An Opposite Pattern of Selection of a Single T Cell Antigen Receptor in the Thymus and among Intraepithelial Lymphocytes

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Summary

The differentiation of intestinal intraepithelial lymphocytes (IEL) remains controversial, which may be due in part to the phenotypic complexity of these T cells. We have investigated here the development of IEL in mice on the recombination activating gene (RAG)-2−/− background which express a T cell antigen receptor (TCR) transgene specific for an H-Y peptide presented by Db (H-Y/Dd × RAG-2−/− mice). In contrast to the thymus, the small intestine in female H-Y/Dd × RAG-2−/− mice is severely deficient in the number of IEL; TCR transgene+ CD8αα is virtually absent. This is similar to the number and phenotype of IEL in transgenic mice that do not express the Db class I molecule, and which therefore fail positive selection. Paradoxically, in male mice, the small intestine contains large numbers of TCR1 CD8αβ IEL that express high levels of CD8αα homodimers. The IEL isolated from male mice are functional, as they respond upon TCR cross-linking, although they are not autoreactive to stimulator cells from male mice. We hypothesize that the H-Y/Db TCR fails to undergo selection in IEL of female mice due to the reduced avidity of the TCR for major histocompatibility complex peptide in conjunction with the CD8αα homodimers expressed by many cells in this lineage. By contrast, this reduced TCR/CD8αα avidity may permit positive rather than negative selection of this TCR in male mice. Therefore, the data presented provide conclusive evidence that a TCR which is positively selected in the thymus will not necessarily be selected in IEL, and furthermore, that the expression of a distinct CD8 isoform by IEL may be a critical determinant of the differential pattern of selection of these T cells.

Key words: T cells • intraepithelial lymphocytes • positive selection • coreceptors

The development of T cells in the thymus ultimately results in one of three fates: negative selection leading to deletion of autoreactive clones, positive selection leading to thymocyte survival and emigration, or a lack of selection that also results in programmed cell death of thymocytes (1). Although there is significant evidence consistent with the extrathymic differentiation of intestinal intraepithelial lymphocytes (IEL), it has been difficult to clearly define the requirements for the development and selection of these cells (2–5). Part of this challenge arises from the phenotypic complexity of IEL, and the still undefined lineage relationships between some of the phenotypic subsets (6).

Besides the TCR γδ+ IEL, five TCR αβ+ phenotypes have been reported in IEL based on their expression pattern of coreceptor molecules (6). In addition to the two subsets predominant in circulating T lymphocytes outside of the intestine, CD4+ and CD8αβ+ single positive cells, IEL also contain unique TCR αβ+ subsets that express CD8αα either alone or in combination with CD4 (CD4+, CD8αα+ double positive cells). Finally, as found elsewhere in peripheral lymphoid tissues, there is a small population of double negative (DN) IEL that do not express either CD4 or CD8. Notably, in contrast to other peripheral T cell populations, IEL of the small intestine are composed predominantly of CD8αβ single positive T cells, with approximately equal proportions of cells that express either CD8αβ heterodimers or CD8αα homodimers among the TCR αβ+ cells, whereas the TCR γδ+ IEL are nearly exclusively CD8αα+ (7, 8).
Perhaps the best evidence in favor of some type of selection of TCR αβ IEL comes from the analysis of β2 microglobulin knockout mice (9). In these class I-deficient mice, thymic selection of TCR αβ+ CD8 single positive T cells is almost completely inhibited. In the intestine of these mice, normal numbers of TCR γδ+ CD8ααα IEL can be found, although the vast majority of CD8ααα single positive and CD8αβ+ single positive TCR αβ+ IEL are absent (10-12). These data suggest that all TCR αβ+ CD8+ IEL require class I molecules for their positive selection, like their counterparts in the spleen and LN, although the data do not determine if this positive selection occurred in the thymus or elsewhere.

TCR transgenic mice can be used to study the positive and negative selection of individual TCRs in the thymus and in IEL. A useful and widely studied transgenic model for the selection of CD8+ T cells involves expression of the TCR derived from a clone reactive to a male-derived peptide (H-Y) presented by the Db class I molecule (H-Y/Db mice; reference 13). In male mice that express the Dα class I gene, this TCR is autoreactive. As a result, thymocytes in Dα+ male mice undergo extensive deletion, and these mice have a small thymus with few double or single positive T cells (14, 15). Interestingly, H-Y/Db male TCR transgenic mice do have significant numbers of TCR transgene+ cells in the periphery, but these T lymphocytes are either DN or CD8 single positive T cells (16). It has been proposed that the TCR transgene+ cells in male mice may be derived from thymocytes that have downregulated CD8 expression in order to avoid negative selection, or that they may be γδ lineage cells that have been forced to express an αβ TCR (17). By contrast, female Dα+ transgenic mice lack the male antigen, but in the thymus, they must express a peptide or peptides that can positively select this TCR. As a consequence, the thymus of female TCR transgenic mice is approximately normal or somewhat larger than normal in size, and it has a greatly increased number of CD8αβ+ single positive thymocytes (18-20). In either male or female H-Y/Db TCR transgenic mice that do not express Dα, the TCR transgene does not undergo positive selection, and thymocyte differentiation is blocked at the double positive stage (18, 20).

