

The timing of TCR α expression critically influences T cell development and selection

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Sequential rearrangement of the T cell receptor for antigen (TCR) β and α chains is a hallmark of thymocyte development. This temporal control is lost in TCR transgenics because the α chain is expressed prematurely at the CD4⁻CD8⁻ double negative (DN) stage. To test the importance of this, we expressed the HY α chain at the physiological CD4⁺CD8⁺ double positive (DP) stage. The reduced DP and increased DN cellularity typically seen in TCR transgenics was not observed when the α chain was expressed at the appropriate stage. Surprisingly, antigen-driven selection events were also altered. In male mice, thymocyte deletion now occurred at the single positive or medullary stage. In addition, no expansion of CD8 $\alpha\alpha$ intestinal intraepithelial lymphocytes (IELs) was observed, despite the fact that HY transgenics have been used to model IEL development. Collectively, these data establish the importance of proper timing of TCR expression in thymic development and selection and emphasize the need to use models that most accurately reflect the physiologic process.

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Abbreviations used: CFSE, carboxyfluorescein diacetate succinimidyl ester; DN, double negative; DP, double positive; HP, homeostatic proliferation; IEL, intraepithelial lymphocyte; SP, single positive.

During T cell development, progenitors seed the thymus from the blood and begin a sequential program of maturation marked by changes in cell surface phenotype (for review see reference 1). The earliest progenitors lack expression of the CD4 and CD8 coreceptors and are therefore termed double negative (DN). DN thymocytes can be further subdivided based on the expression of CD44 and CD25 into DN1–DN4 stages. The DN3 stage of development is where thymocytes must pass their first test of fitness, β -selection. If TCR β gene rearrangement is successful, the polypeptide chain pairs with an invariant pre-TCR α and signals the thymocyte to undergo further differentiation. The events of β -selection include survival, proliferation, differentiation, and allelic exclusion at the TCR β locus. At this point, the progenitor also up-regulates CD4 and CD8 to become double positive (DP) and initiates rearrangement at the TCR α gene locus. If a productive TCR α gene rearrangement occurs, the α chain can pair with the already expressed TCR β chain and be expressed on the surface. All subsequent selection events are based on the antigen binding site formed by this heterodimer. It is currently held that thymocytes bearing a TCR with high affinity for self-MHC–peptide complexes

are deleted from the repertoire, whereas those with a low affinity are positively selected. If the TCR has negligible affinity for self-MHC, the thymocyte undergoes death by neglect. A key feature of these selective events and a hallmark of T cell development is the ordered and sequential rearrangement and expression of the TCR β and TCR α chains, respectively. This highly regulated process ensures the production of a clonally expressed repertoire with a minimum of energy expenditure.

Despite the normally sequential expression of TCR β and α in normal mice in most TCR transgenic model systems both TCR α and β chains are expressed early in development. This early expression of TCR α has been suggested to affect β -selection even in the presence of the pre-TCR because TCR β has a higher affinity for TCR α than it does for pre-T α (2). Although the $\alpha\beta$ TCR heterodimer can mediate β -selection if expressed at the DN stage, it is highly inefficient (3). In addition, early expression may affect $\alpha\beta/\gamma\delta$ lineage commitment, resulting in a large population of mature DN TCR⁺ cells both in the thymus and the periphery (4–7). In the thymus, these cells are thought to represent a terminally differentiated population without the ability to seed the DP compartment (6). In the periphery, DN TCR⁺ cells display properties consistent with a $\gamma\delta$ -lineage cell (7). These lineage-

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misdirected cells are not observed in wild-type mice or mice that express a transgenic TCR β chain. Therefore, it has been suggested that early TCR α expression results in the previously mentioned abnormalities.

To directly test this, we sought to create a model in which TCR α expression would be delayed until the DP stage (as is the case in normal animals). Using a Cre/lox-based conditional strategy, we expressed the HY TCR α at the DP stage of development (HY^{cd4} mice). In this model, the defects in β -selection and lineage commitment observed in conventional HY transgenics were corrected. Other developmental characteristics, including positive selection and lymphopenia-induced proliferation, were unchanged. Interestingly, in HY^{cd4} male mice, clonal deletion did not occur until the single positive (SP) stage, despite antigen encounter at the DP stage. In addition, the prominent expansion of CD8 α^+ intraepithelial lymphocytes (IELs) observed in conventional HY male mice was not apparent in HY^{cd4} mice. These observations suggest that certain properties of conventional TCR transgenics are nonphysiologic and demonstrate that T cell selection is critically influenced by the appropriate timing of TCR α expression.

RESULTS

Conditional expression of HY TCR

To conditionally express the HY TCR α chain at the DP stage, we used the CD4 promoter-enhancer. However, because this promoter is not active in mature CD8 T cells, we combined it with a Cre/lox-based strategy. The HY TCR α was cloned immediately downstream of a transcriptional and translation “STOP” cassette flanked by loxP sites. After removal of the STOP cassette by Cre-mediated recombination, a constitutively active promoter, pCAGGS, drives transcription of the HY TCR α (Fig. 1 A). By this strategy, expression of HY TCR α should be completely dependent on Cre expression from the CD4 promoter but not extinguished in CD8 T cells. Transient cotransfection of the conditional HY TCR α construct and a Cre expression vector into the TCR⁻BW5147 58^{-/-} hybridoma cell line indicated that expression of HY TCR α was dependent on the presence of Cre (unpublished data). Previous data indicated that in CD4-Cre mice, Cre-mediated recombination was initiated at the late DN3 stage and completed at the DN4 stage (8, 9). Therefore, by using CD4-driven Cre, we predicted that HY TCR α would not be expressed until after β -selection, which fairly accurately mimics when endogenous TCR α rearrangement and expression occur. Conditional HY TCR α mice were created, crossed to CD4-Cre mice, and bred to HY TCR β transgenic mice. Mice bearing all three transgenes are referred to as HY^{cd4} mice. Although the construct encodes a bicistronic message containing GFP, no GFP expression was observed (unpublished data), similar to a conditional construct reported previously (10). HY^{cd4} mice showed no gross abnormalities in the CD4/CD8 thymic profile (Fig. 1 B, top). The CD44/CD25 profile of DN thymocytes from HY^{cd4} mice

was similar to HY β -only mice, which display a slight acceleration through the DN subsets (Fig. 1 B, bottom). To determine when the HY $\alpha\beta$ TCR is expressed at the cell surface of thymocytes, thymic subpopulations were electronically gated and examined for HY TCR α expression using the T3.70 antibody (11). T3.70 staining was observed in the DP and CD8SP subsets and to a lesser extent in CD4SP cells (Fig. 1 C). As predicted, no T3.70 expression was observed in the bulk DN compartment (Fig. 1 C) or in any DN subcompartment

