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 D^b (H-Y/D^b × RAG-2^- mice). In contrast to the thymus, the small intestine in female H-Y/D^b × RAG-2^- mice is severely deficient in the number of IEL; TCR transgene$^+$ CD8αα and CD8αβ are virtually absent. This is similar to the number and phenotype of IEL in transgenic mice that do not express the D^b class I molecule, and which therefore fail positive selection. Paradoxically, in male mice, the small intestine contains large numbers of TCR$^+$ 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/D^b 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),[1] 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 CD8$^+$ 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).

[1]*Abbreviations used in this paper:* IEL, intestinal intraepithelial lymphocyte(s); DN, double negative; LPL, lamina propria lymphocyte(s); RAG, recombination activating gene; TAP, transporter associated with antigen processing.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/07/255/11 $2.00
Volume 188, Number 2, July 20, 1998 255–265
http://www.jem.org

Perhaps the best evidence in favor of some type of selection of TCR $\alpha\beta$ IEL comes from the analysis of β2 microglobulin knockout mice (9). In these class I–deficient mice, thymic selection of TCR $\alpha\beta^+$ CD8 single positive T cells is almost completely inhibited. In the intestine of these mice, normal numbers of TCR $\gamma\delta^+$ CD8$\alpha\alpha$ IEL can be found, although the vast majority of CD8$\alpha\alpha$ single positive and CD8$\alpha\beta$ single positive TCR $\alpha\beta^+$ IEL are absent (10–12). These data suggest that all TCR $\alpha\beta^+$ 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 D^b class I molecule (H-Y/D^b mice; reference 13). In male mice that express the D^b class I gene, this TCR is autoreactive. As a result, thymocytes in D^{b+} 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/D^b male TCR transgenic mice do have significant numbers of TCR transgene$^+$ cells in the periphery, but these T lymphocytes are either DN or CD8$\alpha^{low}\beta^{low}$ (15, 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 $\gamma\delta$ lineage cells that have been forced to express an $\alpha\beta$ TCR (17). By contrast, female D^{b+} 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 CD8$\alpha\beta^+$ single positive thymocytes (18–20). Finally, in either male or female H-Y/D^b TCR transgenic mice that do not express D^b, 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/D^b TCR transgenic mouse model (21–23). These investigations have resulted in partially discordant outcomes. In one report, it was concluded that the CD8$\alpha\alpha$ 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 CD8$\alpha\alpha$ IEL development was able to occur not only in male H-Y/D^b TCR transgenic mice, but also in female H-Y/D^b TCR transgenic mice on either the H-2D^b or D^d background (22). Most importantly, it was concluded that positive selection of TCR transgene$^+$ CD8$\alpha\beta^+$ IEL occurs via an extrathymic pathway in these female transgenic mice (22). The issue of positive selection of TCR $\alpha\beta^+$ CD8$\alpha\beta^+$ 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/D^b–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 $\gamma\delta$ TCRs on cells expressing one or both of the H-Y/D^b–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/D^b–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 D^b (H-Y/D^b 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 Rothenberg, 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/D^b × RAG-2$^-$ mice were from the F2 generation of an intercross between H-Y/D^b transgenic mice and RAG-2$^{-/-}$ mice. Identification of H-Y/D^b × 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/D^b × RAG-2$^-$ × TAP-1$^-$ mice were obtained from the F3 generation of an intercross between H-Y/D^b × 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′, GACATTGAGCTGTAATCAGAC and 3′, ACAGCGTTTCTGCACTGTTATCACC. H-Y/D^b × RAG-2$^-$ × D^d mice were obtained from the F3 generation of an intercross be-

