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7. PUT3 and PPR1 deletion derivatives and all chimeric proteins were constructed with the polymerase chain reaction (oligonucleotide sequences are available on request). Amplified DNA fragments were cloned into pET16b (Novagen, Madison, WI) cut with Nco I–Bam HI. The resulting plasmids contained the fusion gene under the direct control of the T7 promoter. All plasmids were subjected to DNA sequencing analysis to ensure that the correct fusion had been made and that no other mutations had arisen. Plasmids were transformed into *Escherichia coli* strain BL21-(DE3)pLysS (Novagen), and cells were grown at 37°C in Luria broth media containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) until an absorbance of 0.3 at 595 nm was reached. We then added $ZnSO_4$ to the cultures to a final concentration of 20 μM, and the cells were induced with isopropyl-β-D-thiogalactopyranoside (BRL) to a final concentration of 2 mM. Growth was continued for a further 2 hours, after which the cells were harvested by centrifugation (5000*g* for 15 min). The cell pellet was resuspended in 2.5% of the original culture volume of buffer A [20 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 1.4 mM β-mercaptoethanol, and 20 μM $ZnSO_4$], and the cells were sonicated and centrifuged at 15,000*g* for 15 min. The supernatant was added to an equal volume of buffer A and applied to a 3-ml S Sepharose Fast Flow column (Pharmacia) equilibrated in buffer A. The column was washed with 100 column volumes of buffer A and developed with a 30–column volume gradient of buffer A to buffer A + 1 M NaCl. Peak fractions (followed by absorbance at 280 nm) were analyzed by SDS–polyacrylamide gel electrophoresis, pooled, and dialyzed against buffer A. With the use of this single column, each of the fusion proteins was isolated with greater than 95% purity.
8. Other experiments (*15*), either in vivo or with cell extracts, have suggested that a GAL4 or LAC9 molecule, whose six-cysteine region has been replaced by that of PPR1, binds DNA with the specificity of GAL4 but with at most one-tenth its affinity. In further experiments, when the PPR1 zinc cluster and the 14 amino acids to the COOH-terminal side were included in the swap, PPR1 specificity was conferred on the resultant protein but DNA binding activity was weak. Our results indicate that the reduced binding affinity observed in those studies was probably due to the suboptimal placement of the chimeric junction.
9. We have failed in attempts to construct active chimeras that contain just the PUT3 zinc cluster and linker fused to the dimerization region of GAL4. For example, PUT3(31–65)+GAL4(49–100), PUT3(31–67)+GAL4(53–100), PUT3(31–68)+GAL4(54–100), PUT3(31–74)+GAL4(53–100), and PUT3(31–76)+GAL4(62–100) were all inactive in DNA binding in our assays. However, we do not know the structure of PUT3, and it is possible that chimeric junctions were not appropriately positioned. Also, attempts to characterize a GAL4 molecule in which just the linker and start of the dimerization element have been replaced by PUT3 sequences [GAL4(1–38)+PUT3(61–79)+GAL4(58–100)] have been hampered by the insolubility of the protein.
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19. We thank R. Marmorstein for the gift of purified PPR1(29–123) and many useful discussions. We also thank R. Marmorstein, H. Himmelfarb, N. Lehming, J. Brickman, and J. Robinson for critically reading the manuscript. R.J.R. was supported by fellowships initially from the European Molecular Biology Organization (ALTF 475-1990) and latterly from the Science and Engineering Research Council (B/92RF/1595). This work was supported by grant GM32308 from NIH.

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Development of Mature CD8$^+$ Thymocytes: Selection Rather Than Instruction?

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The role of major histocompatibility complex (MHC) molecules in T cell differentiation was investigated by comparison of thymocyte subpopulations in wild-type mice and β_2-microglobulin (β_2M) mutant mice deficient in MHC class I expression and mature CD8$^+$ cells. On the basis of surface markers, glucocorticoid resistance, in vitro differentiation capacity, and absence in $\beta_2M^{-/-}$ mice, CD4^{intermediate}CD8^{hi} cells with high expression of αβ T cell receptor (TCRαβ) were identified as having been positively selected by MHC class I for development into mature CD8$^+$ T cells. Activated CD4^{int}CD8^{hi} cells bearing intermediate rather than high amounts of TCR were present in both wild-type and $\beta_2M^{-/-}$ animals. These data suggest that recognition of MHC class I molecules is required for full maturation to CD8$^+$ T cells, but not for receptor-initiated commitment to the CD8$^+$ lineage, consistent with a stochastic (selection) model of thymocyte development.