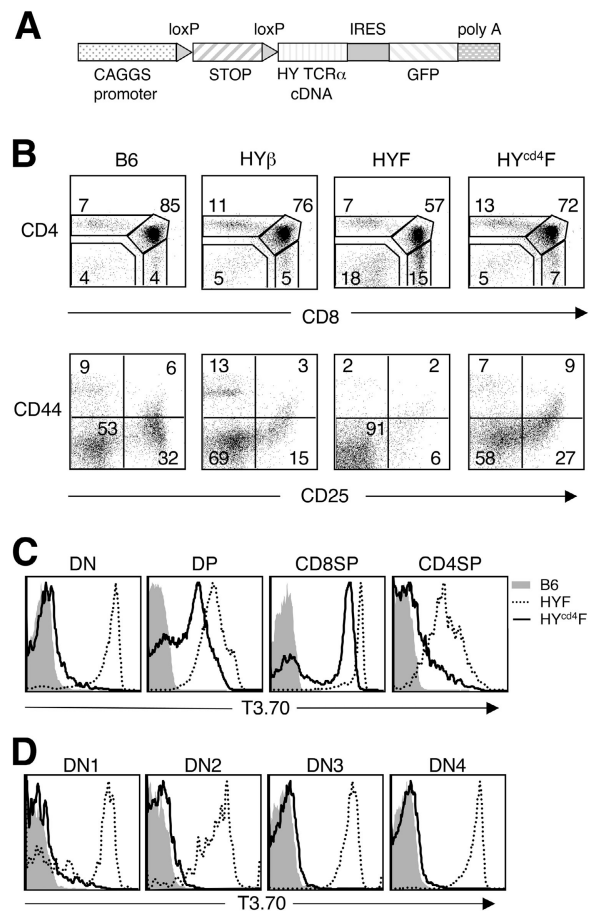


Figure 1. Conditional expression of the HY TCR beginning at the DP stage in HY^{cd4} mice. (A) Schematic representation of the conditional HY TCR α transgene. (B) Thymocytes from the indicated mice were stained with either anti-CD4 and anti-CD8 (top) or with anti-CD4, -CD8, -B220, and -NK1.1 to exclude lineage-positive cells and with anti-CD44 and anti-CD25 (bottom). Cells were analyzed by flow cytometry. Numbers represent the percentage of cells in each gate. (C) Thymocytes from B6 (shaded region), HY female (dotted line), and HY^{cd4} female (continuous line) were stained with anti-CD4, anti-CD8, and T3.70 and analyzed by flow cytometry. Individual subpopulations were gated, and T3.70 expression is depicted. (D) Thymocytes from B6 (shaded region), HY female (dotted line), and HY^{cd4} female (continuous line) were stained with anti-CD4, -CD8, -B220, and -NK1.1 to exclude lineage-positive cells and with anti-CD44, anti-CD25, and T3.70 to allow electronic gating of the DN subpopulations. T3.70 expression is depicted for the individual DN subpopulations. The y axes in C and D represent the percentage of maximum expression.

ment (Fig. 1 D). This is in contrast to conventional HY TCR transgenic mice that express the TCR α chain as early as the DN2 stage (Fig. 1 D). Although there was expression of conventional HY TCR in DN1 phenotype cells, this subset is heterogeneous (12), and it is unclear if canonical DN1 progenitors express it. We also observed a slightly reduced level of surface TCR in all subsets of HY^{cd4} mice when compared with conventional HY mice (Fig. 1 C), which could be caused by the strength of the ubiquitous promoter chosen. When comparing with wild-type thymocytes, the HY^{cd4} DP TCR levels were closer: HY DPs express 4–5-fold more receptor, whereas HY^{cd4} DPs express only 2.5–3-fold more receptor (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20050359/DC1>). In the CD8SP compartment, HY^{cd4} thymocytes expressed approximately half the level of TCR as the wild type, whereas HY thymocytes express similar levels (Fig. S1). Overall, as predicted, the HY TCR complex was expressed beginning at the DP stage and continued through the CD8SP stage in HY^{cd4} mice.

Corrected β -selection and lineage commitment in HY^{cd4} mice

Early expression of a mature TCR was proposed to result in impaired β -selection and altered lineage commitment in HY and other TCR transgenics (5, 6, 13). Therefore, we wished to determine whether delaying TCR α expression until the DP stage corrected these events. Because little or no cell division occurs in the DP and SP compartment, the total number of thymocytes present in the thymus largely reflects the proliferative burst that accompanies β -selection. The thymic cellularity of HY^{cd4} female mice was equivalent to that of B6 mice, whereas conventional HY female mice showed a two- to threefold reduction in total thymocyte numbers (Fig. 2 A, left). Additionally, β -selection mediated by an $\alpha\beta$ TCR, as opposed to a pre-TCR, resulted in an increase in annexin V⁺ cells in the DN4 compartment (3). By expressing HY TCR α at the DP stage, we found a similar percentage of annexin V⁺ DN4 cells as in wild-type mice, whereas the early TCR α expression observed in conventional HY mice resulted in a 5–10-fold increase in annexin V⁺ DN4 cells (Fig. 2 A, right). These results provide further evidence that the early expression of an $\alpha\beta$ heterodimer impairs β -selection.

It has additionally been suggested that $\alpha\beta/\gamma\delta$ lineage commitment is disrupted by early expression of TCR α , resulting in mature $\alpha\beta$ TCR⁺ DN cells in both the thymus and periphery (5, 7, 13). In examining the thymic DN compartment, conventional HY female mice had a large number of resident $\alpha\beta$ TCR⁺ cells, whereas this population was substantially reduced in wild-type and HY^{cd4} mice (Fig. 2 B, left). As cells mature, the expression of CD24 (heat-stable antigen) decreases with the most mature thymic cells being CD24^{lo}. No difference in CD24 levels between wild-type and HY^{cd4} mice was observed (Fig. 2 B, right). However, the vast majority of conventional HY mice had lower levels of CD24, suggesting that they are more mature and may