To study the selection of a single TCR within IEL, several groups have previously used the H-Y/Db TCR transgenic mouse model (21-23). These investigations have resulted in partially discordant outcomes. In one report, it was concluded that the CD8ααα IEL subset was able to develop exclusively in the male intestine of transgenic mice (21). In a different report, this finding was challenged, as the investigators found CD8ααα IEL development was able to occur not only in male H-Y/Db TCR transgenic mice, but also in female H-Y/Db TCR transgenic mice on either the H-2Db or Db background (22). Most importantly, it was concluded that positive selection of TCR transgene+ CD8αβ+ IEL occurs via an extrathymic pathway in these female transgenic mice (22). The issue of positive selection of TCR αβ+ CD8αβ+ IEL was not addressed in the other investigation (21). Therefore, because of discordant results, or the lack of corroborating results, the possibility of positive selection of TCR transgene+ IEL in these mice remains unresolved.

Despite the use of transgenic TCR models aimed at generating monoclonal T cell populations in the two studies cited above, the rearrangement of endogenous TCR genes, particularly the rearrangement of α genes (21, 22, 24), provides a significant factor complicating the interpretation of results. The H-Y/Db-specific TCR transgene may be particularly leaky, since in the above cited studies, most of the IEL expressed endogenous TCR α chains (20, 25), and up to 30% coexpressed γδ TCRs on cells expressing one or both of the H-Y/Db-specific TCR transgene chains (21). In such a context, it is difficult to rigorously determine that expression of the transgenic TCR leads to the selection of a particular lymphocyte, despite expression of detectable levels of both the transgene-encoded α and β chains by that cell. The degree of endogenous TCR expression could be one reason for the discrepancies described above. Therefore, in this report, we have revisited this TCR transgenic model of T cell selection, except that we have analyzed truly monoclonal H-Y/Dα-specific TCR transgenic mice by crossing these mice onto the recombination activating gene (RAG)-2-deficient background. The data we have obtained demonstrate that a TCR positively selected in the thymus is not necessarily also selected among the IEL. The data also suggest that the CD8 isoform expressed may be an important factor in the different patterns of selection of single TCRs in the thymus and in the small intestine epithelium.

**Materials and Methods**

Mice.

Mice transgenic for a TCR specific for a male-derived peptide presented by Dα (H-Y/Db transgenic mice [13]) were obtained from Dr. Wendy Shores at the National Institutes of Health (Bethesda, MD). RAG-2−/− mice (26) were obtained from Dr. Ellen Rotherenberg, California Institute of Technology (Pasadena, CA). Transporter associated with antigen processing (TAP)-1−/− mice (27) were obtained from Dr. Luc Van Kaer, Vanderbilt University (Nashville, TN). H-Y/Db × RAG-2− mice were from the F2 generation of an intercross between H-Y/Db transgenic mice and RAG-2−/− mice. Identification of H-Y/Db × RAG-2− mice was done by staining peripheral blood with mAbs directed against Vβ8 for the TCR transgene and CD4. Mice with only Vβ8−, CD4+ lymphocytes were selected, because all CD4 T cells are absent in RAG-2−/− mice with (Vβ8+) or without the transgene. Doubly deficient and TCR transgenic H-Y/Db × RAG-2− × TAP-1−/− mice were obtained from the F3 generation of an intercross between H-Y/Db × RAG-2− mice and TAP-1−/− mice. Typing of these mice required two steps: (a) identification of RAG-2− and TAP-1−/− deficiencies was done by staining peripheral blood with mAbs directed against K b and CD4. K b+ (TAP-1−/−) and CD4− (RAG-2−/−) mice were selected, and (b) identification of the H-Y/D b Vβ8 transgene was done by PCR typing. Primers used to identify the transgene β chain spanned the V-D-J region. Sequences of the primers used are 5′, GACATGGAGCTTGATAACGAC and 3′, AACAGCGTTTCCTGCACGTGTTACCC, H-Y/Db × RAG-2− × D4 mice were obtained from the F3 generation of an intercross be-
between H-Y/D\textsuperscript{b} × RAG-2\textsuperscript{−/−} mice and BALB/c mice. Staining of peripheral blood from the progeny with mAbs to K\textsuperscript{d} and K\textsuperscript{b} class I molecules confirmed the MHC haplotype, and negative results from staining with mAbs to CD4 confirmed the RAG-2\textsuperscript{−/−} status. PCR typing was used to identify the TCR transgene as described above. All mice were housed under specific pathogen-free conditions in the UCLA Center for Health Sciences vivarium. Mice were analyzed between 8 and 20 wk of age.

Preparation of lymphocytes. Thymus and LN\'s were excised, and single cell suspensions were prepared by grinding the organs between the frosted ends of two glass slides in complete RPMI 1640 medium with 5% FCS. Isolation of IEL was performed as described previously (28), with minor modifications. In brief, small and large intestines were removed and separated from mesentery and Peyer's patches. They were cut longitudinally and then into 0.5-cm pieces. The pieces were shaken three times for 20 min in M\textsuperscript{g}2\textsuperscript{−/−}, Ca\textsuperscript{2+}-free HBSS (Life Technologies Inc., Gaithersburg, MD) supplemented with 1 mM dithiothreitol (Sigma Chemical Co., St. Louis, MO). Cells were collected from these washes and passed over a discontinuous 40/70% Percoll (Pharmacia Biotech, Piscataway, NJ) gradient at 900 g for 20 min. IEL were then isolated from the Percoll-gradient interface, washed free of Percoll, and counted by light microscopy.

