in the thymus (13, 15–17). Despite positive selection of CD8αβ+ cells in the female thymus, few CD8αβ+ T cells are present among IEL; in fact, the majority of IEL are CD8α−CD8β− T cells. By contrast, while male mice delete CD8αα+ T cells in the thymus and therefore virtually lack CD8αβ+ T cells in the spleen and gut, a large population of CD8αα+ IEL is present (15, 16).

The origin of CD8αα+ IEL in male H-Y TCR-transgenic mice is not well defined. It has been proposed that post-thymic T cells with low levels of CD8 (CD8low) escape deletion in the thymus and are expanded in the gut as CD8αα+ IEL in the presence of the nominal Ag recognized by this transgenic TCR (17). It has also been proposed, however, that TCR αβ+, CD8+, or CD8low T cells result from the premature expression of α and β H-Y TCR transgenes in the TCR γδ lineage, cells known to be enriched within the compartments of the gut (18, 19). Whether the generation of
CD8α⁺ IEL in H-Y TCR transgenic mice is driven by premature expression of the TCR transgenics or reflects the postthymic differentiation or generation of CD8⁻ T cells in the gut is still a matter of controversy.

In the present study, we used a genetic approach to understand more fully the role of conventional MHC class I molecules in the development of CD8⁺ IEL. We assessed whether alterations in MHC class I allele dosage skewed the development of CD8α⁺ and CD8αβ⁺ IEL in the H-2D⁻⁻/H-Y TCR-transgenic mouse line. Our results imply that deletion of conventional CD8αβ⁺ T cells is tightly constrained by signals generated via peptide/MHC class I in the thymus, while generation of CD8α⁺ IEL, perhaps locally within the gut, is less dependent on these signals. Furthermore, our studies demonstrate that positive selection of CD8αβ⁺ T cells does not abrogate the development of CD8α⁺ IEL. Taken together, our results suggest that the differentiation of CD8α⁺ IEL may involve signals that are overlapping with CD8αβ⁺ IEL, but are not wholly dependent on MHC class I.

Materials and Methods

Mice

Female and male SJL.B6 (H-2Db) and B10.D2 recombinase-activating gene 2 (RAG2⁺⁻) (H-2Dd) mouse strains homozygous for the H-Y TCR β transgene (SJL.B6 H-Y TCR RAG2⁺⁻ and B10.D2 H-Y TCR RAG2⁺⁻, respectively) were obtained from Taconic Farms (Tarrytown, NY) (20, 21). H-Y TCR, RAG2⁻⁻/H-2D⁻⁻/+ mice were also obtained from Dr. M. Vaccaro (National Institutes of Health, Bethesda, MD). Intercrosses of either non-TCR-transgenic Rag-deficient male or female SJL.B6 RAG2⁺⁻/⁻ (H-2Db) or B10.D2 RAG2⁺⁻/⁻ (H-2Dd) mice with either H-Y TCR RAG2⁺⁻/⁻-transgenic mouse strain noted above were used in the generation of F₁ mice under a vendor breeding agreement (Taconic Farms) (22). Mice generated from either cross had one chromosomal complement of the H-Y TCR transgene and were heterozygous for the restricting (H-2Db) and nonrestricting (H-2Dd) MHC class I alleles (H-2Db⁺, MHC F₁). In some cases, F₁ mice were generated from a cross between male or female C57BL/10 RAG2⁺⁻/⁻ (H-2Db⁺) mice expressing the H-Y TCR transgene with non-TCR-transgenic male or female B10.D2 RAG2⁺⁻/⁻ (H-2Dd⁺) Taconic Farms. These mice were similar to the MHC F₁ mice described above, were heterozygous for the MHC class I alleles, H-2Db⁺, and had one chromosomal complement of the H-Y TCR transgenes.

F₂ mice were generated by intercrossing F₁ mice derived as noted above and N2 generation mice were derived from MHC F₁ mice backcrossed with SJL.B6 RAG2⁺⁻/⁻ (H-2Db⁺) mice. F₂ and N₂ mice were selected for carriage of the TCR β transgene by PCR typing. The primers used to identify the HY TCR β transgene that spanned the CDR3 H-Y transgene Vβ3-Dβi-Jβ1-Dβ3 region were in 5'--3' orientation) 5'-GACAATTGACCTGAATACAGAC (forward) and 5'-ACAGCGTTTTCTGCAGCTTATACACC (reverse) (17). N₂ and F₂ mice harboring the TCR β transgene were further typed for MHC class I haplotype by mAb staining of lymphocyte populations (see below).

Mice were housed and bred in a laminar flow barrier facility at the University of Virginia Health Sciences Center vivarium (Charlottesville, VA) under specific pathogen-free conditions. Pregnant mice were identified and monitored daily until delivery. The day of birth was identified as day 0 of life, and individual mice were examined between 4 and 16 wk of age. The animal care and use committee at the University of Virginia approved all animal protocols and procedures.