tween H-Y/D^b × RAG-2$^-$ mice and BALB/c mice. Staining of peripheral blood from the progeny with mAbs to K^d and K^b class I molecules confirmed the MHC haplotype, and negative results from staining with mAbs to CD4 confirmed the RAG-2$^{-/-}$ 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 LNs 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 Mg^{2+}-free, Ca^{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 Vβ8.1 and 8.2 (clone MR5-2), FITC- or PE-labeled or biotinylated anti-CD8α clone 53-6.7, FITC- or PE-labeled anti-CD8β clone 53-5.8, PE-labeled or biotinylated anti-K^b clone AF6-88.5, biotinylated anti-K^d clone SF1-1.1, FITC- or PE-labeled anti-CD4 clone GK1.5, and FITC-labeled anti-α_E integrin clone M290. All of the above were purchased from PharMingen (San Diego, CA). Hybridoma cells producing the clonotypic antibody (T3.70) that recognizes the transgenic TCR α chain were a gift of Dr. B.J. Fowlkes, National Institutes of Health. Secondary reagents used included FITC-labeled anti–mouse IgG1 clone A85-1 (PharMingen) as a secondary antibody against the mouse T3.70 antibody, and streptavidin coupled to tricolor (Caltag Laboratories, Inc., South San Francisco, CA) was used for all biotinylated antibodies. For staining, cells were suspended in buffer composed of PBS, pH 7.3, with 2% (wt/vol) BSA, 0.02% NaN_3. 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® 440 flow cytometer (Becton Dickinson, San Jose, CA) at the Jonsson 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 multiplying the number of total IEL by the percentage obtained by staining after gating on either total or TCR$^+$ lymphocytes. The total number of IEL obtained was determined by counting lymphocytes that excluded trypan blue in a hemacytometer.

Proliferation and IFN-γ Release Assays. IEL isolated from small intestine of male H-Y/D^b × RAG-2$^-$ transgenic mice were stained with anti-CD8α and anti-CD8β mAbs and then sorted with a FACStar® fluorescence-activated cell sorter (Becton Dickinson) in order to obtain a highly enriched (>98% pure) CD8αα$^+$ IEL population. 10^5 CD8αα$^+$ IEL or total lymphocytes isolated from LN of female H-Y/D^b transgenic mice were cultured in RPMI supplemented with 12 U of recombinant human IL-2 and 5% FCS in plates that had been coated with 5 μg/ml of anti-CD3 mAb (2C11; PharMingen) or anti-γδ TCR clone UC7. Cells were cultured for 48 h, pulsed with 1 μCi [^{3}H]thymidine, and harvested 24 h later. Proliferation levels are expressed as mean ± SEM of culture triplicates. For the IFN-γ release assays, cells were cultured in triplicate in a similar manner except that 100 μl of supernatant was withdrawn after 48 h of culture. The IFN-γ levels in the supernatants were detected using a sandwich ELISA with a coated capture mAb (purified R4-6A2) and a biotinylated detection mAb (XMG1.2) according to the manufacturer's protocol (PharMingen). Cytokine levels are expressed as mean OD ± SEM of culture triplicates.

Results

Generation of a TCR Monoclonal IEL Population. H-Y/D^b transgenic mice were crossed onto the RAG-2$^{-/-}$ background in order to completely eliminate rearrangement of endogenous α, β, γ, and δ TCR genes. Expression of the H-Y/D^b–specific TCR was examined by two-color flow cytometry analysis with the T3.70 mAb, which recognizes the transgene-encoded Vα3 chain, and with the M35-2 mAb, which recognizes Vβ8.1- and Vβ8.2-containing TCRs. In contrast to the results obtained from mice not on the RAG-2–deficient background, all of the peripheral LN T cells in both male and female TCR transgenic mice expressed only the H-Y/D^b–specific TCR (Fig. 1 *A*). 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. 1 *B*). 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^b transgenic mice which are not RAG-2$^{-/-}$, heterogeneous levels of expression of the TCR α transgene are found, most likely reflecting the rearrangement and coexpression of endogenous TCR α genes (22).

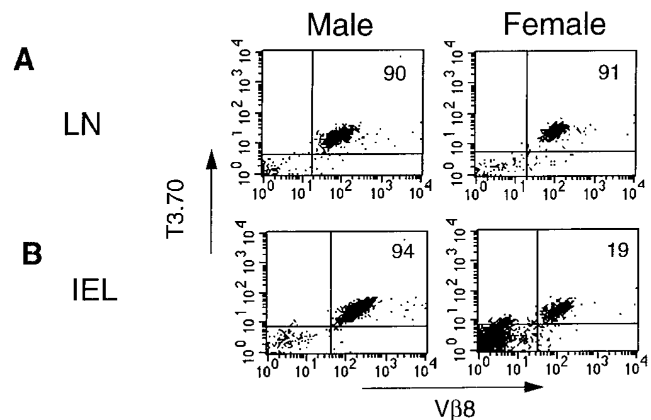

Figure 1. T cells from male and female H-Y/D^b × RAG-2$^-$ mice express only the transgenic TCR. Male and female lymphocytes isolated from LNs (*A*) and IEL (*B*) were stained with the T3.70 mAb followed by anti–mouse IgG1-FITC antibody. Cells were then stained with anti–Vβ8-PE. Data from 15-wk-old mice are shown, representative of all such mice ages 8–20 wk analyzed. The percentage of cells positive for both the TCR transgenes is indicated in the upper right quadrant.