Flow Cytometric Analysis. The following mAbs were used for phenotypic analysis of lymphocytes: PE-labeled or biotinylated V\textsuperscript{8.1} and 8.2 (clone MR5-2), FITC- or PE-labeled or biotinylated anti-CD8\textsuperscript{a} or anti-CD8\textsuperscript{b} clone 53-5.8, PE-labeled or biotinylated anti-K\textsuperscript{d} clone SF1-1.1, FITC- or PE-labeled anti-CD4 clone GK1.5, and FITC-labeled anti-CD3 mAb (2C11; PharMingen). Secondary reagents used included FITC-labeled anti-mouse IgG1 clone A85-1 (Pharmingen) as a secondary antibody against the mouse T3.70 (PharMingen) was used for all biotinylated antibody, and streptavidin coupled to tricolor (Caltag Laboratories, Inc., Gaithersburg, MD) supplemented with 1 mM dithiothreitol (Sigma Chemical Co., St. Louis, MO). mAbs were added at a predetermined concentration that gave optimal staining. Cells were incubated at 4°C for 20 min with the primary mAb, washed twice, and then incubated with secondary reagents for another 20 min at 4°C. Cells were analyzed on a FACScan\textsuperscript{b} 440 flow cytometer (Becton Dickinson, San Jose, CA) at the Jonson Cancer Center Flow Cytometry Core Facility, UCLA (or the La Jolla Institute for Allergy and Immunology). Viable lymphocytes were gated by forward and side angle light scatter parameters, and the number in each subset of IEL was determined by counting lymphocytes that excluded trypan blue in a hemacytometer.

Proliferation and IFN-γ release. T cells from male and female H-Y/D\textsuperscript{b} × RAG-2\textsuperscript{−/−} mice were cultured only the TCR transgenic population. H-Y/D\textsuperscript{b} transgenic mice were crossed onto the RAG-2\textsuperscript{−/−} background in order to completely eliminate rearrangement of endogenous α, β, γ, and δ TCR genes. Expression of the H-Y/D\textsuperscript{b}-specific TCR was examined by two-color flow cytometry analysis with the T3.70 mAb, which recognizes the transgene-encoded V\textsuperscript{α}3 chain, and with the M35-2 mAb, which recognizes V\textsuperscript{β}8.1- and V\textsuperscript{β}8.2-containing TCRs. In contrast to the results obtained from mice not on the RAG-2\textsuperscript{−/−} background, all of the peripheral LN T cells in both male and female TCR transgenic mice expressed only the H-Y/D\textsuperscript{b}-specific TCR (Fig. 1A). Similarly, all of the TCR+ small intestine IEL in male and female TCR transgenic mice expressed only the α and β chains encoded by the TCR transgenes (Fig. 1B). Although the percentage of TCR+ cells obtained from different sources varies, among the TCR+ cells the level of expression of both TCR chains is approximately similar. This is significant, because in small intestine IEL of female H-Y/D\textsuperscript{b} transgenic mice which are not RAG-2\textsuperscript{−/−}, heterogeneous levels of expression of the TCR α transgene are found, most likely reflecting the rearrangement and coexpression of endogenous TCR α genes (22).

![Figure 1](image-url)
Opposite Patterns of T Cell Selection Are Observed in the Thymus and Intestine. As noted above, when D\(^b\) molecules are expressed by thymic epithelial cells, this has been reported to lead to the positive selection of thymocytes expressing the H-Y/D\(^b\) TCR transgene in female mice, and to the negative selection of these same T cells in male mice (22). Consistent with these results, the thymus of male H-Y/D\(^b\) × RAG-2\(^{-}\) transgenic mice contains relatively few cells (Fig. 2), and the double positive population is greatly reduced in number (data not shown). However, opposite to the pattern observed in the thymus, H-Y/D\(^b\) transgenic × RAG-2\(^{-}\) male mice have relatively large numbers of TCR \(^{+}\) cells in IEL obtained from the small intestine, whereas the small intestine epithelium of their female counterparts is severely hypocellular with respect to lymphocytes (Fig. 2). Strikingly, of the few IEL isolated from female H-Y/D\(^b\) × RAG-2\(^{-}\) mice, the majority did not express the TCR transgene (Fig. 1 B). By contrast, in H-Y/D\(^b\) transgenic × RAG-2\(^{-}\) male mice, the proportion of TCR \(^{+}\) cells to small intestine IEL (Fig. 1 B) is equal to or greater than that typical of nontransgenic mice. Male TCR transgenic mice also have increased numbers of lamina propria lymphocytes (LPL) from the small intestine compared with female TCR transgenic mice, the increase averaging 15-fold (n = 3) when age matched mice were analyzed. The difference between male and female TCR transgenic mice was more modest in the LPL than that seen in small intestine IEL, which had \sim 90\ times more TCR \(^{+}\) cells in male transgenic than in female transgenic mice (Table 1). This difference between small intestine IEL and LPL in TCR \(^{+}\) cell numbers may reflect the presence of substantial numbers of circulating T cells in female mice, and the more extensive colonization by these circulating T cells of the lamina propria compared with the epithelium (8). By contrast, in the large intestine, there was little difference in the number of IEL and LPL when male and female TCR transgenic mice were compared, and the total number of lymphocytes in H-Y/D\(^b\) transgenic × RAG-2\(^{-}\) mice tended to be less than the number in nontransgenic mice (data not shown). In summary, the small intestine IEL compartment appeared to be unique in the mucosa with regard to the differentiation and/or expansion of TCR transgene\(^{+}\) lymphocytes in male mice.