Preparation of lymphocyte populations

Mucosal lymphocytes were prepared from the small intestine of individual mice using our previously published procedure (23). Briefly, the small intestine was dissected from the mesentery and washed in RPMI 1640 (Life Technologies, Grand Island, NY). The intestine was cut longitudinally, and the contents were removed before it was cut into 0.5-cm pieces. IEL were released from the epithelium during shaking in calcium- and magnesium-free HBSS (Life Technologies) supplemented with 1 mM DTT (Sigma, St. Louis, MO) three times for 20 min each time at 250 rpm. Mononuclear cells collected from the epithelial layer were filtered through stainless steel mesh (200 by 200 μm) to discontinuously suspend 90% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) gradient at 900 × g for 20 min. The resultant cell populations were washed in RPMI 1640 with 10% FCS and analyzed as outlined below.

Mononuclear cells were prepared from the thymus and spleen following mechanical disruption of the capsules with a glass pestle on stainless steel mesh or between frosted glass slides. Cell suspensions of splenocytes were depleted of RBC by hypotonic lysis as we have described previously (24). The resultant mononuclear cells from the spleen and thymus were suspended in PBS mAb staining buffer containing 5% FCS and 0.02% NaN₃ (both from Sigma) and stored at 4°C until use as noted below.

Antibody staining and flow cytometric analysis of lymphocyte populations

Mononuclear cell populations were suspended at a concentration of 1 × 10⁶ cells/ml in PBS mAb staining buffer. Four-color immunofluorescence cell surface labeling was conducted by adding the optimal concentration of mAb either directly conjugated to FITC, PE, allophycocyanin, biotin, or unconjugated primary mAb as necessary to cell suspensions at 4°C with incubation for 20–30 min. FcR blocking with anti-CD16/CD32 added to a final concentration of 5.0 μg/ml cells was used in all cases where cell populations were incubated with unconjugated primary mAb and in some cases with directly conjugated mAb. After incubation with primary mAb, the samples were washed three times in PBS with 0.02% NaN₃ at 4°C. The fluorochrome-conjugated secondary reagents Tricolor or PE-Cy7-conjugated streptavidin (Caltag Laboratories, South San Francisco, CA) in PBS mAb staining buffer were used to detect biotinylated primary mAb (see below), while anti-mouse IgG1 FITC (A58-1) was added to detect mouse anti-clonotypic TCR α (T3.70) (14). The secondary reactions were incubated for 20–30 min and washed as described above for primary mAb staining reactions. After mAb staining and washing, all samples were fixed in PBS with 0.02% sodium azide with 1% paraformaldehyde and stored at 4°C until analysis by flow cytometry (both from Sigma). The mAb reagents used in this study were anti-CD3e (145-2C11) FITC or allophycocyanin, anti-CD4 (YTS 191.1) FITC or PE, anti-CD8a (53-6.7) PE or allophycocyanin, anti-CD8β (53-5.8) FITC, anti-CD16/CD32 (2.4G2) purified, anti-TCR β (H57-597) FITC, anti-TCR Vβ8.1.2.2 (MRK-5) PE or biotin, anti-CD4 (30-F1) PE, anti-MHC H-2Dd (34-2-12) FITC or biotin, and anti-MHC H-2Dd (28-14-8) PE or biotin (all from BD PharMingen, San Diego, CA). Purified anti-clonotypic TCR mAb (T3.70) was a gift from Dr. H. Cheroutre (La Jolla Institute for Allergy and Immunology, San Diego, CA) (14).

Mononuclear cell suspensions were analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) in the Beirne Carter Center Flow Cytometry Core Facility (University of Virginia). Forward angle and side angle light scatter properties were acquired, and the data were analyzed using CellQuest software (BD Immunocytometry Systems). Single and multiple parameter analyses using dot plots and histograms with corresponding statistics were used. The number of T cells present among IEL, but are not wholly dependent on MHC class I.

Statistical analysis

The computer software program StatView (Abacus Concepts, Berkeley, CA) was used to calculate the SD between experimental samples. Differences between more than two mean values were determined with Student’s two-tailed t test using the analysis features of this program. We considered p < 0.05 to be statistically significant.

Results

Development and expansion of CD8α⁺⁺ IEL requires at least one copy of the restricting MHC class I allele, H-2Dd, in H-Y TCR-transgenic male mice

The phenotype of IEL in H-Y TCR-transgenic mice has been previously documented, although the findings have not always been in full agreement (15–17). Therefore, before examining the development of CD8⁺⁺ IEL subsets in H-Y TCR-transgenic mice bred to be heterozygous for the restricting (H-2Dd) and nonrestricting MHC class I alleles (H-2Dd⁺), we wanted to confirm previous findings in this mouse line on the RAG2⁻⁻ background (17). We examined H-Y TCR-transgenic mice homozygous for H-2Dd and homozygous for H-2D⁺⁺ MHC class I alleles. More than 90% of CD8α⁺⁺
mononuclear cells isolated from the intestinal epithelium of H-2D<sup>b</sup> mice were CD3<sup>+</sup> and expressed both transgenic α and β TCR chains (Fig. 1, A and B). By contrast, <10% of CD45<sup>+</sup> mononuclear cells isolated from the intestinal epithelium of H-2D<sup>d</sup> mice were CD3<sup>+</sup> and expressed the clonotypic TCR chains (Fig. 1, C and D). When CD3<sup>+</sup> IEL from each mouse strain were examined for CD8α and CD8β chain expression, 74% of IEL from the H-2D<sup>b</sup> homozygous mice were CD8α<sup>+</sup>, while only 7% were CD8αβ<sup>+</sup> and 19% were CD8<sup>−</sup> (Fig. 1B). This was in contrast to IEL prepared from mice homozygous for H-2D<sup>d</sup>, which were largely CD8<sup>−</sup> T cells (Fig. 1D).