An extensive series of studies on thymocyte differentiation have led to a proposed scheme of development in which precursor cells that lack surface expression of the TCRαβ-CD3 complex and the CD4 or CD8 coreceptors (triple negative cells) become either CD4$^-$CD8$^+$ or CD4$^+$CD8$^-$ intermediates and then CD4^{hi}CD8^{hi} (double positive, DP), TCRαβ^{lo} blasts. These cells are the substrate for two opposing selection events involving the coordinate activity of TCRs, the CD4 or CD8 coreceptors, and MHC class I or class II molecules: positive selection for differentiation to mature CD4^{hi}CD8$^-$ or CD4$^-$CD8^{hi}, TCRαβ^{hi} (CD4 or CD8 single positive, SP) cells, or negative selection that deletes self-reactive cells (*1*). The mechanism that leads to commitment of DP precursor cells to the CD4 or CD8 lineage, however, remains unclear. Some investigators favor "instructive" models, which postulate that differential signaling through CD4–class II or CD8–class I interactions determines the fate of bipotential precursor cells (*2*, *3*). Others propose "selective" models in which stochastic events determine the lineage commitment, but full maturation requires an appropriate match among the MHC restriction (class I or class II) of the TCR and the coreceptor that was retained (*4*, *5*).

Analysis of developmental intermediates could contribute to resolving this issue. The transition from DP-TCRαβ^{lo} to either CD4 or CD8 SP (TCRαβ^{hi}) mature cells must involve progression through intermediate states with increasing TCRαβ expression and with decreasing amounts of the coreceptor whose expression is to be extinguished. Guidos *et al.* described small thymocyte populations of CD4^{hi}CD8^{int}TCRαβ^{int} or CD4^{int}CD8^{hi}TCRαβ^{int} phenotype that show biased V_β usage associated with positive selection by certain MHC molecules (*6*). On the basis of these results, it was concluded that these cells were the product of positive selection events that led to development of CD4 or CD8 SP mature T lymphocytes. In contrast, other investigators have described a CD4$^+$CD8$^+$ DP subpopulation expressing high amounts of TCRαβ. The biased expression of V_β regions by DP-TCRαβ^{hi} cells in normal mice (*7*) and the presence of such cells only in TCR transgenic mice of the positively selecting haplotype (*3*) suggested that they

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represented thymocytes having undergone TCR-mediated positive selection. Their high expression of TCRαβ, CD4, and CD8 distinguished them from the cells studied by Guidos *et al.*, raising questions about which subpopulation actually represented the differentiating progeny of positively selected precursors. This issue was made even more cogent by a report of $CD4^{hi}CD8^{-/lo}TCR\alpha\beta^{int/hi}$ cells in mice lacking the class II molecules necessary for differentiation into mature $CD4^+$ T cells (*8*).

To better understand MHC molecule–dependent T cell maturation, we undertook a detailed phenotypic analysis of cells in the thymuses of β_2M-mutant, MHC class I molecule–deficient mice lacking significant numbers of mature CD8 SP cells (*9, 10*). We specifically searched for cell populations that could be confirmed as relevant developmental intermediates between DP-$TCR\alpha\beta^{lo}$ precursors and mature CD8 SP T cells, based on the presumption that the first phenotypically distinct population resulting from completed positive selection should be lacking in mice deficient in the corresponding class of MHC molecule.

Figure 1 shows the relations among CD4, CD8, and TCR expression on thymocytes of mice analyzed by three-color flow microfluorimetry (*11*). A substantial proportion of $CD4^-CD8^-$ thymocytes do not express detectable TCRαβ and correspond to committed precursor cells that have recently migrated into the thymus (*12*). A small proportion of the $CD4^-CD8^-$ thymocytes are mature and express relatively high amounts of TCRαβ. A fraction of the cells with low expression of TCRαβ presumably consists of precursors for $CD4^{hi}CD8^{lo}TCR\alpha\beta^{lo}$ and $CD4^{lo}CD8^{hi}TCR\alpha\beta^{lo}$ thymocytes that in turn give rise to the DP population (*13–15*). Most DP thymocytes express low or medium amounts of TCRαβ and include a small percentage of blast-like cells, the remainder being small, nondividing cells. A few DP cells had high TCRαβ expression, as noted (*7, 16*). Finally, there are the characteristic mature CD4 and CD8 SP $TCR\alpha\beta^{hi}$ populations.

Additional populations (about 1% of total thymocytes each) with cell surface CD4, CD8, and TCRαβ phenotypes "intermediate" between those of the precursor populations and mature cells can be identified (*6*). A discrete subpopulation of $CD4^{int}CD8^{hi}$ cells can be seen that expresses more TCRαβ than the DP-$TCR\alpha\beta^{lo}$ cells but less than mature CD8 SP thymocytes ($CD4^{int}CD8^{hi}TCR\alpha\beta^{int}$). A $CD4^{hi}CD8^{int}TCR\alpha\beta^{int}$ population is also present, whose amount of TCRαβ is consistently higher than that of the $CD4^{int}CD8^{hi}TCR\alpha\beta^{int}$ cells but still distinctly different from and lower than that of mature CD4 SP cells. We also noted previously undescribed members of the $CD4^{int}CD8^{hi}$ and $CD4^{hi}CD8^{int}$ populations with TCRαβ expression identical to that on the mature CD8 and CD4 SP thymocytes, respectively.