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^+ 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 ~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.

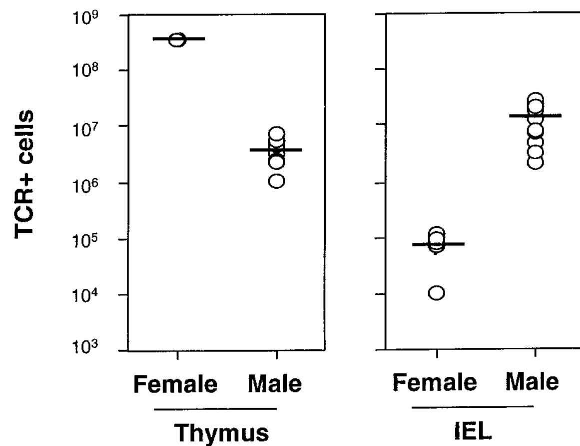

Figure 2. Opposite patterns of T cell selection are observed in the thymus and intestine of H-Y/D^b transgenic × RAG-2^- mice. Thymocytes and small intestine IEL were stained for TCR transgene expression with either Vβ8-PE, or Vβ8-biotin followed by streptavidin-tricolor. The number of TCR transgene$^+$ cells was calculated by multiplying the total number of lymphocytes by the percentage of Vβ8^+ cells as determined by flow cytometry analysis. Each symbol represents the analysis of cells from a single mouse. Values are as follows: female thymus (n = 3), $3.4 \times 10^8 \pm 0.03$; male thymus (n = 7), $4.0 \times 10^6 \pm 0.9$; female small intestine (n = 6) $7.7 \times 10^4 \pm 1.5$; and male small intestine (n = 9), $1.3 \times 10^7 \pm 0.3$.

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$\alpha^{high}\beta^{high}$ T cells in the periphery as a result of positive selection in the thymus, surprisingly, relatively few TCR transgene$^+$ CD8αβ IEL are present in IEL from the small intestines of these mice (Fig. 3 *A*, and Table 1). The number of TCR^+ CD8αβ$^+$ 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
	n = 3	n = 3	n = 2	n = 2
TCR^+ (× 10^3)	8,220 ± 2,428	93 ± 13	11 ± 3.3	7 ± 2.7
CD8αα (× 10^3)	6,556 ± 1,933	3 ± 0.6	4 ± 1.7	2 ± 1.4
CD8αβ (× 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 × 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 α transgene and an endogenous α gene had expanded. Female TCR transgenic mice also contain only a very few TCR^+ $CD8\alpha\alpha^+$ IEL (Fig. 3 *A*, and Table 1). These findings clearly show that the earlier reported selection of TCR $transgene^+$ $CD8\alpha\alpha^+$ 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 α chains (22).

Small intestine IEL from female TCR transgenic mice are predominantly $CD3^-$ cells (Fig. 3 *A*, 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 × 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 $transgene^+$ 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 × 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 Vβ8, CD8β, and the α_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 × RAG-2^- mice (data not shown). Fig. 3 *B* shows two-color flow cytometry data on cells gated for Vβ8 expression. In H-Y/D^b transgenic × 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 α_E negative. This lack of α_E expression in TCR $transgene^+$ 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.