The absolute number of CD3<sup>+</sup> IEL isolated from H-2D<sup>b</sup> homozygous male mice was nearly 100-fold greater, on the average, in the number of IEL isolated from H-2D<sup>d</sup> homozygous male mice (Fig. 2). Our results confirm, as has been shown by others, that the development and expansion of CD3<sup>+</sup>CD8α<sup>+</sup> IEL expressing the clonotypic TCR in male mice requires the MHC class I allele, H-2D<sup>b</sup> (15–17).

**FIGURE 1.** The development of CD8α<sup>+</sup> IEL in male H-Y TCR-transgenic mice requires the restricting MHC class I allele, H-2D<sup>b</sup>. IEL were isolated from male H-Y TCR-transgenic mice homozygous for H-2D<sup>b</sup> (A and B) and H-2D<sup>d</sup> (C and D). Flow cytometry was used to determine the expression of CD3 by CD45<sup>+</sup> IEL (A and C). Anti-CD3, anti-T3.70 (followed by anti-mouse IgG1), and anti-V8.2 mAb were used to determine the expression of the TCR α and β transgenes by CD3<sup>+</sup> IEL, and anti-CD8α and anti-CD8β mAb were used to determine the coreceptor expression by CD3<sup>+</sup> IEL (B and D). Single-fluorescence histogram gating on CD45<sup>+</sup> cells demonstrated the percentage of CD3<sup>+</sup> IEL in each mouse strain (A and C). The expression of H-Y TCR α- and β-chain transgenes and CD8α and CD8β coreceptors are shown in representative two-color dot-plot analysis on IEL electronically gated on CD3 and CD45 expression (B and C). The percentage of CD3<sup>+</sup>CD45<sup>+</sup> cells in each histogram and the percentage of cells in the relevant quadrants of the dot plots are shown in the upper right corners. Data are representative of a minimum of three experiments and represent one experiment from data presented in Fig. 4E.

**FIGURE 2.** One copy of H-2D<sup>b</sup> is required for the expansion of IEL in H-Y TCR-transgenic male mice. IEL were prepared from male and female H-Y TCR-transgenic mice on the MHC backgrounds shown. The number of CD3<sup>+</sup>CD45<sup>+</sup> T cells in each subset was calculated by multiplying the total number of viable cells, as determined by light microscopy, by the percentage of CD45<sup>+</sup>CD3<sup>+</sup> cells, as determined by flow cytometry. Each symbol represents the analysis of cells from a single experiment, which was comprised of a single mouse, n = 7 for male H-2D<sup>b</sup>, n = 11 for male H-2D<sup>d</sup>, n = 4 for male H-2D<sup>d</sup>, and n = 4 female H-2D<sup>d</sup>.. * p < 0.05 when male IEL H-2D<sup>d</sup> were compared with male H-2D<sup>d</sup> or female H-2D<sup>d</sup>; †, p = ns when male IEL H-2D<sup>d</sup> compared with male H-2D<sup>b</sup>. TCR α- and β-chains (Fig. 3, C and D). Similar results were obtained with IEL in MHC F<sub>1</sub> mice generated by crossing nontransgenic C57BL/10 RAG2<sup>−/−</sup> mice with B10.D2 H-Y TCR RAG2<sup>−/−</sup>
Results demonstrate that CD8 isoform expression segregates with the MHC haplotype in H-Y TCR-transgenic mice. The H-Y TCR-transgenic mice used in the generation of MHC F1 mice have several copies of α and β TCR transgenes integrated on the same chromosome and are carried in the homozygous state (25). To evaluate whether CD8 isoform expression in MHC F1 mice was due to quantitative differences in the level of TCR expression in these mice, we examined the expression of transgenic TCR α and TCR β levels in MHC F1 and MHC homozygous mouse lines by flow cytometry. We found that the level of TCR transgenes in MHC F1 mice was similar to that in the H-Y TCR MHC homozygous mouse lines (Figs. 1D, 3D, and 4A and B). Furthermore, TCR α and β transgene levels were similar in the H-Y TCR-transgenic line obtained from Dr. Melanie Vacchio, a line maintained by crossing H-2Dd-nontransgenic RAG2−/− mice with H-Y TCR-transgenic RAG2−/− mice (H-2Dd) (data not shown). These results suggest that the level of TCR transgene expression does not differ significantly between MHC F1 and MHC class I homozygous H-Y TCR-transgenic mice and therefore is an unlikely cause of the retention of CD8αβ+ T cells in male MHC F1 mice.