The $CD4^{hi}CD8^{int}$ and $CD4^{int}CD8^{hi}$ (henceforth called "coreceptor skewed," CRS) populations were further characterized with monoclonal antibodies to surface proteins whose expression increases upon TCR-mediated activation (*17*). The thymocyte activation marker CD5 (*18*) is higher on the CRS-$TCR\alpha\beta^{int}$ and CRS-$TCR\alpha\beta^{hi}$ populations and on SP thymocytes than on DP-$TCR\alpha\beta^{lo}$ cells (Fig. 2). Expression of the early T cell activation antigen CD69 (*19*) is also greater on these populations than on DP

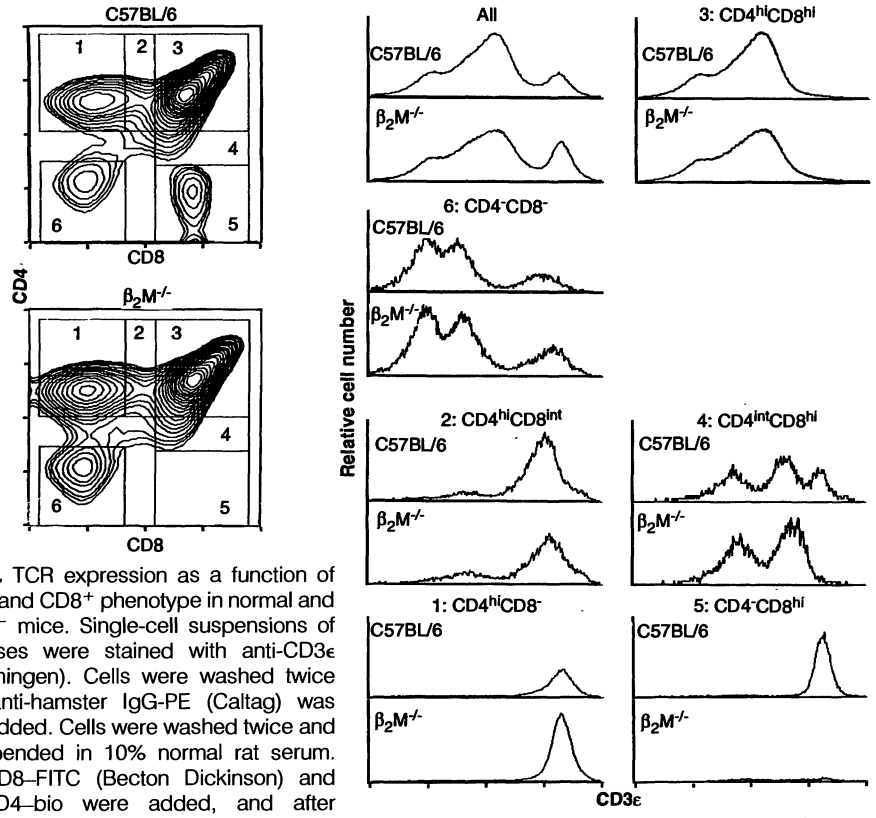

Fig. 1. TCR expression as a function of $CD4^+$ and $CD8^+$ phenotype in normal and $\beta_2M^{-/-}$ mice. Single-cell suspensions of thymuses were stained with anti-CD3ε (Pharmingen). Cells were washed twice and anti-hamster IgG-PE (Caltag) was then added. Cells were washed twice and resuspended in 10% normal rat serum. Anti-CD8–FITC (Becton Dickinson) and anti-CD4–bio were added, and after washing twice StrA-Cytochrome (Pharmingen) was added. Flow-cytometric analyses were done on a FACScan (Becton Dickinson). The histograms represent logarithmic CD3ε fluorescence as a function of cell number of populations electronically gated on their CD8 and CD4 fluorescence as indicated in the contour plots (logarithmic fluorescence scale, 75% logarithmic contours). Note that the linear cell number scales in the corresponding histograms for normal and $\beta_2M^{-/-}$ thymocytes are identical. The same results were obtained with anti-TCRβ—PE instead of anti-CD3ε.