Selection of the TCR Transgene Does Not Occur in IEL of H-Y/D\(^b\) Transgenic × RAG-2\(^{-}\) Female Mice. Although H-Y/D\(^b\) transgenic × RAG-2\(^{-}\) female mice contain circulating TCR transgene\(^{+}\) CD8\(^{\text{alpha}}\text{high}\) T cells in the periphery as a result of positive selection in the thymus, surprisingly, relatively few TCR transgene\(^{+}\) CD8\(^{\text{alpha}}\) IEL are present in IEL from the small intestines of these mice (Fig. 3 A, and Table 1). The number of TCR \(^{+}\) CD8\(^{\text{alpha}}\) IEL

### Table 1. Number of IEL within Each Subset Isolated from H-Y/D\(^b\) × RAG-2\(^{-}\) and H-Y/D\(^b\) × RAG-2\(^{-}\) × TAP-1\(^{-}\) Mice

|          | H-Y/D\(^b\) × RAG-2\(^{-}\) | H-Y/D\(^b\) × RAG-2\(^{-}\) × TAP-1\(^{-}\) |
|----------|----------------------------|---------------------------------|
|          | Male                       | Female                          | Male                       | Female                          |
| TCR \(^{+}\) (× 10\(^{3}\)) | 8,220 ± 2,428              | 93 ± 13                         | 11 ± 3.3                   | 7 ± 2.7                         |
| CD8\(^{\text{alpha}}\) (× 10\(^{3}\)) | 6,556 ± 1,933              | 3 ± 0.6                         | 4 ± 1.7                    | 2 ± 1.4                         |
| CD8\(^{\text{beta}}\) (× 10\(^{3}\)) | 540 ± 186                  | 6 ± 2.6                         | ND                        | ND                              |
| DN (× 10\(^{3}\))      | 1,043 ± 299                | 83 ± 11                         | 6 ± 0.5                    | 4 ± 1.1                         |
| TCR \(^{-}\) (× 10\(^{3}\)) | 140 ± 37                   | 257 ± 63                        | 119 ± 67                   | 45 ± 16                         |

Data from male and female H-Y/D\(^b\) transgenic × RAG-2\(^{-}\) mice were obtained from individual 15-wk-old littermates, all analyzed on the same day. H-Y/D\(^b\) transgenic × RAG-2\(^{-}\) × TAP-1\(^{-}\) mice were analyzed as 8-wk-old individuals on the same day. The number of cells in each subset was calculated as described in Materials and Methods.
isolated from the small intestine of female mice averaged only \(6 \times 10^3\) in the set of animals of matched age analyzed in Table 1. This is \(-100\)-fold less than the number reported in female TCR transgenic mice that are not RAG-2\(^{-/-}\) (22), suggesting that in the presence of a functional RAG-2 gene, IEL coexpressing both the \(\alpha\) transgene and an endogenous \(\alpha\) gene had expanded. Female TCR transgenic mice also contain only a very few TCR\(^{+/}\)CD8\(^{+/}\)IEL (Fig. 3A, and Table 1). These findings clearly show that the earlier reported selection of TCR transgenic CD8\(^{+/}\)IEL in small intestine IEL of H-Y/D\(^b\) transgenic female mice that were not on the RAG-2\(^{-/-}\) background was probably due to the coexpression of endogenous TCR \(\alpha\) chains (22).

Small intestine IEL from female TCR transgenic mice are predominantly CD3\(^+\) cells (Fig. 3A, and Table 1), with some TCR\(^+\)DN cells. The exact number of such CD3\(^+\)IEL is difficult to determine because there may be some contaminating nonlymphoid cells that fall into the lymphocyte light scatter gate. However, the majority of the CD3\(^+\)cells from female H-Y/D\(^b\) transgenic \(\times\) RAG-2\(^{-/-}\) mice stain positively for CD69 (data not shown), and it therefore is likely that the majority belong to the lymphoid/hemopoietic lineages.

Although the very small number of TCR transgenic IEL in female mice is consistent with an inefficient positive selection of these cells, it more likely reflects the complete lack of positive selection. A few peripheral T cells or short-term residents of the epithelium could have been obtained in the IEL preparation. Although proportionally few such cells might be present in IEL from normal mice, in the setting of the severely hypocellular IEL compartment of female H-Y/D\(^b\) \(\times\) RAG-2\(^{-/-}\) transgenic mice, such a population could be numerically very significant. To investigate the possible origin of these cells in female mice, IEL were stained with mAbs specific for V\(\beta\)8, CD8\(\beta\), and the \(\alpha_E\) subunit of the mucosa-specific integrin. This mucosa-specific integrin is expressed on nearly all subpopulations of IEL, but it is expressed much less frequently by circulating T cells (29, 30) and is absent from LN T cells of H-Y/D\(^b\) transgenic \(\times\) RAG-2\(^{-/-}\) mice (data not shown). Fig. 3B shows two-color flow cytometry data on cells gated for V\(\beta\)8 expression. In H-Y/D\(^b\) transgenic \(\times\) RAG-2\(^{-/-}\) female mice, most TCR\(^+\)IEL, including the few that are CD8\(\beta\)+ and that might constitute the positively selected subset derived from the thymus, are \(\alpha_E\) negative. The lack of \(\alpha_E\) expression in TCR transgenic IEL from female mice is consistent with these cells being contaminants from blood, or with them being recent emigrants and transient residents of the epithelial compartment. Thus, in the absence of endogenous TCR rearrangement, female mice cannot support the selection and/or expansion of CD8\(\alpha\)\(\beta^+\)IEL expressing the H-Y/D\(^b\)-specific TCR transgene.