To determine whether CD8 isoform usage segregated with the MHC haplotype we generated additional male lines that were either heterozygous or homozygous for H-2Dd. F2 mice were generated by backcrossing MHC F1 mice with non-H-Y RAG2−/− (H-2Dd) mice. MHC F1 mice were generated using male mice from either the B10.D2 or SJL.B6 strain; therefore, a different strain serves as the Y chromosome donor. IEL derived from F2 and N2 mice demonstrated again that CD8 isoform expression segregated with the MHC haplotype. In the example shown in Fig. 4, 59% of CD3+ IEL in N2, MHC heterozygous mice were CD8αα+, while 40% of IEL were CD8αβ− and 1% of IEL were CD8α−CD8β+ (Fig. 4C). By contrast, IEL in N2 mice homozygous for the selecting MHC class I alleles were largely CD8αα+ IEL (Fig. 4D). The increase in CD8αβ+ IEL in MHC F1 mice was also seen in F2 mice that were heterozygous for the restricting and nonrestricting MHC alleles (Fig. 4E). A large number of experiments derived from individual mice were pooled and compared with H-2Dd homozygous mice and illustrate the same trend (Fig. 4E). In the example shown, CD8αβ+ IEL were increased in MHC heterozygous mice compared with mice homozygous for the restricting MHC (Fig. 4E). Additionally, the strain of the male mouse (Y chromosome) used to generate MHC F1 mice did not alter the appearance of CD8αβ+ T cells (data not shown). Collectively, these data indicate that the appearance of CD8αβ+ IEL segregated with the heterozygous configuration of the MHC class I alleles, H-2Dd and H-2Dd.

CD8αβ+ T cells are more numerous and CD8α−CD8β− T cells are less abundant among IEL and in the spleen of MHC F1, H-Y TCR-transgenic mice

The restoration of CD8αβ+ T cells among IEL in MHC F1 mice suggested that CD8αβ lineage cells were positively selected in the thymus and expanded in the periphery of MHC F1 mice. We hypothesized that MHC F1 mice would, therefore, have a reduction in CD8α−CD8β+ or CD8− T cells among IEL and in the spleen if these cells were generated during deletion in thymus or tolerance in the periphery. We found that while up to 33% of IEL, on the average, were CD8− in H-2Dd homozygous male mice, MHC F1 mice had no >5% of these T cells, a difference that was statistically significant when a large number of mice from individual experiments were compared (Fig. 4E). The reduction in CD8− IEL

FIGURE 3. One copy of the restricting MHC class I allele, H-2Dd, is sufficient for the development of IEL in H-Y TCR-transgenic male mice, but hemizygosity of this allele alters CD8 isoform expression by these T cells. CD45+CD3+ IEL were electronically gated and shown in single-fluorescence histograms for H-Y TCR-transgenic male mice on the nonrestricting MHC background (A), in non-H-Y TCR-transgenic mice on the restricting MHC background (B), and in MHC F1 male progeny (C). IEL isolated from male MHC F1 progeny were analyzed for the expression of the clonotypic TCR α- and β-chains (D) and the expression of the CD8α and CD8β coreceptors (E). CD8 isoform expression by IEL isolated from H-Y TCR-transgenic mice on the restricting MHC background is shown for comparison in F. The percentage of cells in each quadrant is shown in the upper right corner. The data are representative of a minimum of seven experiments derived from the analysis of individual mice.

mice (data not shown). The number of IEL isolated from MHC F1 male mice was not statistically different from the number of IEL isolated from male mice homozygous for H-2Dd (Fig. 2). These results indicate that one copy of H-2Dd is required for the development and expansion of CD3+ IEL in male H-Y TCR-transgenic mice.

Surprisingly, when we examined CD8 expression by IEL in MHC F1 male mice, we found that they were composed of nearly equal proportions of CD8αα+ and CD8αβ+ T cells. In the example shown in Fig. 3E, 55% of CD3+ IEL in MHC F1 were CD8αα+, while 44% were CD8αβ+, and 1% lacked expression of CD8α and CD8β (Fig. 3E). This is in contrast to IEL isolated from H-2Dd homozygous mice, which in the example shown were 74% CD8αα+, 7% CD8αβ+, and 19% CD8α−CD8β− T cells (Fig. 3F). The proportion of CD8 isoforms expressed by IEL was similar whether MHC F1 male mice were derived from a cross between non-TCR-transgenic male or female mice on the MHC-nonselecting background with the H-Y TCR transgenic on the MHC-selecting background or the converse (data not shown). These results demonstrate that CD8αβ+ IEL were restored in MHC F1 male mice.

mice (data not shown). The number of IEL isolated from MHC F1 male mice was not statistically different from the number of IEL isolated from male mice homozygous for H-2Dd (Fig. 2). These results indicate that one copy of H-2Dd is required for the development and expansion of CD3+ IEL in male H-Y TCR-transgenic mice.

Surprisingly, when we examined CD8 expression by IEL in MHC F1 male mice, we found that they were composed of nearly equal proportions of CD8αα+ and CD8αβ+ T cells. In the example shown in Fig. 3E, 55% of CD3+ IEL in MHC F1 were CD8αα+, while 44% were CD8αβ+, and 1% lacked expression of CD8α and CD8β (Fig. 3E). This is in contrast to IEL isolated from H-2Dd homozygous mice, which in the example shown were 74% CD8αα+, 7% CD8αβ+, and 19% CD8α−CD8β− T cells (Fig. 3F). The proportion of CD8 isoforms expressed by IEL was similar whether MHC F1 male mice were derived from a cross between non-TCR-transgenic male or female mice on the MHC-nonselecting background with the H-Y TCR transgenic on the MHC-selecting background or the converse (data not shown). These results demonstrate that CD8αβ+ IEL were restored in MHC F1 male mice.

mice (data not shown). The number of IEL isolated from MHC F1 male mice was not statistically different from the number of IEL isolated from male mice homozygous for H-2Dd (Fig. 2). These results indicate that one copy of H-2Dd is required for the development and expansion of CD3+ IEL in male H-Y TCR-transgenic mice.