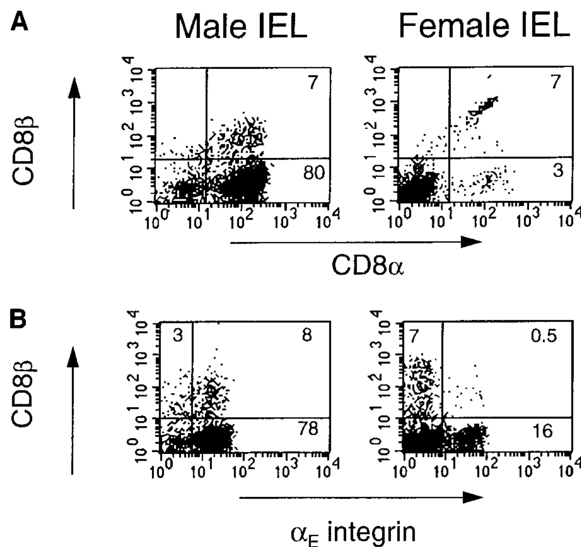

Figure 3. $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ IEL develop more efficiently in male than in female H-Y/D^b transgenic mice. Three-color flow cytometry analysis was used to determine coreceptor expression by TCR^+ cells in small intestine IEL preparations. (*A*) IEL from male transgenic mice express predominantly CD8αα homodimers, whereas the majority of female IEL are DN. The analysis of CD8 coreceptor expression is shown for TCR $transgene^+$ ($V\beta8^+$) cells from individual mice. Representative data from one of many different experiments are shown. (*B*) The TCR^+ IEL that express CD8β in the females do not coexpress the α_E integrin. The analysis of CD8β and α_E expression is shown for $V\beta8^+$ cells from individual mice. Representative data from one of two mice analyzed in this way.

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 × 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 × 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 × 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^d 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 × 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 $transgene^+$ small intestine IEL are greatly reduced in number in either male (Fig. 4 *A*, and Table 1) or female mice (Fig. 4 *B*, and Table 1). The remaining few TCR^+ cells in mice of both sexes are either DN or $CD8\alpha\alpha^+$. The very small population of TCR^+ $CD8\alpha\beta^+$ IEL, described above, which is present in female D^{b+} H-Y/D^b transgenic × 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 × 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^b TCR transgenic × RAG-2$^-$ × TAP-1$^-$ mice (Fig. 4 *C*). Other than the presence of a few CD8β$^+$ 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 × 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 CD8$\alpha^{low}\beta^{low}$ (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 × 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 CD8αα homodimers (Fig. 3 *A*, and Table 1). The level of CD8α expression on these IEL is higher than that expressed by the major population of peripheral CD8$\alpha^{low}\beta^{low}$ 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 CD8$\alpha\alpha^{high}$ phenotype in either spleen or LNs of male mice (Fig. 5 *A*).

Interestingly, in H-Y/D^b TCR transgenic × RAG-2$^-$ male mice, there are nearly 100-fold more small intestine IEL that are CD8αβ$^+$ than in TCR transgenic females (Table 1). This population was not observed previously in

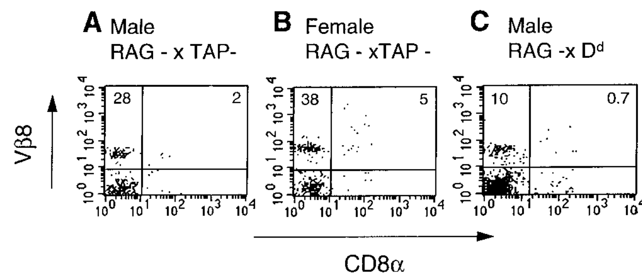

Figure 4. Phenotype of small intestine IEL in TCR transgenic mice that lack expression of the positively selecting class I molecule. (*A*) IEL from an individual H-Y/D^b × RAG-2$^-$ × TAP-1$^-$ 25-wk-old male mouse. (*B*) IEL from an individual H-Y/D^b × RAG-2$^-$ × TAP-1$^-$ 25-wk-old female mouse. (*C*) IEL from an individual 18-wk-old D^{d+} H-Y/D^b × RAG-2$^-$ male mouse. Isolated IEL were stained with anti-CD8α, anti-CD8β, and anti-Vβ8 mAbs, and the two-color dot plots for CD8α and Vβ8 expression are displayed. There were no cells positive for CD8β 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-1$^{-/-}$ mice in Table 1.