Class I Molecules Are Required for the Selection of TCR Transgene IEL. The decreased number and largely CD8\(^{-}\)and/or TCR\(^{-}\)-phenotype of small intestine IEL in H-Y/D\(^b\) transgenic \(\times\) RAG-2\(^{-/-}\) female mice suggested that cells expressing the TCR transgene failed to undergo positive selection. To determine if this is the case, we also analyzed IEL from H-Y/D\(^b\) TCR \(\times\) RAG-2\(^{-/-}\) transgenic mice that lack expression of the D\(^b\) class I molecule, a situation in which positive selection of the TCR transgene should not occur either in male or in female mice. To do this, H-Y/D\(^b\) TCR transgenic \(\times\) RAG-2\(^{-/-}\) mice were bred either to TAP-1\(^{-/-}\) mice, in order to generate TCR transgenic mice doubly deficient for both the expression of classical class I molecules and for endogenous V gene rearrangement, or to the nonselecting H-2\(^b\) background. Consistent with the requisite role of class I molecules in the thymic positive selection of peripheral CD8\(\alpha\)\(\beta^+\) T cells (9), H-Y/D\(^b\) TCR transgenic \(\times\) RAG-2\(^{-/-}\) TAP-1\(^{-/-}\) male and female mice lack TCR transgene\(^+\), CD8\(^+\) thymocytes, although they contain a few TCR\(^+\)DN peripheral T cells (data not shown). Similarly, without the expression of TAP-dependent classical class I molecules, TCR transgenic small intestine IEL are greatly reduced in number in either male (Fig. 4A, and Table 1) or female mice (Fig. 4B, and Table 1). The remaining few TCR\(^+\) cells in mice of both sexes are either DN or CD8\(\alpha\)\(\beta^+\). The very small population of TCR\(^+\)CD8\(\alpha\)\(\beta^+\)IEL, described above, which is present in female D\(^b^+\) H-Y/D\(^b\) transgenic \(\times\) RAG-2\(^{-/-}\) mice, is completely absent in TCR transgenic mice that are also TAP-1-deficient (Table 1). Furthermore, a specific interaction of the TCR with the selecting D\(^b\) molecule is required, as H-Y/D\(^b\) TCR transgenic \(\times\) RAG-2\(^{-/-}\) male mice on the non-
selecting D\(^d\) background also fail to support the development of more than just a very few TCR transgene\(^+\) IEL, similar to the greatly reduced cell numbers observed in IEL from H-Y/D\(^d\) TCR transgenic \(\times\) RAG-2\(^-\) \(\times\) TAP-1\(^-\) mice (Fig. 4 C). Other than the presence of a few CD\(8\)\(^+\) cells in female TCR transgenic D\(^b\) mice, the greatly reduced number and TCR\(^-\) phenotype of the IEL in D\(^b\) female mice is similar to that in mice that do not express selecting class I molecules. However, female D\(^b\) mice do contain increased numbers of TCR transgene\(^+\) DN IEL compared with their D\(^b\) counterparts, but the total cell numbers are quite low in both kinds of animals.

TCR\(^+\) IEL in Male H-Y/D\(^b\) TCR Transgenic \(\times\) RAG-2\(^-\) Mice Have Unusual Patterns of Coreceptor Expression. In male H-Y/D\(^b\) TCR transgenic mice, T cells in the periphery are either TCR transgene\(^+\) and DN, or TCR transgene\(^+\) and CD\(8\)\(^{low}\) or CD\(8\)\(^{low}\) (15, 16). Both of these populations are unresponsive to male antigen (16). By contrast, there are three patterns of coreceptor expression in TCR transgene\(^+\) small intestine IEL of male H-Y/D\(^b\) \(\times\) RAG-2\(^-\) transgenic mice; only one of these, the DN subset, is also found in peripheral lymphoid tissues.

The major cell population in small intestine IEL of TCR transgenic male mice expresses a relatively high amount of CD\(8\alpha\)\(^{high}\) homodimers (Fig. 3 A, and Table 1). The level of CD\(8\alpha\) expression on these cells is higher than that expressed by the major population of peripheral CD\(8\alpha^{low}\) or CD\(8\alpha^{low}\) T cells in these male TCR transgenic mice, and it is nearly comparable to the level seen in female LN or spleen cells (Fig. 5 A). There are no detectable cells with this CD\(8\alpha^{high}\) phenotype in either spleen or LN\(\_\)s of male mice (Fig. 5 A).

Interestingly, in H-Y/D\(^b\) TCR transgenic \(\times\) RAG-2\(^-\) male mice, there are nearly 100-fold more small intestine IEL that are CD\(8\alpha\)\(^+\) than in TCR transgenic females (Table 1). This population was not observed previously in H-Y/D\(^b\) transgenic male mice that are not on the RAG\(^-\)-background (22). However, the majority of these CD\(8\alpha\)\(^+\) cells are unique in that they express high levels of CD\(8\alpha\) (Figs. 3 A and 5 A), comparable to the levels expressed by the CD\(8\alpha^{high}\) cells along with relatively low levels of CD\(8\beta\) (Figs. 3 A and 5 B). Such a combination suggests that CD\(8\alpha^{high}\) IEL express both CD\(8\alpha\) homodimers and CD\(8\alpha\)\(-\)CD\(8\beta\) heterodimers on their cell surface. By contrast to the CD\(8\alpha\)\(^+\) cells in the female small intestine, most of the CD\(8\alpha^{+}\) cells in the male small intestine express \(\alpha\)\(\beta\) integrin (Fig. 3 B), suggesting that they are likely to be long-term residents of the epithelial compartment.