Surprisingly, when we examined CD8 expression by IEL in MHC F1 male mice, we found that they were composed of nearly equal proportions of CD8αα+ and CD8αβ+ T cells. In the example shown in Fig. 3E, 55% of CD3+ IEL in MHC F1 were CD8αα+, while 44% were CD8αβ+, and 1% lacked expression of CD8α and CD8β (Fig. 3E). This is in contrast to IEL isolated from H-2Dd homozygous mice, which in the example shown were 74% CD8αα+, 7% CD8αβ+, and 19% CD8α−CD8β− T cells (Fig. 3F). The proportion of CD8 isoforms expressed by IEL was similar whether MHC F1 male mice were derived from a cross between non-TCR-transgenic male or female mice on the MHC-nonselecting background with the H-Y TCR transgenic on the MHC-selecting background or the converse (data not shown). These results demonstrate that CD8αβ+ IEL were restored in MHC F1 male mice.

mice (data not shown). The number of IEL isolated from MHC F1 male mice was not statistically different from the number of IEL isolated from male mice homozygous for H-2Dd (Fig. 2). These results indicate that one copy of H-2Dd is required for the development and expansion of CD3+ IEL in male H-Y TCR-transgenic mice.

Surprisingly, when we examined CD8 expression by IEL in MHC F1 male mice, we found that they were composed of nearly equal proportions of CD8αα+ and CD8αβ+ T cells. In the example shown in Fig. 3E, 55% of CD3+ IEL in MHC F1 were CD8αα+, while 44% were CD8αβ+, and 1% lacked expression of CD8α and CD8β (Fig. 3E). This is in contrast to IEL isolated from H-2Dd homozygous mice, which in the example shown were 74% CD8αα+, 7% CD8αβ+, and 19% CD8α−CD8β− T cells (Fig. 3F). The proportion of CD8 isoforms expressed by IEL was similar whether MHC F1 male mice were derived from a cross between non-TCR-transgenic male or female mice on the MHC-nonselecting background with the H-Y TCR transgenic on the MHC-selecting background or the converse (data not shown). These results demonstrate that CD8αβ+ IEL were restored in MHC F1 male mice.
pared with a greater proportion of CD8/H11001 derived from the analysis of individual mice. The data are representative of a minimum of four experiments.

FIGURE 4. CD8 isoform expression by IEL segregates with the MHC haplotype in male H-Y TCR-transgenic mice. The level of transgenic TCR β-chain expression by CD3+ IEL was determined following mAb staining of IEL derived from MHC F1 mice (A) and H-Y TCR-transgenic mice homozygous for H-2D\(^b\) (B). The geometric mean fluorescence of TCR Vβ8.2 expression by CD3+ IEL is shown in the upper left corner of each histogram. CD8 isoform expression by CD3+ IEL isolated from a N2 generation H-Y TCR-transgenic mice heterozygous for the restricting and non-restricting MHC class I alleles (C) and mice homozygous for the restricting MHC class I alleles (D) were determined as noted in Fig. 3. The percentage of cells in the relevant quadrants on the dot plots is shown in the upper right corner. The data are representative of a minimum of four experiments derived from the analysis of individual mice. E, Summary data for co-receptor expression by CD3+ IEL derived from H-Y TCR-transgenic mouse lines as a function of their H-2D\(^b\) genotype is shown. CD8α\(^\alpha\), CD8αβ\(^\beta\), or CD8\(^\delta\) IEL populations were determined by three- and four-color flow cytometry as indicated by the graph legend. *, \(p < 0.05\) compared with H-2D\(^b\) homozygous mice; †, \(p < 0.05\) compared with MHC F1 mice. Data were derived from a minimum of three individual experiments, each derived from the analysis of individual mice.

was also seen in the individual FACS dataplots shown in Fig. 3, E and F, and in Fig. 4, C and D. Similar findings are seen among T cells in the spleen of these mice. In the example shown in Fig. 5A, 80% of T cells in the spleen of MHC F1 mice were CD8αβ\(^\beta\), and 13% of T cells were CD8\(^\gamma\). By contrast, 95% of T cells in H-2D\(^b\) homozygous mice were CD8\(^\gamma\), and 3% of T cells had lower levels of CD8\(^\alpha\) and CD8\(^\beta\) (Fig. 5A). These results are consistent with the increased generation or reduced destruction and survival of CD8αβ\(^\beta\) T cells in MHC F1 mice compared with male mice homozygous for H-2D\(^b\).