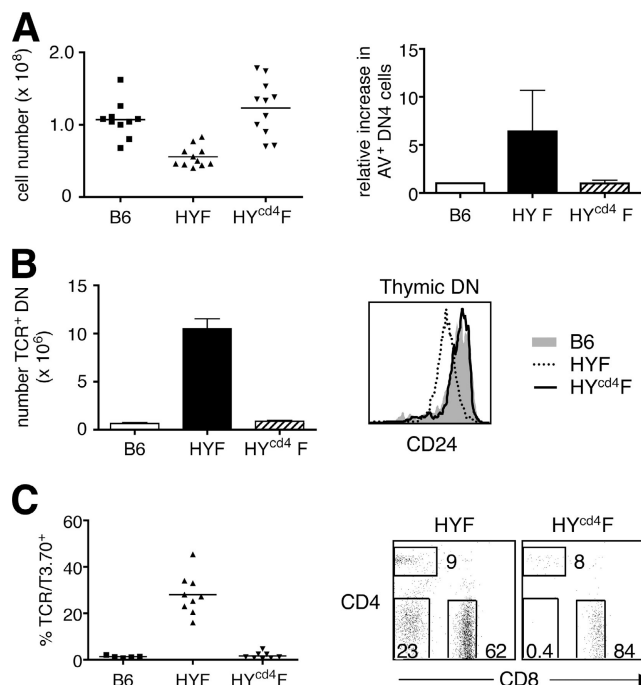


Figure 2. β -selection and lineage misdirection is corrected in HY^{cd4} mice. (A) Total thymocyte numbers from B6 ($107 \pm 25 \times 10^6$), HY female ($56 \pm 14 \times 10^6$), and HY^{cd4} female ($123 \pm 38 \times 10^6$) are shown (left). Thymocytes were stained with anti-CD4, -CD8, -B220, and -NK1.1 to exclude lineage-positive cells. The horizontal line represents the mean. Anti-CD44, anti-CD25, and annexin V staining were performed to identify apoptotic cells in the DN4 compartment (right). Data are expressed as a ratio of the percentage of annexin V⁺ DN4 cells compared with B6. Error bar represents SD. (B) DN thymocytes were analyzed for TCR β or CD24 expression. The number of DN TCR⁺ cells from B6 ($6.7 \pm 1.8 \times 10^6$), HY female ($11 \pm 1.7 \times 10^6$), and HY^{cd4} female ($9.0 \pm 1.8 \times 10^6$) (left; error bar represents SD) or the expression level of CD24 on B6 (shaded region), HY female (dotted line), and HY^{cd4} female (continuous line) (right; y axis represents the percentage of maximum expression) was determined. (C) LN cells from the indicated mice were stained with anti-CD4, anti-CD8, H57-597 (anti-TCR β), and T3.70. The percentage of CD4⁺CD8⁻ cells expressing T3.70 (HY female and HY^{cd4} female) or TCR β (B6) was assessed (left). Additionally, the CD4/CD8 profile of T3.70⁺ LN cells is shown for HY female and HY^{cd4} female mice (right). The numbers within the FACS plots represent the percentage of cells within that gate.

comprise a population of lineage-misdirected cells (so-called “ $\gamma\delta$ -wannabes”; Fig. 2 B, right). Mature $\alpha\beta$ TCR⁺ DN cells are also prominent in the periphery of conventional HY mice and, again, were absent in HY^{cd4} mice (Fig. 2 C). Collectively, these data suggest that delaying TCR α expression in a transgenic mouse allows β -selection and early lineage commitment to occur normally.

Timing of TCR α does not appear to affect positive selection or homeostatic proliferation (HP)

It was unclear at this point whether or not early expression of TCR α would affect positive selection events. By first gat-

ing on T3.70⁺ cells and then examining the CD4/CD8 distribution, we observed a prominent CD8SP population, indicating positive selection in HY^{cd4} female mice, as in the conventional model (Fig. 3 A). The population of T3.70⁺ CD8SPs was somewhat lower in both percentage and number in HY^{cd4} mice compared with conventional HY mice, suggesting a reduced efficiency. The reason for this difference is currently unclear; however, it could be caused by endogenous TCR gene rearrangements, which are likely to be more prevalent when the transgene comes on later. Indeed, the phenotype of HY^{cd4}TCR $\alpha^{-/-}$ mice (see Fig. 5 C) supports this. Positive selection is known to induce changes in gene expression that can be monitored by changes in the cell

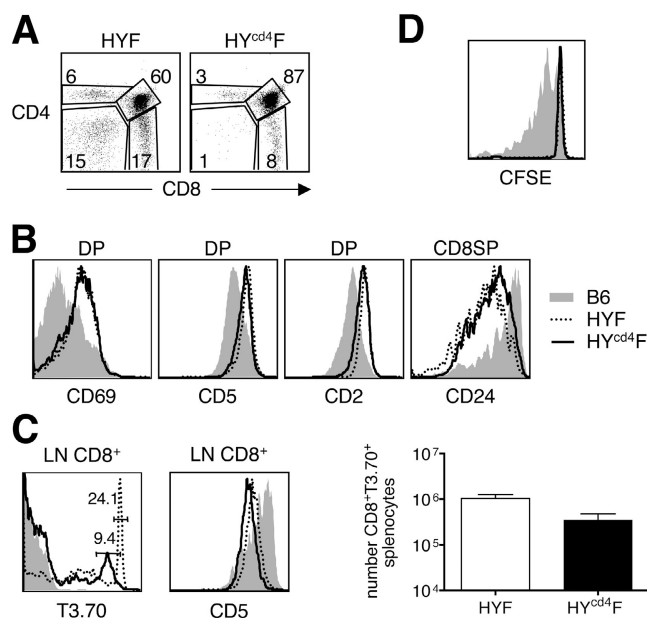


Figure 3. Positive selection and HP are not affected by early TCR α expression. (A) Thymocytes from HY female and HY^{cd4} female mice were stained with anti-CD4, anti-CD8, and T3.70 and analyzed by flow cytometry. The CD4/CD8 profile of T3.70⁺ cells is shown. The numbers within the FACS plots represent the percentage of cells within that gate. (B) T3.70⁺ DP or CD8SP thymocytes from B6 (shaded region), HY female (dotted line), and HY^{cd4} female (continuous line) were assessed for levels of CD69, CD5, CD2, and CD24. (C) LN cells from B6 (shaded region), HY female (dotted line), and HY^{cd4} female (continuous line) mice were stained with anti-CD4, anti-CD8, T3.70, and CD5. The level of T3.70 on gated CD8 cells (left) and CD5 on gated CD8⁺ T3.70⁺ cells (middle) is shown. The numbers in the left panel indicate the percentage of T3.70^{hi} CD8 cells. The right panel shows the absolute number of CD8⁺ T3.70^{hi} splenocytes from HY female ($1.0 \pm 0.6 \times 10^6$) and HY^{cd4} female ($3.5 \pm 3 \times 10^5$) mice. Error bars represent SD. (D) Bulk thymocytes from HY^{cd4} female (continuous line) or a mixture of B6 (shaded region) and HY female (dotted line) thymocytes were CFSE labeled and adoptively transferred into sublethally irradiated B6.SJL mice. Recipient mice were harvested 9 d after transfer and CD45.2⁺ CD8⁺ T3.70⁺ cells were analyzed for CFSE dilution. CD45.2⁺ CD8⁺ T3.70⁻ cells were used as an internal control. Representative data from one recipient mouse out of three receiving either HY^{cd4} female or B6/HY female cells are shown. The y axes in B–D represent the percentage of maximum expression.

surface phenotype of DP thymocytes, including up-regulation of CD69, CD5, and CD2, and down-regulation of heat-stable antigen. Compared with B6 DP thymocytes, both conventional HY and HY^{cd4} DP thymocytes up-regulated CD69, CD5, CD2 (Fig. 3 B), and CD53 (not depicted) to a similar extent. In the CD8SP compartment, both showed a reduction in CD24, similar to wild-type controls (Fig. 3 B). Examination of the LNs from conventional HY and HY^{cd4} mice indicated the presence of CD8⁺ T3.70⁺ cells (Fig. 3 C). Although there was a slight difference in the percentage of CD8⁺ T3.70⁺ cells in the LNs and in the number of T3.70⁺ CD8⁺ cells in the spleens of conventional HY and HY^{cd4} mice (Fig. 3 C), this difference is likely attributable to a reduced number of T3.70⁺ CD8s exiting the thymus in HY^{cd4} mice because not all progenitors express the HY TCR α chain (Fig. 1 C). Additionally, the level of CD5 expressed by T3.70⁺ LN CD8 cells was equivalent in HY and HY^{cd4} mice, suggesting that the “tuning” of the TCR signal is similar in both strains of mice (Fig. 3 C).