In addition, male H-Y/D\(^b\) \(\times\) RAG-2\(^-\) mice on the D\(^b\) background contain significant numbers of TCR transgene\(^+\) DN IEL in the small intestine. The number of DN IEL is increased substantially compared with the numbers present in small intestine IEL of female mice with the same MHC haplotype, or TCR transgenic mice on either one of the nonselecting MHC haplotypes we tested (Table 1). Finally, in the presence of endogenous TCR gene rearrangements, a substantial frequency of CD\(4^+\) and CD\(4^+\), CD\(8\alpha\)\(^+\) IEL were reported in H-Y/D\(^b\) transgenic mice (21, 22). By

![Figure 4](image)

**Figure 4.** Phenotype of small intestine IEL in TCR transgenic mice that lack expression of the positively selecting class I molecule. (A) IEL from an individual H-Y/D\(^b\) \(\times\) RAG-2\(^-\) \(\times\) TAP-1\(^-\) 25-wk-old male mouse. (B) IEL from an individual H-Y/D\(^b\) \(\times\) RAG-2\(^-\) \(\times\) TAP-1\(^-\) 25-wk-old female mouse. (C) IEL from an individual 18-wk-old D\(^b\) H-Y/D\(^b\) \(\times\) RAG-2\(^-\) male mouse. Isolated IEL were stained with anti-CD\(8\alpha\), anti-CD\(8\beta\), and anti-V\(\beta\)\(\_\)mAbs, and the two-color dot plots for CD\(8\alpha\) and V\(\beta\) expression are displayed. There were no cells positive for CD\(8\beta\) staining in these nonselecting TCR transgenic mice. Data from these mice, which are slightly older than the average, were collected on a different day, and were not used in the compilation of TAP\(-\)I\(^-\) mice in Table 1.

![Figure 5](image)

**Figure 5.** Levels of CD8 coreceptor expression by T cells from H-Y/D\(^b\) \(\times\) RAG-2\(^-\) transgenic mice. (A) CD8\(\alpha\) staining. Cells obtained from the indicated sources from individual mice were stained for V\(\beta\)\(\_\)biotin and CD\(8\beta\)-PE, and the single-color CD8 histogram is shown for the V\(\beta\) cells. (B) CD8\(\beta\) staining, determined with a CD8\(\beta\)-PE mAb, for the same individuals as described above for CD8\(\alpha\). Representative data from one of many experiments. MF, Mean fluorescence.

![Figure 6](image)

**Figure 6.** CD4\(^+\) IEL are absent in H-Y/D\(^b\) \(\times\) RAG-2\(^-\) mice. Total IEL, with the lymphocyte gate set according to light scatter parameters, from individual male and female mice were stained with anti-CD4-FITC and anti-V\(\beta\)\(\_\)biotin, followed by streptavidin-tricolor. Representative data from one of many experiments are presented.
contrast, there is no significant expression of CD4 on IEL in either male or female H-Y/D\textsuperscript{b} transgenic × RAG-2\textsuperscript{-/-} mice (Fig. 6). It is possible that the TCR transgene\textsuperscript{+} T cells in IEL of male mice are derived from T cells circulating in the periphery. Although the CD8\textgreek{a} and CD8\textgreek{a}high\textgreek{b}low phenotypes of the male IEL in transgenic mice are not found in circulating T cells, the migration of T cells to the intestine could cause an upregulation in CD8\textgreek{a} corexpression leading to the generation of CD8\textgreek{a}high\textgreek{b}IEL from DN cells, and CD8\textgreek{a}high\textgreek{b}low IEL from the CD8\textgreek{a}low\textgreek{b}low cells. These hypothetical changes in CD8\textgreek{a} expression are analogous to the behavior of splenic or LN CD8\textsuperscript{+} T cells, which after transfer to SCID mice, can migrate to the intestine and acquire CD8\textgreek{a} homodimers (31–33). However, in contrast to the results derived from the transfer of CD8\textsuperscript{+} T cells, when splenic or LN lymphocytes obtained either from male or female transgenic mice were transferred to either male or female D\textsuperscript{b} SCID mice, we could not detect donor-derived IEL in the intestine of multiple SCID recipients (data not shown). However, this is not due to an intrinsic defect in intestinal homing by CD8\textsuperscript{+} T cells after SCID transfer, as polyclonal CD8\textsuperscript{+} splenic T cells can migrate to the intestine after transfer to a SCID host (33). Therefore, we consider it unlikely that circulating T cells in male transgenic mice give rise to the majority of IEL in these animals.

CD8\textgreek{a}IL in male transgenic mice are not anergic. We analyzed the functional state of the CD8\textgreek{a}IL in male TCR transgenic mice, the major small intestine IEL population in these individuals, to determine if these T lymphocytes had been tolerized as a result of exposure to male antigen. Anergy has been reported to be a major mechanism for self-tolerance induction of TCR γδ IL (34), and the oligoclonal TCR αβ\textgreek{a}CD8\textgreek{a}IL from normal mice have been reported to express self-reactive V\beta chains and to be relatively anergic when stimulated in vitro (35–38). The CD8\textgreek{a}IL were enriched by flow cytometry and were >98% pure (data not shown). Upon CD3 cross-linking, these IEL proliferate to a similar degree as do LN T cells isolated from female H-Y/D\textsuperscript{b} transgenic mice (Fig. 7 A). In addition, in comparison to LN cells from female H-Y/D\textsuperscript{b} transgenic mice, CD8\textgreek{a}IL from male transgenic mice are also able to produce similar levels of IFN-γ in response to CD3 cross-linking (Fig. 7 B). Despite their ability to respond to TCR-mediated signals in vitro and in agreement with previously published results (22), CD8\textgreek{a}IL from male transgenic mice are unable to proliferate, even in the presence of added IL-2, in response to H-Y antigen-bearing splenocytes (data not shown).