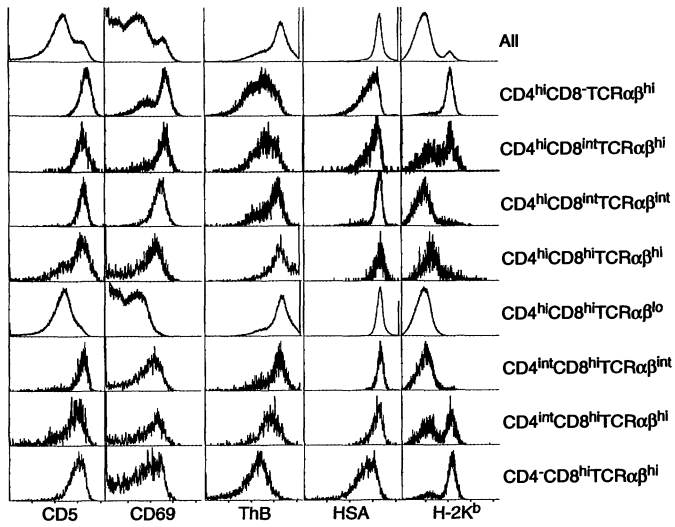

Fig. 2. Expression of CD5, CD69, ThB, HSA, and H-2K^b on thymocytes from normal mice. Thymocytes from female 4- to 6-week-old C57BL/6 mice were stained with labeled antibodies specific for TCRβ, CD4, CD8, and the fourth parameter, either CD5, CD69, ThB, HSA, or H-2K^b. Flow-cytometric analyses were done on a FACStar Plus equipped with an argon and a dye laser (Becton Dickinson). List-mode files were processed by gating on CD4, CD8 (as in Fig. 1), and TCR (as explained in the text) and by display of logarithmic fourth parameter histograms.

cells, as reported (*20*). Thus, the CRS-TCRαβ^{int} and the CRS-TCRαβ^{hi} populations consist of recently activated cells, a conclusion that for the CRS-TCRαβ^{int} cells is consistent with earlier observations (*5*, *6*, *21*) indicating that they have been signaled through their clonotypic receptor.

Antibodies detecting antigens whose expression correlates with T cell maturation rather than activation were also used to analyze these subpopulations (Fig. 2). Most immature thymocytes express large amounts of ThB, whereas mature cells express less (*22*). The CRS-TCRαβ^{int} population has ThB amounts similar to that of most immature DP thymocytes, whereas the CRS-TCRαβ^{hi} populations have less, implying that the latter are more mature. A similar expression pattern was seen for heat-stable antigen (HSA) (*23*). The MHC antigen H-2K is highly expressed on mature SP cells but less well expressed on immature DP thymocytes (*24*). Most CRS-TCRαβ^{int} cells have low H-2K^b, whereas CRS-TCRαβ^{hi} cells are a mixture of H-2K^b low and high cells (Fig. 2). Thus, although both CRS-TCRαβ^{int} and TCRαβ^{hi} populations show evidence of TCR-mediated activation, only the latter seems to have progressed toward a mature phenotype.

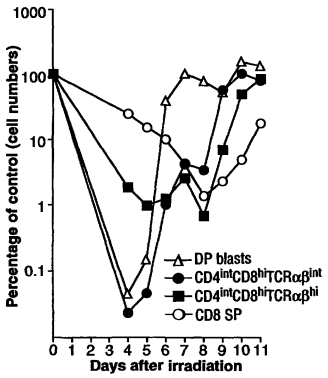

Fig. 3. Reconstitution of the thymus after sublethal γ irradiation. Eight-week-old male C57BL/6 mice were irradiated (750 rad) and thymuses from these mice analyzed as described in Fig. 1 on the indicated numbers of days after irradiation. Percentages of absolute cell numbers are relative to unmanipulated controls.

Evidence for a possible developmental relation among these phenotypically identified subpopulations was obtained from analysis of repopulating thymocytes in adult mice subjected to sublethal γ irradiation (*25*). There was a sequential appearance of DP blasts, followed by CRS-TCRαβ^{int} cells, then CRS-TCRαβ^{hi} cells, and finally SP CD8 T cells (Fig. 3). This order of appearance coincided with the progressive gain of maturation markers in the CRS-TCRαβ^{hi} and SP subpopulations described above for steady-state thymocyte populations.

Thymocytes that have undergone successful positive selection increase their resistance to glucocorticoid-induced cell death (*26*). Most of the thymocytes from mice treated with dexamethasone (*27*) are CD4 and CD8 SP (data not shown). The DP and CRS thymocytes that remain after treatment all express large amounts of TCRαβ (Fig. 4) (*28*). This result suggests that despite their recent TCR-mediated activation, but consistent with their immature phenotype, the CRS-TCRαβ^{int} thymocytes have not completely undergone differentiation events characteristic of positive selection. In contrast, and in agreement with the maturation-marker studies, at least a fraction of the CRS-TCRαβ^{hi} cells appear to have done so. Overnight culture of dexamethasone-resistant CD4^{int}CD8^{hi}TCRαβ^{hi} cells resulted in their substantial conversion into SP CD8$^+$ cells (Fig. 4B), consistent with these cells having been fully positively selected (*29*).