To determine whether the thymus of MHC F1 mice contained a greater proportion of CD8αβ lineage cells than the thymus of H-2D\(^b\) homozygous male mice, we examined the thymocytes from these two mouse lines. Although the total number of thymocytes in MHC F1 was increased only modestly compared with that in H-2D\(^b\) homozygous mice, the phenotypes of cells in the thymus were considerably different between these two mouse lines (Fig. 5 and data not shown). The thymus of MHC homozygous male mice was predominantly CD4+CD8\(^\alpha\) double-negative cells (85%) with a small population of CD8αβ\(^\gamma\) cells (7%), while the MHC F1 thymus had a population of CD4+CD8\(^\alpha\) double-positive (26%) and CD8αβ\(^\beta\) single-positive cells (18%; Fig. 5, B and C). Therefore, CD8αβ lineage precursors are more abundant in the thymus of MHC F1 male mice than in H-2D\(^b\) homozygous mice. These results are consistent with the idea that CD8αβ lineage cells are positively selected in the thymus of MHC F1 male mice.

Positive selection in female mice is not altered by MHC class I allele dosage in H-Y TCR-transgenic mice

Our findings in MHC F1 male mice prompted us to evaluate whether MHC class I allele dosage altered T cell development in female H-2D\(^b\) homozygous H-Y TCR-transgenic mice. Female mice are known to have few CD8\(^+\) IEL despite positive selection of this transgenic TCR in the thymus of H-2D\(^b\) female mice (13). Although we identified differences between T cells in the thymus, spleen, and intestine of MHC F1, male mice, the phenotype of T cells in MHC F1 female mice was similar to that in female H-2D\(^b\) homozygous mice. In the example shown, the majority of T cells among IEL were CD8\(^\gamma\) among both MHC F1 and H-2D\(^b\) female mice (63 and 57%, respectively; Fig. 6). In addition, the number and phenotype of IEL in female mice were similar to what has been described previously for H-2D\(^b\) homozygous mice on the RAG2\(^−/−\) background (Fig. 6) (19). The thymus of MHC F1 and H-2D\(^b\) homozygous mice was composed of CD4+CD8\(^\alpha\) double-positive, CD8αβ\(^\gamma\) single-positive, and CD4+CD8\(^\alpha\), double-negative cells consistent with selection to the CD8αβ\(^\gamma\) T cell lineage in these mice (data not shown). These data imply that one copy of H-2D\(^b\) is sufficient to mediate positive selection of CD8αβ\(^\gamma\) T cells in the thymus of female mice, although CD8αα\(^\alpha\) and CD8αβ\(^\gamma\) T cells remain poorly represented among IEL.

Discussion

We have extended previous studies of IEL development in H-Y TCR-transgenic mice by examining the role of MHC class I allele dosage in thymic selection and the development of IEL. Our study, like that of Cruz et al. (17), confirmed that cognate Ag and the appropriate MHC class I allele, H-2D\(^b\), were required for the development and expansion of CD8αα\(^\alpha\) IEL in H-Y TCR RAG2\(^2−/−\) mice. We found, however, that while MHC F1 male mice had nearly the same number of IEL as male mice homozygous for the H-2D\(^b\) MHC class I allele, IEL in MHC F1 mice were composed of a large population of CD8αβ\(^\beta\) T cells in addition to CD8αα\(^\alpha\) T cells. Furthermore, T cells in the spleen of MHC F1 mice were mostly CD8αβ\(^\beta\) cells. These results demonstrate that deletion of autoreactive CD8αβ\(^\gamma\) TCR αβ\(^\gamma\) lineage cells in H-Y TCR-transgenic male mice requires two copies of the restricting MHC, H-2D\(^b\). By contrast, only one copy of the restricting MHC class I allele was necessary for the development of CD8αα\(^\alpha\), TCR αβ\(^\gamma\) IEL. These data strongly support the hypotheses that the development of CD8αα\(^\alpha\) and CD8αβ\(^\gamma\) IEL are not mutually exclusive and that CD8αα\(^\alpha\) require different signals in the thymus or elsewhere for their development than do CD8αβ\(^\gamma\) lineage T cells.

Our data support a central and a peripheral mechanism for the generation and expansion of CD8αβ\(^\gamma\) T cells in MHC F1 male mice. First, the thymus of MHC F1 mice had a marked increase in thymocytes known to be progenitors of CD8αβ\(^\gamma\) T cells, namely,
data are representative of at least three experiments derived from the analysis of individual mice between 1 and 3 mo of age.

**FIGURE 5.** CD8αβ+ T cells are more numerous in the spleen and CD4+ CD8+ thymocytes are more abundant in the thymus of MHC F1 mice than in mice homozygous for the restricting MHC, H-2Db. The expression of CD8α and CD8β was determined by multicolor flow cytometry of cells derived from the spleen by electronically gating on CD45+ CD3+ T cells (A). Thymocytes were gated on CD45 and the expression of CD4 and CD8 coreceptor was determined (B). H-2Dα homozygous (A, upper panel, and B), and MHC F1 mice (A, lower panel, and C) were analyzed. A, Few T cells in the spleen of MHC H-2Dα mice (A, top panel) are CD8αβ+ or expressed lower levels of CD8α and β than did T cells in the spleen of MHC F1 mice (A, lower panel). B. The expression of CD4 and the CD8 coreceptor by total cells in thymus of H-2Dα homozygous male mice was markedly reduced compared with MHC F1 male mice (C). The percentage of cells in relevant quadrants is shown in the upper right corners of the FACS dot plots. These data are representative of at least three experiments derived from the analysis of individual mice between 1 and 3 mo of age.

