

T Cell Receptor-mediated Negative Selection of Autoreactive T Lymphocyte Precursors Occurs after Commitment to the CD4 or CD8 Lineages

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Summary

To identify the maturational stage(s) during which T cell receptor (TCR)-mediated positive and negative selection occurs, we followed the development of CD4⁺8⁻ and CD4⁻8⁺ T cells from TCR^{lo} CD4⁺8⁺ thymic blasts in the presence of different positive and negative selecting (major histocompatibility complex or Mls) elements. We describe novel lineage-committed transitional intermediates that are TCR^{med} CD4⁺8^{lo} or TCR^{med} CD4^{lo}8⁺, and that show evidence of having been positively selected. Furthermore, negative selection is not evident until after cells have attained one of the TCR^{med} transitional phenotypes. Accordingly, we propose that negative selection in normal mice occurs only after TCR^{lo} CD4⁺8⁺ precursors have been positively selected into either the CD4 or CD8 lineage.

Crucial to preventing immune destruction of self tissues is the capacity of T lymphocytes to direct their destructive potential towards foreign invaders. Self-nonself discrimination occurs at the level of both components of the T cell's bimolecular ligand; normal T cells are activated only by cell surface complexes consisting of nonself peptides bound to self-MHC proteins. That is, they display self-MHC-restricted recognition of foreign peptide antigens but remain self-(peptide) tolerant (1). The immunological definition of self is imprinted by a complex and poorly understood series of events during the intrathymic maturation of T lymphocytes from fetal liver or bone marrow-derived precursors. Several lines of evidence suggest that critical features of "thymic education" are interactions between the TCR complex on immature T cells and MHC proteins on thymic stromal cells (2, 3). Two distinct types of TCR-mediated selective events have been postulated to account for the self recognition properties of mature T cells. Precursors bearing TCR with affinity for self-MHC proteins expressed in the thymus (perhaps complexed with unknown self peptides) (4-6) apparently receive TCR-mediated positive signals resulting in rescue from programmed cell death and commitment to either the CD4 or CD8 mature lineage. Alternatively, self-tolerance may result from negative selection, or clonal deletion, of precursors bearing TCR with high affinity for self-peptide/self-MHC complexes (7). Thus, the specificity of the T cell antigen/MHC receptor (TCR) expressed on immature precursors is a critical determinant of thymocyte cell fate.

Investigation of the molecular and cellular processes in-

involved in TCR-mediated specification of cell fates during intrathymic maturation has been hampered by difficulties in identifying and isolating TCR⁺ precursors that are targets of positive and negative selection. The earliest intrathymic progenitors are TCR⁻, CD4⁻, and CD8⁻; mature TCR⁺ CD4⁺8⁻ and CD4⁻8⁺ cells together with immature CD4⁻8⁻ precursors comprise only 10-20% of neonatal or adult thymocytes (8). The majority of thymocytes are CD4⁺8⁺, 50% of which express low levels of surface TCR- α/β -CD3 (9-11). However, direct tests of in vivo developmental potential have shown that only the blast subset, ~10-15% of total CD4⁺8⁺ thymocytes, contains precursors for mature T cells (12). Surface TCR- α/β expression by this progenitor CD4⁺8⁺ subset has not been specifically examined. Small CD4⁺8⁺ thymocytes, including the TCR- α/β -CD3^{lo} subset, are post-mitotic (nonmature) cells that die intrathymically without giving rise to mature progeny (12).

In addition to identifying TCR⁺ maturational stages, elucidating the role of TCR specificity in thymocyte fate determination requires that precursors expressing a TCR clone of known specificity be followed through all phases of intrathymic development. This has been accomplished experimentally by making mice transgenic for a rearranged α and β TCR gene pair of defined antigen/MHC specificity, such that the majority of thymocytes and mature peripheral T cells express the transgenic, rather than highly diverse, endogenous TCR. In such mice, positive selection into either the CD4 or CD8 lineage requires interactions between the transgenic TCR on CD4⁺8⁺ precursors and MHC proteins