Direct evidence for the involvement of MHC recognition in positive selection of CRS-TCRαβ^{hi} cells came from examination of thymuses from $\beta_2M^{-/-}$ mice (*10*, *30*). Consistent with previous reports, the CD8 SP thymocyte population is strongly reduced in number in these mutant mice (0.1% of all thymocytes in $\beta_2M^{-/-}$ mice as compared with 3 to 4% in controls; Fig. 1). In full accord with the conclusions reached on the basis of surface marker analysis, corticosteroid resistance, repopulation kinetics, and precursor potential, the CD4^{int}CD8^{hi}TCRαβ^{hi} population was virtually absent in MHC class I–deficient mice that cannot mediate positive selection through MHC class I molecules. In contrast, the CD4^{int}CD8^{hi}TCRαβ^{int} population was present in $\beta_2M^{-/-}$ mice in numbers and proportion (1%) indistinguishable from that in wild-type animals. Staining for additional markers (CD5, CD69, HSA, and ThB) did not reveal any differences between this population in $\beta_2M^{-/-}$ and normal mice (Fig. 5).

We have therefore identified a previously undescribed population of cells (CD4^{int}CD8^{hi}TCRαβ^{hi}) whose members are clearly in transition to fully mature CD8 cells as a consequence of positive selection by MHC class I molecules. These data support a scheme of development in which at least a significant fraction of DP-TCRαβ^{lo} precursor cells upregulate their TCR expression to high levels before completing down-regulation of CD4 during maturation to the SP CD8$^+$ phenotype. Our studies have also unexpectedly revealed that CD4^{int}CD8^{hi}TCRαβ^{int} cells, previously considered to have undergone MHC molecule and TCRαβ-dependent positive selection, do not depend for their generation on the expression of surface class I molecules required for the development of CD8 SP mature T cells. Further, they lack both the surface markers and glucocorticoid resistance characteristic of cells that have been successfully selected. Thus, although their activated surface phenotype is consistent with the interpretation that they have been stimulated through their TCR (*6*, *21*), we conclude that these cells have not received the necessary signals for differentiation to mature T cells. This indicates that receptor

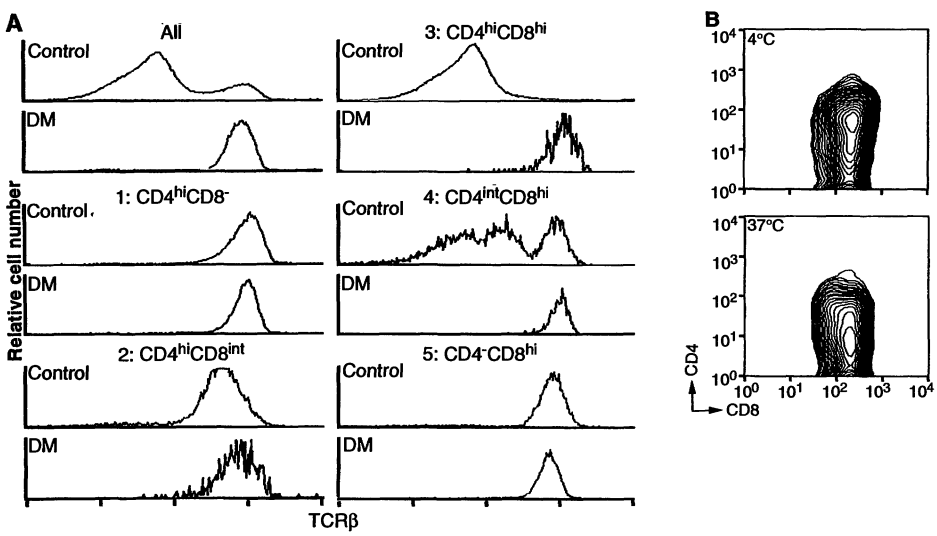

Fig. 4. (**A**) Glucocorticoid-resistant DP, CRS, and SP cells are TCRαβ^{hi}. Mice were injected with dexamethasone and their thymocytes analyzed 2 days later. Analysis was as in Fig. 1, except that anti-CD3ε was replaced by anti-TCRβ–PE. The thymuses from control mice contained 5 × 10^7 cells; those from dexamethasone-treated animals (DM) had 2 × 10^6 to 3 × 10^6 cells. (**B**) A substantial number of glucocorticoid-resistant CD4^{int}CD8^{hi}TCRαβ^{hi} cells become CD8 SP in in vitro overnight culture. Electronically sorted CD4^{int}CD8^{hi}TCRαβ^{hi} thymocytes from dexamethasone-treated mice were incubated in serum-supplemented Dulbecco's modified eagle's medium at either 4° or 37°C overnight and stained with anti-CD8–FITC, anti-CD4–PE, and anti-TCRβ–Red670 the next day. Seventy-five percent logarithmic contour plots of TCRαβ^{hi} cells are shown.

engagement of precursor thymocytes can result in partial activation without delivery of either a fully adequate positive or (death-inducing) negative selection signal.