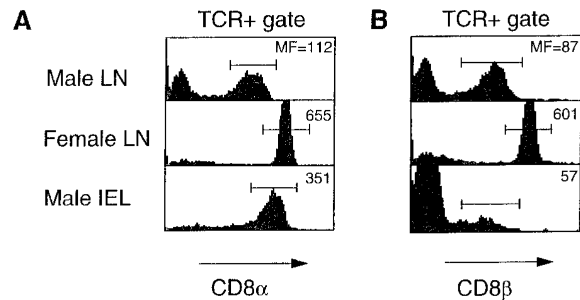

Figure 5. Levels of CD8 coreceptor expression by T cells from H-Y/D^b × RAG-2$^-$ transgenic mice. (*A*) CD8α staining. Cells obtained from the indicated sources from individual mice were stained for Vβ8-biotin and CD8α-PE, and the single-color CD8 histogram is shown for the Vβ8$^+$ cells. (*B*) CD8β staining, determined with a CD8β-PE mAb, for the same individuals as described above for CD8α. Representative data from one of many experiments. MF, Mean fluorescence.

H-Y/D^b transgenic male mice that are not on the RAG-2$^{-/-}$ background (22). However, the majority of these CD8αβ$^+$ cells are unique in that they express high levels of CD8α (Figs. 3 *A* and 5 *A*), comparable to the levels expressed by the CD8αα$^+$ cells, along with relatively low levels of CD8β (Figs. 3 *A* and 5 *B*). Such a combination suggests that CD8$\alpha^{high}\beta^{low}$ IEL express both CD8αα homodimers and CD8αβ heterodimers on their cell surface. By contrast to the CD8αβ$^+$ cells in the female small intestine, most of the CD8αβ$^+$ cells in the male small intestine express α_E 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 × 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 CD4$^+$ and CD4$^+$, CD8αα$^+$ IEL were reported in H-Y/D^b transgenic mice (21, 22). By

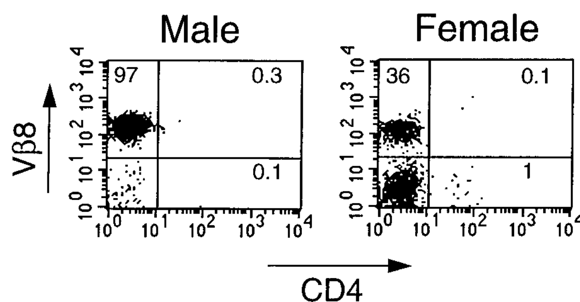

Figure 6. CD4$^+$ IEL are absent in H-Y/D^b × RAG-2$^-$ mice. Total IEL, with the lymphocyte gate set according to light scatter parameters, from individual male and female mice were stained with anti-CD4–FITC and anti–Vβ8-biotin, followed by streptavidin-tricolor. Representative data from one of many experiments are presented.

contrast, there is no significant expression of CD4 on IEL in either male or female H-Y/D^b transgenic × RAG-2^- mice (Fig. 6).

It is possible that the TCR transgene$^+$ T cells in IEL of male mice are derived from T cells circulating in the periphery. Although the CD8$\alpha\alpha^+$ and CD8$\alpha^{high}\beta^{low}$ 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 CD8α coreceptor expression leading to the generation of CD8$\alpha\alpha^+$ IEL from DN cells, and CD8$\alpha^{high}\beta^{low}$ IEL from the CD8$\alpha^{low}\beta^{low}$ cells. These hypothetical changes in CD8α expression are analogous to the behavior of splenic or LN CD4$^+$ T cells, which after transfer to SCID mice, can migrate to the intestine and acquire CD8$\alpha\alpha$ homodimers (31–33). However, in contrast to the results derived from the transfer of CD4$^+$ T cells, when splenic or LN lymphocytes obtained either from male or female transgenic mice were transferred to either male or female D^{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 T cells after SCID transfer, as polyclonal CD8$^+$ 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$\alpha\alpha^+$ IEL in Male Transgenic Mice Are Not Anergic. We analyzed the functional state of the CD8$\alpha\alpha^+$ IEL 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 $\gamma\delta^+$ IEL (34), and the oligoclonal TCR $\alpha\beta^+$ CD8$\alpha\alpha^+$ IEL from normal mice have been reported to express self-reactive Vβ chains and to be relatively anergic when stimulated in vitro (35–38). The CD8$\alpha\alpha^+$ IEL 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^b transgenic mice (Fig. 7 *A*). In addition, in comparison to LN cells from female H-Y/D^b transgenic mice, CD8$\alpha\alpha^+$ IEL 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$\alpha\alpha^+$ IEL 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^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-$2^{-/-}$ 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-$2^{-/-}$ background, we found three significant subpopulations of TCR transgene$^+$ cells, including DN, CD8$\alpha\alpha^+$, and CD8$\alpha^{high}\beta^{low}$ 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^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^b transgenic mice on the D^{b+} selecting background are similar in number and phenotype to the IEL numbers in H-Y/D^b transgenic mice that are D^b negative. The paucity of IEL in female transgenic mice is also consistent with a possible negative selection of TCR transgene$^+$ 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$^+$ CD8$\alpha\beta^+$ 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 circulat-