expressed in the thymic cortex (13–17). In some TCR- α/β transgenic mice, CD4⁺8⁺ thymocytes also appear to be targets of negative selection (18, 19), whereas, in others, a later maturational stage was implicated (20, 21). This lack of uniformity, which may depend on quantitative and temporal variation in expression of the α and β TCR transgenes (20), makes it difficult to extrapolate these conclusions to maturational events in normal mice. Nonetheless, both positive and negative selection of particular TCR-V β during intrathymic maturation in normal mice have been recently demonstrated, using antibodies and DNA hybridization probes specific for V β segments that preferentially recognize certain polymorphic major (e.g., MHC I-E) or minor (e.g., Mls-1, Mls-2) histocompatibility molecules. For example, positive selection of V β 17⁺ precursors into the CD4 lineage occurs relatively efficiently in mice of the H-2^a and H-2^s MHC haplotypes, but positive selection into the CD8 lineage occurs much more efficiently in H-2^s mice (22–24). In addition, TCRs utilizing V β 6, V β 7, and V β 8.1 are negatively selected in Mls-1^a mice, whereas those using V β 5, V β 11, and V β 17 are negatively selected in MHC I-E⁺ mouse strains (25–28). The cellular target of positive selection in normal mice has not been identified, and although several studies concluded that clonal deletion of self-reactive precursors occurs at a CD4⁺8⁺ maturational stage (29–31), this finding is not universal (7, 25, 32).

Of potential importance to these questions is the heterogeneity in developmental potential of CD4⁺8⁺ thymocytes (12). Because 90% or more of these cells are not immature precursors, and are committed to die within 3–4 d of their last cell division (33), studies that specifically examine the frequency of self-reactive TCR-V β , as well as the influence of TCR specificity on the developmental fate of CD4⁺8⁺ blasts, may help to resolve these issues. These considerations prompted us to assess whether CD4⁺8⁺ blasts might serve as targets of repertoire selection in normal mice. Consistent with this idea, we found that the majority of these precursor cells express functional surface TCR- α/β , but at \sim 1/20 the level (designated TCR^{lo}) found on mature T cells (TCR^{hi}). We then used the intrathymic (IT)¹ transfer system and V β -specific mAbs to follow the development of V β 17⁺ or V β 6⁺ mature T cells from CD4⁺8⁺ blasts in the presence or absence of different positive and negative selecting elements. The results suggest that positive selection of TCR^{lo} CD4⁺8⁺ blast cells induces commitment to the CD4 or CD8 lineage that is manifested by maturation to a TCR^{med} and CD4⁺8^{lo} or CD4^{lo}8⁺ transitional stage. These lineage-committed TCR^{med} transitional cells appear to be the targets of I-E- and Mls-1-mediated negative selection in non-TCR transgenic mice.

Materials and Methods

Mice. (SWR \times DBA/2)F₁ \times SWR backcross progeny of the genotype H-2^{a/d}; V β ^a/V β ^a were mated to produce SWR.DBA/2 mice (V β ^a and H-2^d homozygous), as described (Ruberti, G., A. Livingstone, and C. G. Fathman, manuscript in preparation). C57L

and C57BR/cdj mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All other mice were bred and maintained in our departmental animal facility.

Flow Cytometric Analyses. The sources and fluorochrome modifications of mAbs specific for CD4, CD8, Thy-1.1, and Thy-1.2 have been described (12, 34). Sources and specificities of other antibodies were: 500A2 (anti-CD3e) (11); 44-22-1 (anti-V β 6) (26); KJ23 (anti-V β 17) (35); PE-GK1.5 (Becton Dickinson & Co., Mountain View, CA); biotinylated goat anti-hamster IgG; and avidin-allophycocyanin (Caltag Laboratories, South San Francisco, CA). After staining of freshly isolated thymocytes, fluorescence was analyzed on a highly modified dual laser FACS IV (Becton Dickinson & Co.) with four-decade logarithmic amplifiers, as described (12, 34). For four-color analyses, the excitation wavelength of the dye laser was raised from 598 to 605 nm. The FACS/DESK software program was used to analyze fluorescence data and to generate histograms or two-parameter (5%) probability plots with numerical axis labels that are directly proportional to flow cytometer channel number. Thus, a population with mean fluorescence intensity of 100 is 10-fold brighter than a population with a mean fluorescence intensity of 10.

Intrathymic Injections. The details of this procedure have been described elsewhere (12). Briefly, thymocyte cell suspensions were prepared from 3–4-wk-old mice and enriched for large cells by unit gravity sedimentation. CD4⁺8⁺ blast cells were then isolated by cell sorting, and 5×10^5 to 1×10^6 cells in 10 μ l were injected into each thymic lobe of anesthetized, unirradiated 3–5-wk-old host mice. 3–5 d later, thymocyte suspensions made from individual thymic lobes were stained with biotinylated antibody specific for the donor Thy-1 allele. Cells were washed and resuspended in 1 ml staining medium plus 25 μ l avidin-conjugated paramagnetic beads (Advanced Magnetics, Inc., Cambridge, MA). After 30 min at 4°C with constant mixing, cell-bead complexes were removed from free cells using a magnetic separator (BioMag; Advanced Magnetics, Inc., Cambridge, MA), as described (12), resulting in a 50–200-fold enrichment for donor-derived cells. The enriched cell suspension was then stained with avidin-Texas Red and other antibodies, as indicated in the legend for Table 1.