To survive in the periphery, CD8 cells must receive a tonic signal through the TCR (14). The ability to undergo HP has an effect on the number of cells present in the periphery and has been suggested to correlate with TCR affinity and, thus, the level of CD5 expressed on peripheral CD8 T cells (15). HY transgenic CD8 T cells have a notable inefficiency in this homeostasis, particularly in lymphopenic recipients (16), that could either be caused by an inherently low affinity for self-antigen or by some other nonphysiologic aspect of the conventional HY transgenic model, such as early expression of the TCR α chain in development. Thus, we evaluated the capacity of CD8SP thymocytes to undergo HP in lymphopenic recipients. CD8SP thymocytes were used in this assay because this cell population is phenotypically equivalent between the two mouse strains, and the cells had not yet undergone any competition with other peripheral T cells. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled bulk thymocytes from HY^{cd4} or conventional HY thymocytes mixed with B6 thymocytes were injected into sublethally irradiated congenic recipients and parked for 9 d. B6 thymocytes were mixed with conventional HY thymocytes to provide a reference population for determining the extent of HP because the CD8SP pool of thymocytes from HY^{cd4} mice contain T3.70⁻ cells with a polyclonal repertoire. Neither the T3.70⁺ CD8 cells from conventional HY mice nor those from HY^{cd4} mice underwent HP (Fig. 3 D), as was previously reported (17). Control polyclonal cells did undergo division as measured by CFSE dilution. Overall, these data suggest that positive selection and HP are not affected by early expression of TCR α .

Antigen sensitivity of DP and CD8SP thymocytes are identical in conventional HY and HY^{cd4} mice

Because of the reduction in TCR levels, we wanted to examine the antigen sensitivity of T3.70⁺ CD8SP cells from HY^{cd4} mice. Total thymocytes were cultured at a 1:1 ratio

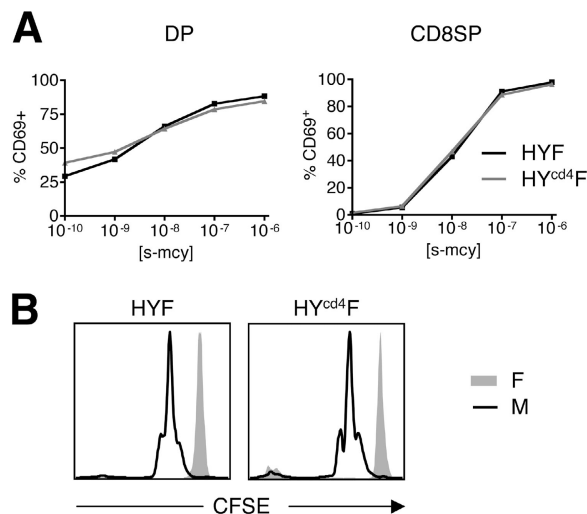


Figure 4. In vitro and in vivo responsiveness of HY and HY^{cd4} CD8 thymocytes is equivalent. (A) Bulk thymocytes from HY female (black line) or HY^{cd4} female (gray line) mice were mixed at a 1:1 ratio with female B6 splenocytes and increasing concentrations of agonist smcy peptide were added to the culture and incubated for 20 h. Cells were harvested and stained with anti-CD4, anti-CD8, T3.70, and anti-CD69 and analyzed by flow cytometry. CD4⁺CD8⁺T3.70^{hi} (left) and CD4⁻CD8⁺T3.70⁺ (right) cells were gated, and the induction of CD69 was measured. Data are expressed as a percentage of cells that maximally up-regulated CD69. (B) HY female (left) or HY^{cd4} female (right) bulk thymocytes were CFSE labeled and injected i.v. into intact female (shaded region) or male (continuous line) recipients. 48 h after injection, spleens were harvested, and the CFSE dilution of CD8⁺ T3.70⁺ cells was measured. The y axis represents the percentage of maximum expression.

with B6 splenocytes and increasing doses of agonist peptide, smcy, in vitro for 20 h, and the response of T3.70⁺ CD8SP cells was evaluated by CD69 induction. Both T3.70⁺ DP and CD8SP thymocytes from conventional HY and HY^{cd4} mice responded equivalently to agonist peptide at all concentrations (Fig. 4 A). Additionally, the T3.70⁺ CD8SP divided similarly in response to 100 nM smcy as measured by CFSE dilution (unpublished data). To compare the functional potential of HY and HY^{cd4} cells in vivo, bulk thymocytes were CFSE labeled and injected i.v. into intact male or female recipients. 2 d after transfer, the LNs and spleens of the recipient mice were harvested and the CFSE dilution of the CD8SP T3.70⁺ cells was determined. Both HY and HY^{cd4} cells diluted CFSE equivalently in male recipients (Fig. 4 B). Therefore, despite a twofold difference in surface TCR levels, the antigen responsiveness of cells from HY and HY^{cd4} mice appears equivalent.

Negative selection occurs late in development in HY^{cd4} mice

Some discrepancy exists in the literature regarding the timing of negative selection during development. In wild-type mice, superantigen-mediated deletion generally occurs late in development, as DP mature into SP cells, whereas in other models, particularly some transgenic models (including

the conventional HY model), deletion occurs early, either right at or preceding DP generation. Examination of the CD4/CD8 profile of T3.70⁺ cells in the thymus revealed striking differences between conventional HY and HY^{cd4} male mice. The overwhelming majority of T3.70⁺ cells in conventional HY male mice were of the DN phenotype with virtually no DP, CD4SP, or CD8SP populations (Fig. 5 A). In contrast, in HY^{cd4} male mice, T3.70⁺ cells were predominantly DP thymocytes with very few CD8SP cells (Fig. 5 A). Comparing HY^{cd4} female to HY^{cd4} male T3.70⁺ thymocytes, there was no difference in the percentage of DP cells, whereas there was a dramatic loss of CD8SP cells in HY^{cd4} male mice (Fig. 5 A). To confirm that this phenotype was dependent on the timing of HY TCR expression and not some other variable of the conditional expression strategy, we also examined HY^{lck} animals. In this strain, Cre is driven by the lck promoter and results in HY TCR expression in the early DN stage, which is similar to conventional HY mice (unpublished data). Indeed, the thymus of the HY^{lck} male animal resembled the conventional HY male thymus, where the majority of T3.70⁺ cells were DN (Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20050359/DC1>). The difference between the conventional HY and HY^{cd4} strains was further highlighted when comparing the number of T3.70⁺ cells in the DP and CD8SP populations from the various mice. There was an ~500–1,000-fold reduction in DP thymocytes when comparing conventional HY male and female mice, whereas only a 2-fold reduction in the numbers of DP thymocytes is observed between HY^{cd4} male and female mice (Fig. 5 B). Again, very few T3.70⁺ DP thymocytes were present in HY^{lck} males (Fig. S2 B), suggesting that this difference is caused by the timing of TCR α gene expression. The reduction in HY^{cd4} mice was predominantly seen in the CD8SP compartment, which is 15-fold smaller in HY^{cd4} male compared with female mice (Fig. 5 B). Thus, although male antigen-mediated deletion occurs at the DN–DP transition in the conventional HY model, it occurred at the DP–SP transition in the HY^{cd4} model, and this difference was caused by the timing of TCR α expression.