Most if not all CD4 SP mature T cells have undergone intrathymic TCR signaling by occupancy with MHC class II ligand, whereas CD8 T cells have been stimulated by MHC class I ligand (*8–10, 31*). Current debate centers on whether this reflects coreceptor-dependent "instructive" differentiation of uncommitted precursors or "selection" for proper TCR-coreceptor-ligand matching after stochastic commitment of precursors to the CD4 or CD8 lineage (*32*). Although the instructional model has been given support by several studies in mice bearing TCRαβ and coreceptor transgenes (*2, 3*), our data on TCRαβ^{int} cells support a selection-based scheme of lineage commitment. The low numbers of intermediate cells as compared with DP-TCRαβ^{lo} cells, the presence of activation markers, the V_β bias reported by Guidos *et al.* (*6*), and the presence of TCRαβ^{int} cells only in TCR-transgenic mice of the positively selecting haplotype (*21*) all indicate that the CRS-TCRαβ^{int} cells have undergone clonotypic receptor signaling events. The presence of both CD4^{hi}CD8^{int} and CD4^{int}-CD8^{hi} CRS-TCRαβ^{int} populations in β_2M-mutant mice implies that TCR engagement by either MHC class I or class II molecules can give rise to both of these populations, as recently demonstrated (*5*). This argues for a model in which the change in coreceptor expression pattern is predetermined and independent of the class of MHC ligand involved in this initial signaling event. The coordination of TCR and coreceptor MHC ligand specificity seen in mature cells must then be achieved at a subsequent stage of differentiation, that is, by subsequent selection.

These data thus suggest the following model for later stages in thymocyte differentiation. A DP-TCRαβ^{lo} thymocyte will, upon TCR engagement with MHC molecules on thymic stroma cells, simultaneously increase its TCRαβ expression to intermediate amounts and down-modulate either its CD4 or CD8 coreceptor. The choice of the coreceptor that is to be maintained is either preprogrammed or occurs stochastically at the moment of activation but is clearly independent of the class of MHC involved in the TCR signaling event. Successful progress through this CRS-TCRαβ^{int} stage depends on the "match" between the chosen lineage (CD4 or CD8) and the MHC restriction of the TCR.

Although this model is consistent with the results presented here and is similar to other models proposed to explain findings with coreceptor transgenic (*4*) or MHC-deficient mice (*5*), in neither the latter study nor our own has evidence for a direct lineage relation between the activated but immature TCRαβ^{int} cells and mature SP cells been obtained. Stronger evidence for a selection scheme of thymocyte development will require direct demonstration by cell transfer that CRS-TCRαβ^{int} cells correspond to a partially selected state between receptor-unselected DP-TCRαβ^{lo} cells and positively selected SP T cells. Our present studies have identified another, previously undescribed population of transitional cells, of indistinguishable CD4 and CD8 phenotype (CRS-TCRαβ^{hi}), which we show contains thymocytes already fully selected for differentiation into mature SP CD8 cells. Therefore, such analysis of the differentiation potential of CRS-TCRαβ^{int} thymocytes by cell transfer awaits the development of methods for obtaining these CRS-TCRαβ^{int} cells free of any contaminating CRS-TCRαβ^{hi} cells. At present, this cannot be accomplished without the use of antibodies that might alter subsequent development by interaction with the TCR or the CD4 and CD8 coreceptors. Examination of the fate of such purified cells after reintroduction into the thymic environment in vivo or in vitro will unequivocally determine whether such cells are the precursors for final selection of the typical CD4 and CD8 T cells, supporting the selection scheme of differentiation described here, or whether they represent receptor-engaged thymocytes undergoing delayed negative selection or even development toward an alternative TCRαβ lineage (*33*).