PCR. cDNA was prepared from poly(A)⁺ RNA (oligo-dT primed; Fig. 1 a) (36, 37), or from total cellular RNA using a α primer plus 20 μ g 5S RNA carrier (Fig. 1 b) (38, 39). PCR amplification using the consensus V α and a perfectly homologous α primer was done using a thermal cycler (Perkin-Elmer Corp., Norwalk, CT) for either 30 (Fig. 1 a) or 60 (Fig. 1 b) cycles. The PCR method, as well as sequences and PCR capabilities of the consensus TCR- α primers, are described in detail elsewhere (39). For Southern blot analysis, PCR products were subjected to electrophoresis on a 1.2% agarose gel, transferred to nylon membrane (Gene Screen Plus; DuPont Co., Wilmington, DE), and hybridized to ³²P hexamer-labeled α DNA (40). Membranes were washed and autoradiographed for 14 h at -70°C using intensifying screens. For dot blot analysis, the total PCR products were phenol/chloroform extracted, ethanol precipitated, resuspended in denaturing buffer and applied to nylon membranes using a Bio-Dot apparatus (Bio-Rad Laboratories, Cambridge, MA), as previously described (41). After UV cross-linking (1200J; Stratagene, La Jolla, CA), the filters were hybridized to ³²P hexamer-labeled α DNA (10^6 cpm/ml), washed, and autoradiographed for 50 h at -70°C with intensifying screens.

Results

TCR Expression by Precursor CD4⁺8⁺ Thymocytes. Intrathymic processes governing selection of the mature α/β

¹ Abbreviation used in this paper: IT, intrathymic.

T cell repertoire presumably act only on maturational intermediates bearing surface TCR. CD4⁺8⁺ thymocytes found mainly, if not exclusively, in the thymic cortex are ideal candidates; they are reportedly the first cells to express TCR- α/β (at low levels) during fetal ontogeny (9), and ~50% of adult CD4⁺8⁺ thymocytes are CD3/TCR^{lo} (10, 11). However, 90% or more of TCR^{lo} CD4⁺8⁺ thymocytes die without further maturation (12, 33), and TCR expression by the precursor (blast) subset of CD4⁺8⁺ thymocytes has not been critically examined. Because rearrangement and expression of the TCR- α locus is thought to be the rate-limiting step for cell surface expression of TCR- α/β during intrathymic development (42), we used the sensitive PCR to ascertain whether TCR- α transcripts are expressed in purified CD4⁺8⁺ blasts. The oligonucleotide primers were designed to amplify TCR- α transcripts containing V, J, and C segments from cDNA, without prior knowledge of the V α segment being used. Due to the dispersal of many J α segments over a 50-kb region and the location of the primers, only cells that have

undergone V-J rearrangement can potentially make transcripts that could serve as PCR substrates. Previous work has shown that this pair of primers can be used in the PCR reaction to amplify VJ α transcripts containing all known V α family members from clonally heterogeneous lymphocyte populations (39). Using this procedure with RNA extracted from purified CD4⁺8⁺ blasts, we detected a DNA segment of appropriate length containing C α sequences from 250 cell equivalents of PCR-amplified material (Fig. 1 a) VJ α transcripts were also reproducibly detectable when RNA was extracted from individual pools of 10 or 100 cells (Fig. 1 b), demonstrating that the frequency of TCR- α -transcribing CD4⁺8⁺ blasts is at least 1 in 10. Using a sensitive three-step staining technique, we found that the majority of CD4⁺8⁺ blasts express surface TCR- α/β (Fig. 1 c). The mean TCR-specific fluorescence of this subset was two- to fourfold lower than that of small CD4⁺8⁺ thymocytes, and ~20-fold lower than that of mature CD4⁺8⁻ thymocytes. Interestingly, CD4⁺8⁺ blasts increased intracellular [Ca²⁺] in response to