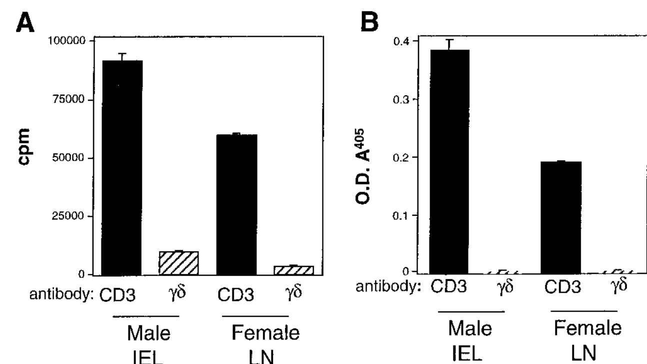

Figure 7. The CD8$\alpha\alpha$ IEL subset from male H-Y/D^b × RAG-2^- mice can respond to CD3 cross-linking. CD8$\alpha\alpha$ IEL were highly purified by cell sorting as described in Materials and Methods from a pool of two mice. Small intestine IEL from male mice, or total lymphocytes isolated from LNs of female H-Y/D^b transgenic mice, were cultured in plates that had been coated either with 5 μg/ml of anti-CD3 mAb (2C11) or with a control hamster mAb (anti-$\gamma\delta$ clone UC7) as described in Materials and Methods. Data are from triplicate wells in each case, with the SE of the mean indicated. (*A*) Proliferation assay. Cells were cultured for 48 h, pulsed with [^{3}H]thymidine, and harvested 24 h later. (*B*) IFN-γ release. Cells were cultured for 48 h as described above, and then supernatants were assayed for cytokine release by ELISA as detailed in Materials and Methods.

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 large numbers of TCR transgene$^+$ 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 2C TCR transgenic model, which demonstrated that at least some CD8$\alpha\alpha^+$ IEL are able to proliferate and secrete cytokines in response to clonotypic antibodies (41).

However, it remains possible that extrathymic $\alpha\beta$ T cells, including IEL, do not require a true positive selection process, as has been proposed for differentiating $\gamma\delta$ T cells (42). According to this view, maturing IEL might be allowed to survive if they expressed any $\alpha\beta$ TCR, and these cells could then be subjected to antigen-driven expansion as mature T cells. This model would account for the near complete absence of TCR transgene$^+$ cells in female mice, as well as for their expansion in D^{b+} male mice. However, it should be noted that we and others could not detect reactivity by TCR transgene$^+$ CD8$\alpha\alpha^+$ IEL for male stimulator cells in vitro, and the male transgenic mice do not show any histologic evidence for small intestine inflammation (data not shown), despite the presence of very large numbers of potentially autoreactive T cells in their intestine. Although these findings argue collectively against the antigen-driven expansion model described above, it is formally possible that a very weak antigenic stimulation in vivo causes the gradual accumulation of large numbers of TCR transgene$^+$ small intestine IEL in the absence of an overt inflammatory condition. Therefore, other than in the discussion of models, we have used the word "selection," which encompasses both positive selection as well as antigen-driven expansion, to describe the presence of large numbers of functional TCR$^+$ CD8$^+$ IEL in male transgenic mice.