To rule out the possible contribution of endogenous TCR α chains to our observations, we constructed bone marrow chimeras with HY^{cd4} TCR α^0 female bone marrow. We created mixed chimeras with either B6.PL male or female bone marrow and injected them into male or female B6.PL recipients, respectively, to provide male antigen-expressing, bone marrow-derived, antigen-presenting cells. The only difference in the CD4/CD8 profile of T3.70⁺ cells from the thymus of HY^{cd4} mice on a TCR α -sufficient or -deficient background was a slightly enhanced CD8 percentage in the females (Fig. 5 C). A large population of T3.70⁺ DP, with an absence of CD8SP, was still observed in the male. Additionally, the relative chimerism of the DP and CD8SP compartments in male and female recipients supported late deletion (unpublished data).

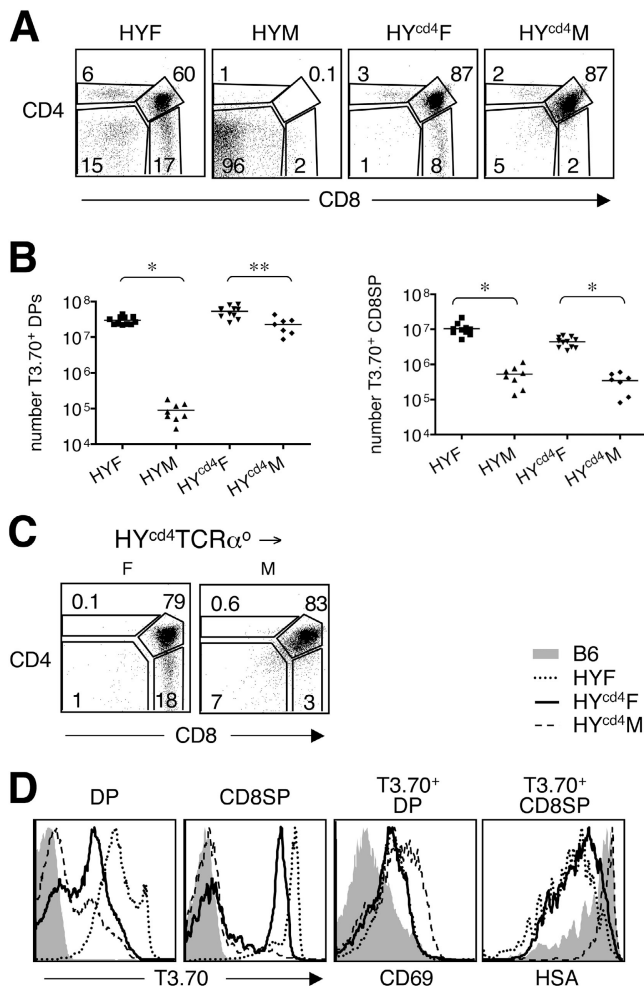


Figure 5. Deletion occurs late in HY^{cd4} male mice. (A) Thymocytes from the indicated mice were stained with anti-CD4, anti-CD8, and T3.70 and analyzed by flow cytometry. The T3.70⁺ thymocytes were gated and the CD4/CD8 profile is indicated. The numbers within the FACS plots represent the percentage of cells falling within that gate. (B) Comparisons of the absolute number of DP (HY female, $30 \pm 7.6 \times 10^6$; HY male, $9.0 \pm 5 \times 10^4$; HY^{cd4} female, $53 \pm 18 \times 10^6$; HY^{cd4} male, $23 \pm 12 \times 10^6$), and CD8SP (HY female, $10 \pm 4 \times 10^6$; HY male, $5.3 \pm 3.3 \times 10^5$; HY^{cd4} female, $4.4 \pm 1.4 \times 10^6$; HY^{cd4} male, $3.4 \pm 1.9 \times 10^5$) T3.70⁺ thymocytes from the different mouse strains. (*, $P < 0.0001$; **, $P < 0.002$). The horizontal lines represent the means. (C) Bone marrow from HY^{cd4} TCRα⁰ female mice was mixed with either female or male B6.PL bone marrow, and 7×10^6 cells were injected i.v. into lethally irradiated female or male B6.PL recipients, respectively. The female (left) and male (right) recipients were harvested 5–8 wk after transfer. CD4/CD8 profile of Thy1.2⁺ cells is indicated. (D) DP and CD8SP thymocytes from B6 (shaded region), HY female (dotted line), HY^{cd4} female (continuous line), and HY^{cd4} male (dashed line) mice were analyzed by flow cytometry for T3.70 expression (far left and left). T3.70⁺ DP (right) and T3.70⁺ CD8SP (far right) were assessed for CD69 up-regulation and CD24 down-regulation, respectively. The y axis represents the percentage of maximum expression.

No difference in the level of T3.70 expression was observed between HY^{cd4} male and female DP cells (Fig. 5 D). Thus, it is possible that T3.70⁺ DP cells are present in HY^{cd4}

male mice because they have yet to encounter antigen (e.g., because of a lack of presentation by cortical epithelial cells). However, this does not appear to be the case because CD69 was expressed at a high level in HY^{cd4} T3.70⁺ male DP cells, indicating that those cells had in fact responded to antigen but were not yet deleted (Fig. 5 D). Additionally, an examination of the few remaining T3.70⁺ CD8SP cells in HY^{cd4} male mice revealed a high expression of CD24, indicating an immature state (Fig. 5 D). Collectively, these data indicate that early expression of HY TCRα in conventional HY male mice leads to immediate and early deletion, whereas HY TCRα expression at the DP stage leads to a later and delayed deletion, which occurs as cells transition from DP to SP.

CD8αα IEL expansion occurs less in HY^{cd4} male mice

There is a unique population of lymphocytes in the gut that express αβTCR and CD8αα homodimers. A great deal of debate has surrounded the issue of how this population develops. Although it was thought that CD8αα IELs develop extrathymically, this might only be the case in lymphopenic conditions (18); other evidence suggests a thymic origin (19). Additionally, this population is thought to be self-reactive and to serve a regulatory function in the gut (20). Indeed, consistent with this idea, conventional HY male mice display at least a 100-fold expansion in CD8αα IELs (21, 22). Nevertheless, it remains controversial as to whether CD8αα IELs develop from DN or DP progenitors. We felt that our model would be a good means to evaluate this issue.