**Discussion**

We have used transgenic mice that are monoclonal for the expression of a TCR specific for an H-Y peptide presented by D\textsuperscript{b} to analyze the development and selection of IEL. As noted above, previous analyses of these same TCR transgenic mice, which were not on the RAG-2\textsuperscript{-/-} background, led to conclusions that were not entirely in agreement (21–23). This is probably due to the significant level of rearrangement of endogenous TCR α genes in this TCR transgenic model, and the phenotypic complexity of mouse small intestine IEL subpopulations. Phenotypic complexity is inherent to IEL, as even in truly monoclonal male TCR transgenic mice on the RAG-2\textsuperscript{-/-} background, we found three significant subpopulations of TCR transgene\textsuperscript{+} cells, including DN, CD8\textgreek{a}IL, and CD8\textgreek{a}high\textgreek{b}low IEL. Nevertheless, analysis of lymphocytes in these TCR monoclonal mice has allowed us to resolve inconsistencies in the prior studies and to provide an explanation for the distinct selective processes in IEL. A major finding in this report is that the pattern of selection of a single TCR is different when thymus-selected peripheral lymphocytes are compared with small intestine IEL. Although the H-Y/D\textsuperscript{b} TCR is positively selected in the thymus of female mice, it fails to be selected in their IEL, IEL in female H-Y/D\textsuperscript{b} transgenic mice on the D\textsuperscript{b} selecting background are similar in number and phenotype to the IEL numbers in H-Y/D\textsuperscript{b} transgenic mice that are D\textsuperscript{b} negative. The paucity of IEL in female transgenic mice is also consistent with a possible negative selection of TCR transgene\textsuperscript{+} cells, but it is very difficult to believe this could occur in female mice. It is noteworthy that lymphocytes from the population of circulating TCR transgene\textsuperscript{+} CD8\textgreek{a}β\textgreek{a} T cells in female transgenic mice do not migrate to the IEL compartment. Because of the absence of male antigen in these mice, this observation is consistent with recent findings indicating that antigenic stimulation is likely to be an important determinant fostering the migration of circul-
ing T cells to the intestinal epithelium and their long-term residence in that location (39, 40).

In contrast to the IEL in female transgenic mice, in male mice, IEL expressing the TCR transgene seem to undergo a selection process that could be similar to positive selection. Positive selection is a process in which immature T cells are tested for the expression of TCRs with an appropriate specificity, with cells undergoing programmed cell death unless they express the correct coreceptor and have a low affinity recognition of a self-MHC molecule plus selecting self-peptide in the absence of foreign antigen (1). After maturation, the selected T cells do not respond to doses of the positively selecting peptide(s) equivalent to those in the thymus. Positive selection in IEL of male transgenic mice is consistent with the large numbers of IEL that express the TCR transgene, the majority of which also have relatively high levels of CD8\(^{\alpha\alpha}\) homodimers. The presence of these CD8\(^{\alpha\alpha}\) IEL requires both D\(^{b-}\) and the male antigen, suggesting that these are positively selected cells rather than immature precursors. Consistent with this, in vitro studies indicate that the CD8\(^{\alpha\alpha}\) IEL from male transgenic mice are capable of responding to TCR-mediated signals, although they do not respond to H-Y\(^{+}\) stimulator cells. Therefore, although in some studies the oligoclonal CD8\(^{\alpha\alpha}\) IEL from normal mice were relatively poor responders to TCR-mediated stimulation (35-38), these data clearly demonstrate that some CD8\(^{\alpha\alpha}\) IEL can respond vigorously to such stimulation. Our findings on the TCR responsiveness of CD8\(^{\alpha\alpha}\) IEL are more consistent with those from a recent study of the thymic maturation. Although it has been proposed that the CD4\(^{+}\) CD8\(^{\alpha\alpha}\) double positive IEL are precursors of single positive IEL, CD4\(^{+}\) and CD4\(^{+}\) CD8\(^{\alpha\alpha}\) IEL are nearly completely absent in the H-Y/D\(^{b-}\) RAG-2\(^{-}\) mice. By contrast to IEL, double positive thymocytes can be found in abundance in female D\(^{b+}\) transgenic mice, as well as on thymocytes from mice of both sexes with a nonselecting MHC haplotype (48). The lack of double positive IEL in all mice studied, including those in which positive selection of the TCR transgene cannot occur and those where it might be fostered, suggests that the double positives are not precursors of TCR transgene CD8 single positive IEL. The population of CD8\(^{\alpha\alpha}\) high IEL in male transgenic mice has not been reported previously in studies of these TCR transgenic mice. They are found only in D\(^{b+}\) male TCR transgenic mice, and they are less numerous in these mice than the CD8\(^{\alpha\alpha}\) IEL. Interestingly, CD8\(^{\alpha\alpha}\) high IEL were reported recently in normal mice, and compared with CD8\(^{\alpha\alpha}\) high IEL, these cells were not highly sensitive to the absence of B2 integrins or ICAM-1 (49). There are several possible explanations for the origin of these CD8\(^{\alpha\alpha}\) high IEL, but the implication is that high levels of both CD8\(^{\alpha}\) and CD8\(^{\beta}\) lead to negative selection, whereas the CD8\(^{\alpha}\) high phenotypes permits survival.