The experiments in this report do not determine if the TCR transgene$^+$ IEL in male mice arise via an extrathymic route, although we favor this possibility. In fact, years of investigation on the origin of IEL have not fully resolved this issue (43), which is complicated by the likely thymic influence on the extrathymic differentiation pathway (44, 45). However, the CD8$\alpha\alpha^+$ phenotype of the major population of IEL in male mice is consistent with a possible extrathymic origin. The thymus-independent origin of the CD8$\alpha\alpha$ IEL subpopulation is supported by studies in which adult thymectomized mice were given a source of stem cells, including two more recent studies that avoided irradiation of the host by using either RAG-2$^{-/-}$ recipients or *W/W^v* recipients with reduced c-Kit receptor function (46, 47). Furthermore, we could not generate these CD8$\alpha\alpha^+$ IEL by transfer of circulating T cells from TCR transgenic mice into SCID recipients. Therefore, we consider it unlikely that the TCR and CD8$^+$ IEL in male transgenic mice arise from circulating T cells that have entered the intestine, although it remains possible that the CD8$\alpha\alpha^+$ and CD8$\alpha^{high}\beta^{low}$ cells in these mice have a thymic origin, and that these cells are transported directly to the intestine after thymic maturation.

Analysis of the expression of the CD4 and CD8 coreceptors in TCR monoclonal mice gives further insight into IEL differentiation. 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 $\times$ 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$\alpha^{high}\beta^{low}$ 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$\alpha^{high}\beta^{low}$ IEL were reported recently in normal mice, and compared with CD8$\alpha^{high}\beta^{high}$ IEL, these cells were not highly sensitive to the absence of β2 integrins or ICAM-1 (49). There are several possible explanations for the origin of these CD8$\alpha^{high}\beta^{low}$ cells, but the implication is that high levels of both CD8α and CD8β lead to negative selection, whereas the CD8$\alpha^{high}\beta^{low}$ phenotype 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

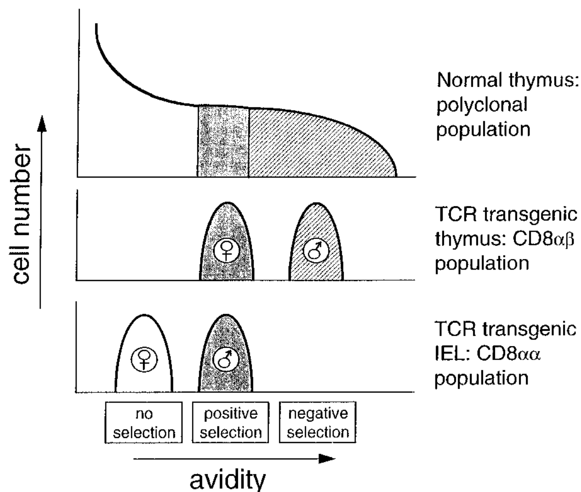

Figure 8. A model for the opposite pattern of selection of the H-Y/D^b TCR in thymocytes and IEL. *Top*, Hypothetical plot of thymocyte number versus TCR avidity for self-MHC in a normal mouse. Most cells are ignored and undergo programmed cell death as their TCRs fall into the no selection avidity range (*open area*), while only a minority fall into the range for positive selection (*solid gray area*). In the thymus of H-Y/D^b × 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/D^b TCR in female mice in the no selection range, and in male mice in the positive selection range.

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 mainstream 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 the 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 that it can increase the avidity 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 CD8$^+$ 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/D^b 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 D^b. 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/D^b 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/D^b TCR transgenic mice crossed onto a CD8β$^{-/-}$ background, as in the absence of CD8β, positive selection of the H-Y/D^b TCR transgene is not supported (55). However, it should be noted that male H-Y/D^b 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 CD8$^+$ 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 oligoclonal 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.

We thank the Jonsson Cancer Center Flow Cytometry Core Facility for help with cell sorting, Drs. Richard Aranda and Chetan Panwala for helpful discussions, and Drs. Laurent Gapin and Tesu Lin for review of the manuscript. This is manuscript 225 from the La Jolla Institute for Allergy and Immunology.

This work was supported by National Institutes of Health grant PO1 DK 46763 (to M. Kronenberg), National Institutes of Health Medical Scientist Training Program grant GM 08042, and by grants from the Crohn's and Colitis Foundation of America (to B.C. Sydora and G. Yakoub).

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Received for publication 2 March 1998 and in revised form 15 April 1998.

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