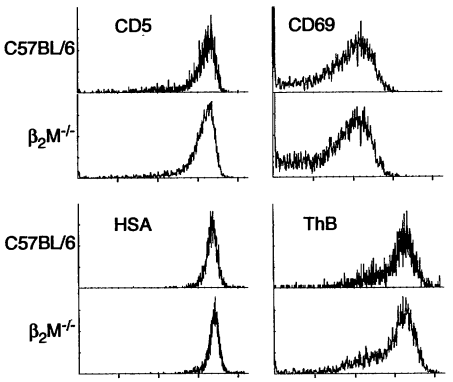

Fig. 5. Expression of CD5, CD69, HSA, and ThB on CD4^{int}CD8^{hi}TCRαβ^{int} cells is indistinguishable in normal and $\beta_2M^{-/-}$ mice. Analyses were done as in Fig. 2. Histograms displaying the logarithmic fluorescence of the indicated parameters of the electronically gated CD4^{int}CD8^{hi}TCRαβ^{int} population are shown for C57BL/6 and $\beta_2M^{-/-}$ mice (*10*). All other populations showed identical expression of these antigens in normal and $\beta_2M^{-/-}$ mice, apart from the CD4^{int}CD8^{hi}TCRαβ^{hi} and CD4^{-}-CD8^{hi}TCRαβ^{hi} populations, which are absent (see Fig. 1).

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17. Four-color analysis was done on a FACStar Plus equipped with argon and dye lasers (Becton Dickinson). Analyses of CD5, CD69, ThB, and HSA were done as follows. Single-cell suspensions of thymocytes were stained with antibody to CD8 (Becton Dickinson) followed by Texas red–labeled antibody to rat immunoglobulin G (Caltag). After washing twice, FITC-labeled antibody to TCRβ (H57-597, Pharmingen), PE-labeled antibody to CD4 (RM4-5, Pharmingen), and biotinylated fourth parameter antibody [CD5, 53-7.3; CD69, H1.2-F3; ThB, 49-h4; HSA, M1/69 (all from Pharmingen)] were added, followed after two washes by allophycocyanin (APC)-labeled streptavidin (Caltag). H-2K^{b} was detected by FITC-labeled AF6-88.5 (Pharmingen) (anti-CD8/anti–rat immunoglobulin G–Texas red, anti-TCRβ–PE, anti-CD4–biotin/StrA-APC). Populations were gated in a manner similar to that depicted in Fig. 1, and fourth parameter histograms were displayed.
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28. Analysis of HSA expression on thymocytes remaining 2 days after dexamethasone treatment revealed that mainly HSA^{lo} cells survived, indicating that only the most mature cells in each population are resistant to glucocorticoids.
29. Thymocytes from mice treated with dexamethasone 2 days previously were stained with anti-CD8–FITC and anti-CD4–PE (Becton Dickinson), and $CD4^{int}$-$CD8^{hi}$ cells were electronically sorted. Cells were incubated in complete medium at either 4° or 37°C overnight and then stained with anti-CD8–FITC, anti-CD4–PE, and anti-TCRβ–Red670 (Gibco-BRL) the next day. Seventy-five percent logarithmic contour plots of $TCR\beta^{hi}$ cells are shown. Using a forward and side scatter gate to exclude dead cells, we observed no cell loss (6.6×10^4 cells recovered in the experiment shown).
30. Mice deficient in surface MHC class I expression as a result of β_2M gene inactivation by homologous recombination were obtained from B. Koller and bred in our facilities. The original stock of mutant animals had been crossed to C57BL/6, and homozygous progeny were obtained by intercrossing heterozygous offspring. The line was maintained by intercrossing homozygous mutant offspring. Control mice were C57BL/6 mice bred in our facilities.
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Reconstitution of T Cell Receptor ζ–Mediated Calcium Mobilization in Nonlymphoid Cells

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T cell antigen receptor (TCR) activation involves interactions between receptor subunits and nonreceptor protein tyrosine kinases (PTKs). Early steps in signaling through the ζ chain of the TCR were examined in transfected COS-1 cells. Coexpression of the PTK $p59^{fynT}$, but not $p56^{lck}$, with ζ or with a homodimeric TCRβ-ζ fusion protein produced tyrosine phosphorylation of both ζ and phospholipase C (PLC)–γ1, as well as calcium ion mobilization in response to receptor cross-linking. CD45 coexpression enhanced these effects. No requirement for the PTK ZAP-70 was observed. Thus, $p59^{fynT}$ may link ζ directly to the PLC-γ1 activation pathway.

Lymphocyte activation through the TCR induces calcium ion mobilization, cell proliferation, and release of lymphokines and cytolytic factors. Early effects of TCR stimulation include tyrosine phosphorylation of the ζ chain (*1*) and CD3 subunits of the TCR (*2*); activation of cytoplasmic PTKs appears crucial in TCR-mediated activation (*3–6*). Unlike the tyrosine kinase growth factor receptors that activate similar signaling pathways (*7*), TCR subunits have no intrinsic enzymatic activities. However, two *src*-family PTKs, $p59^{fynT}$ (*8–10*) and $p56^{lck}$ (*11–15*), may link the TCR to signaling mechanisms. Cell lines and animals deficient in these kinases exhibit TCR signaling defects, as do T cell variants lacking the phosphotyrosine phosphatase CD45, a likely physiologic activator of the *lck* and *fyn* kinases (*16*).