To this end, we purified IELs according to standard protocols and evaluated the number and percentage of T3.70⁺ CD8αα IELs in the different transgenic strains. As previously reported, the number of T3.70⁺ CD8αα IELs was ~500-fold higher in conventional HY male mice compared with females (Fig. 6 A; references 21 and 22). In contrast, there was only a fivefold change in the number of these cells between male and female HY^{cd4} mice, which was not significant with the number of animals analyzed ($P > 0.05$; Fig. 6 A). Examination of HY^{lck} mice showed a 200-fold increase in T3.70⁺ CD8αα IELs in males over females, similar to conventional HY transgenics, further suggesting that the timing of TCRα expression is critical for the expansion of these cells (Fig. S2 D). Additionally, the T3.70⁺ IELs from HY^{cd4} mice showed a similar percentage of CD8αβ⁻ and CD8αα-expressing cells in both males and females, whereas there was a dramatic increase in CD8αα-expressing cells in conventional HY and HY^{lck} male mice (Fig. 6 B and Fig. S2 D). Curiously, the IELs from HY^{cd4} male mice display a small CD8αβ^{hi} population that is not present in HY or HY^{lck} males, of which we do not understand the importance at this time.

To determine whether early versus late TCR expression has an impact on the phenotype of T3.70⁺ CD8αα IELs, we examined the expression pattern of several molecules reported to be differentially expressed on CD8αβ versus CD8αα IELs by the gene profiling (23). CD8αα and CD8αβ IELs from B6 display distinct patterns of CD5,

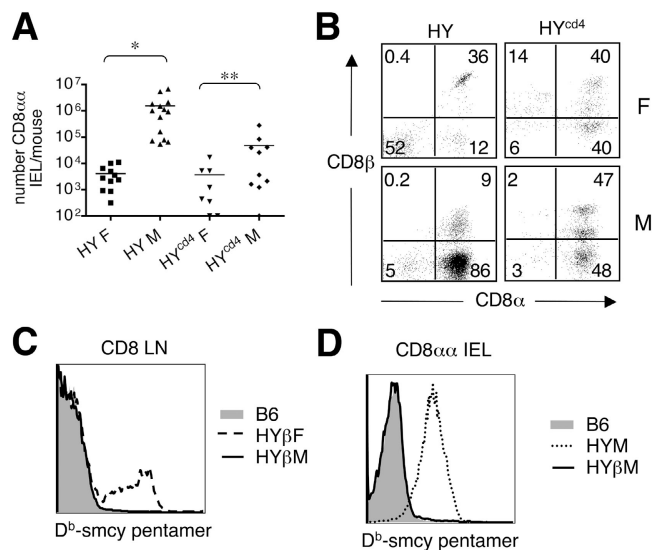


Figure 6. Early TCR α expression is required for expansion of gut CD8 $\alpha\alpha$ IELs. (A) IELs were isolated and stained with anti-CD8 β , -CD8 α , -CD3, and T3.70 and analyzed by flow cytometry. CD3 $^+$ T3.70 $^+$ cells were electronically gated and the absolute number of CD8 $\alpha\alpha$ $^+$ cells was quantified by comparison to the acquisition of a known number of latex beads included in the sample (HY female, $4.1 \pm 3.6 \times 10^3$; HY male, $1.5 \pm 2.1 \times 10^6$; HY cd4 female, $3.7 \pm 5.8 \times 10^3$; HY cd4 male, $4.8 \pm 8.6 \times 10^4$). The horizontal lines represent the means. *, $P < 0.05$; **, $P > 0.05$. (B) CD8 β /CD8 α profile of the CD3 $^+$ T3.70 $^+$ IELs is shown for the indicated mice. The numbers within the FACS plots represent the percentage of cells falling within that gate. (C) LN cells from B6 (shaded region), HY β female (dashed line), and HY β male (continuous line) mice were stained with anti-CD8 α , -CD8 β , -CD3, and D b -smcy pentamer. D b -smcy pentamer staining for CD3 $^+$ CD8 $\alpha\beta$ $^+$ cells is indicated. (D) IELs were harvested from B6 (shaded region), HY β male (continuous line), and HY male (dotted line) mice and stained with anti-CD8 α , -CD8 β , -CD3, and D b -smcy pentamers. D b -smcy pentamer staining from CD3 $^+$ CD8 $\alpha\alpha$ $^+$ cells is indicated. The y axes in C and D represent the percentage of maximum expression.

CD122, and CD11a (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20050359/DC1>). Interestingly, the level of expression of these markers on T3.70 $^+$ CD8 $\alpha\alpha$ IELs from both conventional HY and HY cd4 males was not similar to either normal population (Fig. S3). In fact, for all three markers, the level of expression was intermediate between CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IELs from B6 (Fig. S3). The reason for this difference is also currently unclear. Together, these data suggest that the expansion of T3.70 $^+$ CD8 $\alpha\alpha$ IELs in response to high affinity ligand is dependent on TCR expression early in development. Furthermore, CD8 $\alpha\alpha$ IELs that do develop in conventional HY or HY cd4 male mice have an unusual phenotype when compared with nontransgenic CD8 $\alpha\alpha$ IELs.

If CD8 $\alpha\alpha$ IEL expansion requires TCR gene rearrangement and expression at the DN stage, then one would predict that such cells would not be prominent in HY β transgenic mice. HY β transgenic mice express only the TCR β

chain early in development. The TCR α chain is derived from the endogenous repertoire, which does not undergo gene rearrangement and expression until the DP stage. Thus, potential male reactive receptors are not generated until the DP stage. Female HY β transgenic mice displayed a prominent population of male reactive CD8 $^+$ cells in the LNs as judged by their ability to bind D b /male antigen tetramers (Fig. 6 C; reference 24), which are presumably derived by positive selection at the DP stage. If self-reactivity were to result in the positive selection of CD8 $\alpha\alpha$ IELs in HY β mice, then CD8 $\alpha\alpha$ IELs from male mice should display an increase in male reactivity. This was not the case, as CD8 $\alpha\alpha$ IELs from male HY β mice do not show detectable D b -smcy pentamer binding (Fig. 6 D). This does not reflect a technical inability of CD8 $\alpha\alpha$ cells to bind pentamers because the CD8 $\alpha\alpha$ IELs from HY conventional mice do bind the pentamer (Fig. 6 D). This result confirms the findings from the HY cd4 mouse and suggests that development of CD8 $\alpha\alpha$ IELs in HY mice depends on early rearrangement and expression of the TCR.

DISCUSSION

Our data indicate that early expression of the TCR affects several properties in TCR transgenic mice. This is important because TCR transgenics represent the predominant tool used to study lymphocyte development, particularly as it relates to the specificity and affinity of the rearranged antigen receptor. When the male-specific HY TCR was expressed at the physiological DP stage of development, the β -selection defect and lineage misdirection seen in conventional TCR transgenics was corrected. Furthermore, deletion occurred late in development and expansion of the CD8 $\alpha\alpha$ $^+$ gut IELs was lost. Positive selection and HP were unaffected by early TCR expression. In general, we feel this model represents a more faithful recapitulation of T cell development processes.