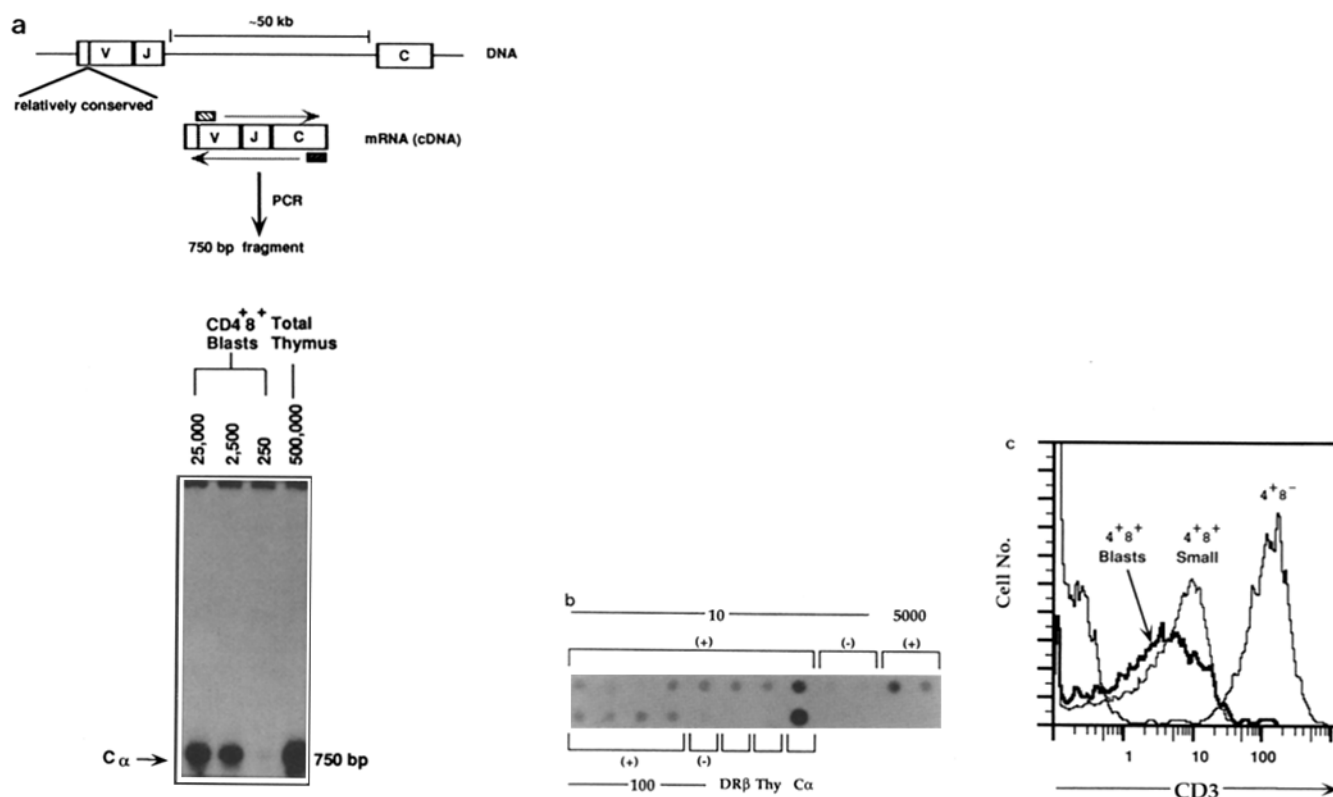


Figure 1. TCR- α/β expression by CD4⁺8⁺ blasts. (a) Detection of TCR- α transcripts by PCR amplification of cDNA with consensus V α oligonucleotide primers. Poly(A)⁺ RNA extracted from 5×10^5 (lane 1) or 5×10^4 (lanes 2 and 3) FACS-sorted CD4⁺8⁺ thymic blasts (C57Bl/Ka), or from 10^7 (lane 4) unfractionated C57Bl/Ka thymocytes, served as a template for first strand cDNA synthesis. PCR amplification was performed with 50% (lanes 1, 2, and 4) or 5% (lane 3) of the cDNA reaction products, and 10% of the PCR reaction products were then subjected to Southern analysis using a C α hybridization probe. Thus, lane 3 contains PCR-amplified material from 250 cell equivalents of RNA. (b) cDNA was prepared from total cellular RNA extracted from multiple pools of 10, 100, and 5,000 purified CD4⁺8⁺ thymic blasts. cDNA reactions were performed with (+) and without (-) reverse transcriptase to control for contaminating template in the subsequent PCR reactions. 500-pg aliquots of purified Thy-1 (TM8) and C α insert DNA were included as negative and positive hybridization controls, respectively. In addition, 5% of an irrelevant PCR reaction (human genomic DNA PCR amplified with HLA-DR β -specific primers, [47]) was processed in parallel to the experimental samples. (c) Surface CD3/TCR expression by CD4⁺8⁺ and CD4⁺8⁻ thymocyte subsets. AKR thymocytes were stained with anti-CD3 (500A2/biotinylated goat anti-hamster/AV-TR), anti-CD4 (allophycocyanin-GK1.5), and anti-CD8 (FITC-53-6.7), and analyzed by flow cytometry. Anti-V γ 3 (536/biotinylated goat anti-hamster/AV-TR) served as an isotype-matched control for anti-CD3 (far left profile). Fluorescence signals were computer gated to display CD3 fluorescence profiles on the indicated subsets.