CD4+CD8+ double-positive cells and CD8 single-positive cells expressing high levels of the CD8α and CD8β chains and TCR α and β transgenes (Fig. 5 and data not shown). Cells in the thymus of H-2Dα homozygous mice were, however, almost exclusively CD4-CD8- double-negative cells with lower levels of CD8α and the TCR transgenes, consistent with negative selection (Fig. 5 and data not shown). Taken together, these results suggest that CD8αβ+ T cells found among IEL and in the spleen were positively selected in the thymus of MHC F1 male mice. By contrast to IEL in male mice, T cells in the spleen and among IEL in female MHC F1 mice were similar in number and phenotype to those in female mice homozygous for H-2Dα. These data imply that the window of positive selection in the thymus is functionally larger than the window of negative selection; the latter is drastically altered in MHC F1 male mice. Second, the selective pressure to down-regulate CD8 α and β expression by T cells in the periphery of MHC F1 mice may be less than that in MHC homozygous mice, hence accounting for the preservation of this T cell subset despite the potential autospecificity in MHC F1 male mice. In support of this we found fewer CD8α- and CD8β- T cells among IEL and in the spleen of MHC F1 male mice compared with H-2Dα homozygous mice. It can be inferred, however, that lower levels of CD8α and CD8β expression by CD8αβ+ T cells in the periphery of MHC F1 male mice compared with female mice may reflect a negative influence on CD8 coreceptor expression in male mice (Figs. 5A and 6B). Notably, MHC F1 male mice, like H-Y TCR-transgenic mice homozygous for the restricting MHC, have no evidence of inflammatory disease in the gut or elsewhere (17). CD8αβ+ T cells were, therefore, not autoreactive by virtue of their development in the thymus and their presence in peripheral organs replete in the cognate Ag. Although CD8α- and CD8β- T cells may derive from either central or peripheral mechanisms, as discussed above, these T cells may derive following the early expression of the H-Y TCR transgenes during ontogeny (18, 19). This pathway could certainly explain the presence of largely CD8α- T cells in H-Y TCR-transgenic mice on the nonrestricting MHC background (H-2Dα) and in female H-Y TCR-transgenic mice (18, 19). The reduction of CD8α- and CD8β- T cells that we documented in MHC F1 mice, however, suggests that CD8α- and CD8β- T cells in male mice homozygous for the restricting MHC normally derive from MHC class I-restricted negative selection in the thymus or following their generation in the periphery. The reduction in these populations in MHC F1 mice would not be expected if their generation was solely due to events independent of MHC-restricted selection in the thymus. We cannot exclude, however, that events secondary to this TCR-transgenic model account for the aberrant generation of T cells in the H-Y TCR-transgenic mouse line. Taken together, our data suggest that CD8αβ+ lineage T cells are selected in the thymus of MHC F1 mice, while in H-2Dα homozygous mice this T cell lineage is efficiently deleted in the thymus and perhaps in the periphery.

The mechanism by which CD8αβ+ T cells are generated in MHC F1 mice was not directly elucidated by our studies. We determined, however, that the generation of CD8αβ+ T cells was dependent on at least one copy of the selecting MHC class I allele, H-2Dα, regardless of whether F2 or N2 H-Y TCR generation male

**FIGURE 6.** Positive selection of H-Y TCR-transgenic IEL in female mice is not altered by MHC class I gene dosage. The expression of CD8α and CD8β by T cells among IEL (A) and in the spleen (B) of female H-Y TCR MHC F1 mice (left panels, A and B) female H-2Dα homozygous mice (right panels, A and B). The expression of CD8α and CD8β was determined following electronic gating on CD45+ CD3+ T cells in each compartment. The majority of IEL on both MHC backgrounds were CD8+, while T cells in the spleen were predominantly CD8αβ+, expressing high levels of CD8 α and β (B). The percentage of T cells present in the relevant quadrants is shown in the upper right corners. The data are representative of a minimum of three experiments derived from the analysis of individual mice.

[Image 72x147 to 257x255]
FIGURE 7. MHC class I allele dosage disrupts negative selection in the thymus and contributes to the development of CD8αβ+ T cells in male H-Y TCR-transgenic mice. The relationship between cumulative signal strength and selection bias in the thymus with respect to the development of CD8+ T cells is shown. The presence of one or two copies of the restricting MHC class I allele, H-2D^d or H-2D^b, results in the generation of intrinsically different signals to the developing thymocyte. In the case of two copies of the restricting MHC class I allele, H-2D^d, a strong signal is generated, and negative selection occurs. By contrast, one copy of the selecting MHC class I allele generates a lower signal and allows positive selection to occur in male mice. Altered signal strength, therefore, allows positive selection of CD8αβ+ T cells in the thymus, resulting in the restoration of CD8αβ+ T cells among IEL and in the spleen of MHC F1 male mice. Figure adapted from Smyth et al. (34).