β -selection and lineage misdirection

β -selection is a process initiated by the pre-TCR at the DN3 stage. It signals the successful completion of TCR β chain gene rearrangement and results in cellular expansion, CD4 and CD8 expression, termination of further TCR β chain gene rearrangement, initiation of TCR α chain gene rearrangement, migration into the cortex, and development of positive and negative selection “competence” factors (25, 26). Although a mature $\alpha\beta$ TCR is capable of inducing β -selection at the DN stage, it is much less efficient than that mediated by the pre-TCR (3). In HY cd4 mice, the β -selection impairment seen in conventional HY mice did not occur. This finding is consistent with a pre-T α competition model, where a higher affinity of TCR β for TCR α , compared with pre-T α , results in less efficient β -selection. In support of this hypothesis, studies by Lacorazza et al. have indicated that premature TCR expression impairs the proliferative burst of DN thymocytes and increases the number of terminally differentiated DNs (6).

In the periphery of conventional HY mice, there is a large population of DN T3.70⁺ cells. These cells have been shown to represent lineage-misdirected cells, arising because of signals from the $\alpha\beta$ TCR at the DN stage as would be the case if a $\gamma\delta$ TCR were expressed (4, 13). When the HY TCR was not expressed until the DP stage, this population of cells was absent. Importantly, some studies have suggested that these DN TCR⁺ cells have regulatory potential (27, 28). Because the DN cells have the same specificity as the CD8 or CD4 cells in the periphery, they have the potential to influence the immune response in a nonphysiologic manner if intact TCR transgenics are used to evaluate an antigen-specific response. This, however, is not a concern when the adoptive transfer of purified CD8 or CD4 transgenic cells is used to evaluate an antigen-specific response.

Positive selection and homeostasis

Early TCR expression does not seem to affect positive selection or homeostasis. We found no differences in the phenotypes of T3.70⁺ DP or CD8SP cells in HY^{cd4} mice compared with conventional HY mice. In both cases, markers shown to be up-regulated in response to TCR stimulation, including CD69, CD2, CD5, and CD53, were all induced to the same extent. Additionally, T3.70⁺ CD8SP cells responded equivalently *in vitro* and *in vivo* to agonist ligand. Evaluation of the HP potential of T3.70⁺ CD8SP thymocytes revealed that cells from neither model were able to divide in response to lymphopenic conditions, which has been previously demonstrated for conventional HY cells (17). The reason for this lack of HP is currently unclear; however, at least two different explanations can be offered. First, it is possible that the selecting ligand in the thymus is either absent or reduced in quantity in the periphery. Work by Santori et al. identified, through homology-based searches, a peptide that could select HY TCR thymocytes in fetal thymic organ culture, but it is unclear if, where, and in what quantities this peptide is presented in the periphery (29). Alternatively, it has been suggested that the failure of HY T cells to undergo HP is caused by the low affinity of the HY TCR for the selecting ligand (15). This reasoning stems from the observation that both peripheral CD8s and CD8SP thymocytes express lower levels of CD5 than do bulk B6 thymocytes or other TCR transgenics that do undergo HP. We find this possibility somewhat unlikely since positive selection of HY thymocytes does occur and appears to be quite efficient. We favor a combination of these two hypotheses in which the affinity of the HY TCR for the selecting ligand is somewhat lower than for other TCR transgenics, as illustrated by reduced CD5 levels, but still high enough to mediate positive selection, and the expression of this ligand is limited in the periphery. Further experiments are necessary to understand this issue fully.

Clonal deletion

Perhaps some of the most intriguing results were obtained when examining T cell development in male HY^{cd4} mice.

We observed that negative selection in HY^{cd4} male mice occurs late in development, at the DP to CD8SP transition. Discrepancies exist in the literature regarding the timing of negative selection. Some models, including the conventional HY and other TCR transgenic models, find that negative selection occurs immediately at or preceding DP generation. However, deletion in other transgenic models (30–32), as well as endogenous superantigen-mediated deletion (33, 34), occurs at the DP–SP transition, as is found in the HY^{cd4} model. In addition, analysis of the anatomy of apoptosis in MHC-sufficient versus -deficient mice suggests that deletion is primarily cortico-medullary (35), corresponding to a late deletion at the DP or SP stage. Based on the findings here, we hypothesize that the early deletion seen in some TCR transgenic models of negative selection is caused by the nonphysiologic early TCR expression. Clearly, the anatomical location of self-antigen can be a factor in when negative selection occurs. However, because the male self-antigen is ubiquitously expressed in the thymus, and its presentation does not vary between conventional HY, HY^{lck}, and HY^{cd4} models, the timing of TCR expression appears to be a more critical factor in when negative selection occurs.

One surprising feature of the response to male antigen in the HY^{cd4} model is the presence of a large population of activated DP progenitors. In conventional HY male mice, it is difficult to find progenitors (DN or DP) with an activated phenotype, presumably because they had undergone immediate apoptosis. This implies that the death induced in the more physiologic HY^{cd4} model is somehow delayed. Previous studies have suggested that the medulla and cortico-medullary junction is the primary site for clonal deletion to superantigen (35) or to the complement component C5 in A18 TCR transgenic animals (36), and genetic studies suggest that costimulatory molecules that are expressed in the medulla, but not the cortex, are important for clonal deletion (37–40). Therefore, an interesting possibility is that self-antigen-specific progenitors normally do not undergo clonal deletion until they migrate to the cortico-medullary junction, a possibility that could be evaluated by determining the site of deletion in the HY^{cd4} model.

In addition to there being discrepancies about the stage of deletion, there is also controversy about the molecular signaling pathways leading to deletion. For example, the role of CD40–CD40L interaction has been demonstrated to be necessary in some models of deletion, yet appears to be dispensable in others (including the conventional HY model; reference 41). Other molecules, including TNFR, CD28, and Fas, also have controversial roles in negative selection (42). Because deletion occurs late in the HY^{cd4} model it may be interesting to reevaluate the role of these molecules in central tolerance to male antigens.

CD8 $\alpha\alpha$ IELs

Although clonal deletion is an important central tolerance mechanism, recent evidence suggests that progenitors can

also be positively selected into a regulatory T cell lineage upon encountering high affinity ligand in the thymus (43, 44). This process has been referred to as “agonist selection” and applies to three different T cell populations: CD4⁺CD25⁺ regulatory T cells, NKT cells, and CD8 α IELs (45). Many questions remain about the factors that lead to agonist selection. Early work in the field of CD8 α IEL development suggested that this cell type arose extrathymically. However, recently, it was demonstrated that the extrathymic pathway might only operate under conditions of lymphopenia, and that the thymus is the primary developmental site for CD8 α IELs (18, 19). In addition to where these cells develop, the progenitor cell giving rise to CD8 α IELs has been controversial. Using TCR transgenic models for CD8 α IEL development, it was suggested that a DN progenitor gives rise to CD8 α IELs, and that in cases where there are few DP cells (such as in conventional HY male mice) there are many CD8 α IELs (21, 22). Gene profiling studies also showed striking commonalities between $\alpha\beta$ TCR CD8 α IELs and $\gamma\delta$ TCR DN IELs (23, 46). Because $\gamma\delta$ TCR-expressing cells arise from DN progenitors and do not develop through a DP intermediate, it was suggested that DN thymocytes are the immediate precursors of CD8 α IELs. Indeed, we found that although CD8 α IELs were present in HY^{cd4} male mice, there was little agonist ligand-dependent expansion, suggesting that the large CD8 α IEL population in conventional HY male mice is an unnatural consequence of the early expression of the receptor at the DN stage. This result was further confirmed using the HY β transgenic model in which there was no male reactivity in the CD8 α IEL population of male mice. In an elegant fate mapping experiment, Eberl et al. demonstrated that CD8 α IELs arose from at least a DN4 stage thymocyte and likely a DP thymocyte (47). Additionally, Yamagata et al. were able to generate CD8 α ⁺ cells from in vitro culture of nonselected HY DP with agonist-expressing stroma in a reaggregate culture (48). Our experiments do not preclude DP thymocytes as intermediates in the generation of CD8 α IELs; however, they do suggest that early TCR expression (within the DN compartment) is required. It is unclear why early TCR expression is necessary for the generation of CD8 α IELs, but one could postulate that signals received by a DN thymocyte from a mature $\alpha\beta$ TCR, as opposed to a pre-TCR, could in some manner imprint the cell to develop along this pathway when faced with agonist ligand.