All of the results on the selection of IEL that we have presented in this report can be explained by a relatively simple model, outlined in Fig. 8, which incorporates four assumptions. First, the model presumes that some IEL are derived from a separate group of cells than the mainstream CD4\(^{+}\) and CD8\(^{\alpha\beta}\) single positive T cells that mature in
the thymus and that are found in spleen and LNs. It is further presumed that mainstream T cells will only enter the IEL compartment after antigenic stimulation. Second, as discussed above, the model hypothesizes that the T cells in this separate IEL lineage, distinct from the main stem T cells, require a true positive selection process, which may occur either in the thymus or elsewhere. Third, the model assumes that in these TCR transgenic mice, positive selection in female and male mice can be explained by the differential avidity model, with high avidity leading to negative selection, low avidity leading to lack of selection, and intermediate avidity leading to positive selection. Fourth, the model assumes that it is the differences in the amount and type of CD8 coreceptor expressed that are the major determinants of the differences between TCR selection in male and female IEL and thymocytes. Indeed, it has been demonstrated that the affinity of the CD8 coreceptor can affect the avidity threshold for positive selection (50). Furthermore, the results from several experiments indicate an important role for CD8β in increasing the strength of the CD8α-mediated signal, including data showing directly in increasing the strength of the TCR-MHC class I interaction (51, 52), that coexpression of CD8β can increase the reactivity of mature CD8α+ T cells for antigen (53, 54), and that CD8β is required for the positive selection of most CD8α+ T cells (55-57).

Interpreted in the context of this model, it is possible that the expression of the CD8αα coreceptor in IEL leads to a decrease in the overall avidity of the interaction of the H-Y/Dβ TCR with peptide plus class I. In male mice, this would shift the avidity of this TCR from the range in which negative selection occurs to the intermediate range in which positive selection might occur (Fig. 8). Furthermore, CD8αα IEL would be able to persist in great numbers in the male intestine without any signs of autoreactivity, as a result of a decreased avidity of the TCR transgene for H-Y peptide plus Dβ. By a similar reasoning, in female mice, the expression of CD8αα coreceptors in the IEL-specific lineage would decrease the overall avidity of this H-Y/Dβ TCR from the positive selection range to an avidity too low to support positive selection. The female transgenic mice remain virtually devoid of IEL because, as noted above, mainstream thymus-derived CD8αβ T cells in the female transgenic mice are not likely to enter the intestine without antigenic stimulation. This interpretation emphasizing the importance of the CD8β chain is consistent with the analysis of female H-Y/Dβ TCR transgenic mice crossed onto a CD8β-/- background, as in the absence of CD8β, positive selection of the H-Y/Dβ TCR transgene is not supported (55). However, it should be noted that male H-Y/Dβ TCR transgenic mice crossed onto a CD8β-/- background do not positively select large numbers of TCR transgene+, CD8αα+ thymocytes (55). This indicates that besides differences in coreceptor expression, additional factors might contribute to the differential selection of this TCR transgene in IEL. Because the CD8αα IEL express CD3 complexes containing FcεRIγ chains, either as heterodimers with CD3ζ/η, or as homodimers, although such CD3 complexes are not found on CD8αβ IEL (58, 59), FcεRIγ expression could be one such factor important for the differentiation of the CD8αα subpopulation.

In conclusion, the data presented here from female transgenic mice demonstrate that CD8α+ T cells positively selected in the thymus will not necessarily be present in small intestine IEL. We also provide data from male mice strongly suggesting the opposite, namely that a CD8+ TCR well represented among IEL need not be efficiently selected in the thymus. Furthermore, the data demonstrate that CD8αα IEL need not be anergic. The results from both male and female mice give rise to a relatively simple model for the differential selection of thymocytes and IEL, based upon the expression of CD8αα coreceptors by IEL and the expression of CD8αβ coreceptors by thymocytes and peripheral T cells derived from CD8 single positive thymocytes. This differential selection model is consistent with a previous analysis indicating that the predominant clones in polyclonal CD8αα IEL populations are different from the predominant clones in CD8αβ IEL (60). Despite these insights, the specificity and function of the oligonclonal TCR αβ CD8αα+ IEL repertoire in normal mice, as well as the reason for the expression of this form of CD8 in the intestine, remain to be determined.

Figure 8. A model for the opposite pattern of selection of the H-Y/Dβ TCR in thymocytes and IEL. Top, Hypothetical plot of thymocyte numbers versus TCR avidity for self-MHC in a normal mouse. Most cells are ignored and undergo programmed cell death as their TCRs fall into the no selection avidity range (open area), while only a minority fall into the range for positive selection (solid gray area). In the thymus of H-Y/Dβ × RAG-2-/- mice (middle), the avidity range of the monoclonal TCR population that expresses CD8αβ is very narrow, and falls into the positive selection range for females and the high avidity negative selection range for males (diagonal stripes). By contrast, in CD8αα+ IEL (bottom), the overall avidity of the TCR interaction is shifted to the left because of the lower avidity of the homodimeric form of CD8. This places the H-Y/Dβ TCR in positive selection in female mice in the no selection range, and in male mice in the positive selection range.

Normal thymus: polyclonal population
TCR transgenic thymus: CD8αβ population
TCR transgenic IEL: CD8αα population

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