The cytoplasmic domains of ζ and CD3ε can each transmit signals independently of other TCR subunits (*17, 18*). Three evenly spaced tyrosine pairs in the ζ tail and one pair in the CD3ε tail (*19*), lying within so-called tyrosine-based activation motifs (*20*), can become phosphorylated on T cell activation (*1, 2, 8, 12*). The role of these phosphorylations in TCR signaling awaits full elucidation.

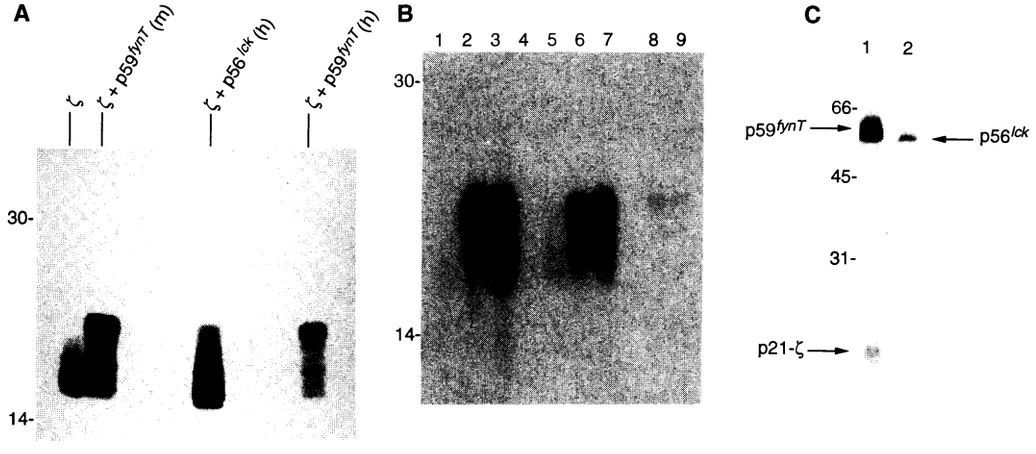

Fig. 1. Modification of ζ by $p59^{fynT}$ but not $p56^{lck}$ in COS-1 cells. Cells (1×10^6 to 2×10^6 per plate) were transfected by the DEAE-dextran method (*21*) and split 12 hours later. After 56 hours, transfectants (10^7 cells per sample) were lifted nonenzymatically (with 5 mM EDTA), washed, permeabilized on ice with L-α-lysolecithin (Sigma; 0.285 mg/ml) for 1 min, and then incubated with [γ-^{32}P]ATP (50 μCi per sample) for 15 min on ice (*12*). Cells were lysed with 1% NP-40 (Pierce) lysis buffer and centrifuged to remove nuclei. The supernatants were immunoprecipitated with 1D4.1 mAb (raised to a peptide comprising human ζ residues 153 to 164). Washed immunoprecipitates (10^7 cells per lane) were subjected to reducing SDS-PAGE (12.5% gels) and visualized by autoradiography. Molecular size standards (in kilodaltons) are at left. (**A**) COS-1 cells transfected with cDNAs encoding ζ only, ζ + (murine) $p59^{fynT}$(m), ζ + (human) $p56^{lck}$(h), and ζ + (human) $p59^{fynT}$(h). (**B**) The $p59^{fynT}$- and $p56^{lck}$-specific phosphorylations are enhanced by CD45. COS cells were cotransfected with cDNAs encoding ζ (lane 1), ζ + $p59^{fynT}$ (lane 2), ζ + $p59^{fynT}$ + CD45 (lane 3), ζ + $p56^{lck}$ (lane 4), ζ + $p56^{lck}$ + CD45 (lane 5), ζ + $p59^{fynT}$ + $p56^{lck}$ (lane 6), and ζ + $p59^{fynT}$ + $p56^{lck}$ + CD45 (lane 7). To examine $p56^{lck}$-specific effects, we cotransfected a 5′ antisense $p59^{fynT}$ cDNA segment (*22*) in samples 1, 4, and 5. The p21-ζ protein immunoprecipitated from activated peripheral blood T cells (lane 8) and from activated Jurkat leukemic T cells (lane 9) are shown for comparison. (**C**) Detection of a $p59^{fynT}$-p21ζ phosphoprotein complex. COS cells were triply transfected (ζ + $p59^{fynT}$ + $p56^{lck}$). Immunoprecipitates of $p59^{fynT}$ (lane 1) and $p56^{lck}$ (lane 2) were prepared with rabbit antisera [anti–human fyn and anti–human lck kinase (CT); UBI, Lake Placid, New York] from a divided lysate.