Mouse lines were examined. Furthermore, CD8αβ+ T cells among IEL and splenocytes were not dependent on the strain of male mouse (SJL.B6, B10.D2, or C57BL/10) used to generate the MHC F1 mouse lines, suggesting that possible differences in the SmcY-derived peptide were not responsible for our results. This is in contrast to 5F TCR-transgenic mice expressing either a high or low affinity ligand for this TCR. Mice expressing the high affinity ligand had mostly CD8αα^- and CD8^- IEL, similar to H-Y TCR-transgenic male mice homozygous for 5F H-2D^d, whereas mice expressing a low affinity antagonist ligand had both CD8αβ+ and CD8αα^- IEL, similar to the MHC F1 mice in our study (26). However, unlike MHC F1 mice, CD8αβ+ T cells were absent in the spleen of 5F TCR-transgenic mice, suggesting that these T cells were not conventionally derived in the thymus (26). By contrast, our data support the hypothesis that CD8αβ+ T cells in MHC F1 male mice were derived via a conventional thymus-dependent pathway and that these T cells established residence within the thymus and within the intestinal epithelium.

We propose that the signals driving negative selection are reduced in the thymus of MHC F1 mice, as the probability of interaction between the cognate Ag in the context of the restricting MHC class I molecule, H-2D^d, with its TCR is reduced by virtue of the haploid state. Therefore, an individual cell is less likely to receive a negative signal or receives a cumulative signal that is not sufficient to drive negative selection, thereby allowing the paradoxical selection of CD8αβ+ lineage cells in the thymus of MHC F1, male mice. The generation of CD8αβ+ lineage cells in MHC F1, male mice can be explained by the quantitative affinity model of TCR selection in the thymus (26–28). One model to show the relationship between signal strength and selection bias in the thymus is depicted in Fig. 7. A high avidity signal normally generated in H-Y TCR-transgenic male mice homozygous for the restricting MHC, H-2D^b, is sufficient to trigger negative selection and deletion of a majority of CD8αβ+ lineage cells in the thymus. By contrast, mice heterozygous for the restricting MHC allele, H-2D^d, generate a signal below the threshold required for negative selection, but sufficient to allow positive selection of CD8αβ+ lineage cells in the thymus. In fact, several TCR-transgenic models demonstrate that selection bias in the thymus is directly proportional to the density of the relevant MHC proteins (29–31). In the TCR-transgenic model the level of the restricting MHC was varied using transgenic constructs expressing H-2L^d, which is normally deleting in the wild-type 2C TCR-transgenic mice. Mice expressing lower levels of this allele allowed positive selection, while higher levels promoted negative selection of CD8αβ+ lineage cells in the thymus (31). It is possible, however, that H-2D^d in the haploid state would not support the positive selection of CD8αβ+ T cells, in which case the nonrestricting MHC class I molecule, H-2D^d, or some other MHC molecule would provide an accessory signal. H-2D^d in the homozygous state is evidently not sufficient to support the development of CD8αβ+ T cells, as these mice have few T cells, most of which are CD8^-.

The colocalization of CD8αβ+ and CD8αα^- IEL in our study demonstrates that positive selection of CD8αβ+ T cells in MHC F1 mice does not abrogate the development of CD8αα^- IEL, suggesting that their developmental pathways are not mutually exclusive (32). The presence of CD8αα^- and CD8αβ+ IEL in naive lymphocytic choriomeningitis virus-specific TCR-transgenic mice also supports the idea that these pathways are not mutually exclusive (33). Although in contrast to the H-Y TCR-transgenic mice that were RAG deficient, some CD8^- T cells in lymphocytic choriomeningitis virus TCR-transgenic mice expressed endogenous α-chains, making their developmental lineage more difficult to assess (33). The reported independence of TCR αβ-, CD8αα^- IEL from conventional MHC class I K and D locus genes and their selective augmentation in Qa-2^- mice may be difficult to reconcile with the clear dependence of CD8αα^- IEL in H-Y TCR-transgenic mice on H-2D^d (4–6). However, when mice expressing high levels of Qa-2 were crossed with a Qa-2-deficient mouse strain, both CD8αα^- and CD8αβ+ T cell subsets were reduced, suggesting that the development or differentiation of these CD8 subsets may be in some way interrelated (12). One possible interrelationship may be that CD8αα^- IEL derive from a subset of conventional CD8αβ+ T cells within the intestinal epithelium. In this case, then, CD8αα^- IEL would be dependent on conventional peptide/MHC class I selection in the thymus, but their differentiation in the gut may involve signals derived independently or coordinately from nonclassical and classical MHC I molecules or some other differentiation Ag restricted to the intestine.

In summary, our data support a crucial role for the level of MHC class I expression as a control point in T cell development in the thymus and gut. The negative selection of conventional T cells in the thymus has a higher threshold than does positive selection of CD8αβ+ T cells or the generation of CD8αα^- IEL. Variations in the level of MHC during ontogeny, particularly in cases where the level of cognate Ag is constant, alterations in MHC expression in response to cytokines or in the context of inflammation may be important in TCR repertoire selection in the thymus or in the generation of CD8αα^- T cells in the gut. Model systems such as ours may provide significant insight into the differentiation Ags responsible for the generation of CD8αα^- and CD8αβ+ IEL and in elucidating their function in immune homeostasis in the gut.

Acknowledgments
We thank Dr. David Camerini (University of Virginia) and Dr. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology) for helpful suggestions and critical reading of this manuscript.
References