In conclusion, the HY^{cd4} model has several advantages over other TCR transgenic and nontransgenic models of development, but it also has a few drawbacks. First, it contains a high frequency of monoclonal cells, which facilitates analysis, but may lead to unnatural effects (as in other TCR transgenics). Second, in order to create the HY^{cd4} mouse, three transgenes are required. This makes it quite laborious and time consuming when wanting to introduce other transgenes or breed onto a gene-deficient strain. Finally, the level of TCR expression on mature T cells in these animals is

lower than what is observed in wild-type mice. Although this issue did not appear to affect the sensitivity of the cells to ligand, there could be cases in which this would be a problem. It would be of substantial benefit to engineer a system where the TCR was expressed at the appropriate time developmentally, expressed similar amounts of surface TCR to wild-type mice, and is facile enough to allow the study of multiple genetic factors.

Overall, the HY^{cd4} model allowed us to define those properties of TCR transgenic lymphocyte development that are nonphysiological and caused by the early expression of the TCR. Our findings suggest that late (DP to SP) models of negative selection may be the most appropriate models to evaluate molecular pathways of clonal deletion, and that improved models are needed to study the development of CD8 α IELs.

MATERIALS AND METHODS

DNA constructs. pCAGGS STOP HY TCR α was generated using standard molecular biology techniques. In brief, the pEF321 ShcFFF plasmid containing a floxed transcriptional and translational STOP cassette was obtained from K. Ravichandran (University of Virginia, Charlottesville, VA; reference 10). The internal ribosomal entry site (IRES)-GFP portion of pEF321 ShcFFF was replaced with an IRES-GFP cassette provided by S. Casola (Harvard University, Boston, MA). The HY TCR α cDNA was amplified from a cDNA library generated from bulk HY thymocytes and replaced the ShcFFF cDNA by directional cloning. The floxed STOP HY TCR α IRES-GFP fragment was inserted into the pCAGGS vector, generating pCAGGS STOP HY TCR α .

Animals. C57BL/6 (B6), Thy 1 congenic C57BL/6.PL (B6.PL), CD45 congenic C57BL/6.SJL (B6.SJL), and TCR α ^{-/-} mice were purchased from the Jackson Laboratory. CD4-Cre and Ick-Cre mice were purchased from Taconic. The conditional HY TCR α -expressing mice were generated by microinjection of the Sal-Not fragment from pCAGGS STOP HY TCR α into preimplantation C57BL/6 embryos by the Mouse Genetics Laboratory at the University of Minnesota. Founders were identified by PCR using primers directed against the 3' region of the STOP cassette. Nine founders were identified, of which only two appeared to express HY TCR α . Data from founder 8820 are presented. Conventional HY and HY β mice were a gift of H. von Boehmer (Harvard University, Boston, MA). Mice containing the HY β , CD4-Cre and conditional HY TCR α (HY^{cd4}) or HY β , Ick-Cre, and conditional HY TCR α (HY^{ick}) transgenes were obtained by breeding. All animals were maintained and treated in accordance with federal guidelines approved by the University of Minnesota Institutional Animal Care Committee.

Antibodies and flow cytometry. All fluorochrome and biotinylated antibodies were purchased from BD Biosciences, eBioscience, or Biolegend. Cells were stained with antibody for 30 min on ice in FACS buffer (PBS, 1% FCS, and 0.02% azide, pH 7.2) and washed two times in FACS buffer after each antibody incubation. Cell events were collected using a cytometer (FACSCalibur or LSRII; BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.). D^b-smcy pentamers were purchased from ProImmune. Pentamer staining was performed in FACS buffer at room temperature for 30 min before staining with other antibodies.

Purification of IELs. Lymphocytes from the small intestine were purified using a protocol described by Podd et al. (49). In brief, the small intestine was isolated, cut longitudinally, and the contents were rinsed out with ice-cold HBSS. The intestine was then cut into 0.5-cm pieces and incubated three times at 37°C for 20 min in HBSS and 1 mM dithiothreitol with

shaking. After each round of shaking, the supernatant was strained with a 70- μ M nylon filter. Cells were pelleted and lymphocytes were enriched by centrifugation over a 70 \times /40 \times Percoll gradient, and the band at the 70 \times /40 \times interface was collected. Cells were washed in RP10 before staining.

In vitro and in vivo stimulation. Responder HY or HY^{cd4} female thymocytes or bulk LN cells were mixed at a 1:1 ratio with female B6 splenocytes and the indicated concentration of agonist smcy peptide in vitro (50). Cultures were incubated for 20 h at 37°C and stained with anti-CD4, anti-CD8, T3.70, and anti-CD69. T3.70⁺ DP or CD8SP cells were electronically gated, and the expression of CD69 was measured. Data are represented as the percentages of indicated population that maximally up-regulated CD69. Bulk thymocytes from HY and HY^{cd4} female mice were CFSE labeled (Molecular Probes) and injected i.v. into unmanipulated male or female recipients. LNs and spleens were harvested 48 h after injection, stained with anti-CD4, CD8, CD69, and T3.70, and analyzed on a cytometer (LSRII; BD Biosciences). Data are presented as the CFSE profile for CD8⁺ T3.70⁺ cells from the spleen from male or female recipients.

HP. Bulk thymocytes from HY, HY^{cd4}, and B6 female mice were isolated. HY thymocytes were spiked with B6 thymocytes to provide a reference population for measurement of HP (HY^{cd4} mice have a population of CD8SP that do not express the HY receptor and thus provide a preexisting internal control). Thymocytes were then CFSE labeled and injected i.v. into sublethally irradiated congenic B6.SJL mice. LNs and spleens were harvested 9 d after injection, and CFSE dilution was measured in the B6, HY, and HY^{cd4} CD8 populations.

Online supplemental material. Fig. S1 depicts a quantitative comparison of TCR levels in CD69⁺ (positively selected) DP thymocytes from B6, HY, and HY^{cd4} mice. Fig. S2 shows a phenotypic and quantitative analysis of thymocytes (A and B) and IELs (C and D) from HY^{lck} female and male mice. Fig. S3 depicts a phenotypic analysis of IEL populations (CD8 $\alpha\alpha$ and CD8 $\alpha\beta$) from various mice. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20050359/DC1>.

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