TCR crosslinking, and the net $[Ca^{2+}]$ increase was only threefold lower than in $CD4^+8^-$ thymocytes (data not shown). These results suggest that the majority of $CD4^+8^+$ blasts possess functional surface TCR, and thus are potential targets of TCR-mediated intrathymic repertoire selection.

Evaluation of $CD4^+8^+$ Thymocyte Subsets as Targets of Negative Selection. Based on evidence from TCR- α/β transgenic mice and bone marrow-reconstituted radiation chimeras, it has been previously concluded that clonal deletion of precursors specific for H-Y, Mls-1^a, and MHC I-E occurs at a $CD4^+8^+$ precursor stage (3). If this also applies to normal unirradiated mice, it would be predicted that $CD4^+8^+$ blasts from Mls-1^a mice would contain fewer $V\beta6^lo$ (Mls-1^a-reactive) cells than in Mls-1^b mice. However, using quantitative three-color flow cytometry, as shown in Fig. 2 *a*, we found that $CD4^+8^+$ blasts from H-2^k mice contain very similar frequencies of $V\beta6^lo$ cells, regardless of Mls-1 genotype (Fig. 2 *b* top). Similarly, the frequency of $V\beta17^lo$ (I-E-reactive) $CD4^+8^+$ blasts was not consistently lower in I-E⁺ strains (Fig. 2 *b*, bottom). In addition, no Mls-1^a- or I-E-specific differences in the frequency of $V\beta6^lo$ or $V\beta17^lo$ cells were discernable within the small $CD4^+8^+$ subset. Thus, we could find no phenotypic evidence for clonal deletion of either the small or blast $CD4^+8^+$ subsets using two different $V\beta$ /self antigen combinations.

The results displayed in Fig. 2 do not rule out the possibility that self-reactive thymocytes receive negative signals at the $CD4^+8^+$ precursor stage that result in physical dele-

tion at later maturational stages. This question was addressed by intrathymic adoptive transfer of purified AKR/J (H-2^k, Mls-1^a, Thy-1.1) $CD4^+8^+$ blasts into unirradiated B10.BR hosts (H-2^k, Mls-1^b, Thy-1.2). The data in Table 1 show that AKR/J $CD4^+8^+$ blasts generated up to 10-fold higher frequencies of $V\beta6^hi$ mature T cells in the "permissive" B10.BR thymic microenvironment than in the "nonpermissive" AKR microenvironment. Conversely, intrathymic transfer of BALB/K (H-2^k, Mls-1^b, Thy-1.2) $CD4^+8^+$ blasts into AKR/J hosts prevented the development of Mls-1^a-reactive ($V\beta6^hi$) mature T cells from BALB/K $CD4^+8^+$ precursors. Similar numbers of $CD3^hi$ mature thymocytes were produced in both cases, but the $V\beta6$ frequency closely approximated that of the host, rather than the donor thymus. Thus, no Mls-1^a/ $V\beta6$ -determined restrictions in developmental potential were demonstrable at the $CD4^+8^+$ maturational stage.

TCR Expression Among Transitional Thymocyte Subsets. To define more precisely TCR⁺ maturational stages during which positive and negative selection might take place in normal mice, we used four-color flow cytometry to characterize TCR/CD3 expression as thymocytes mature from $CD4^+8^+$ precursors into $CD4^+8^-$ or $CD4^+8^+$ cells. We have previously observed cells with TCR^{med} and $CD4^+8^lo$ or $CD4^+8^+$ "transitional" phenotypes as $CD4$ or $CD8$ T cells developed from $CD4^+8^+$ blasts after IT transfer into Thy-1 congenic hosts (12, and data not shown). Thus, commitment to either the $CD4$ or $CD8$ lineage can be observed

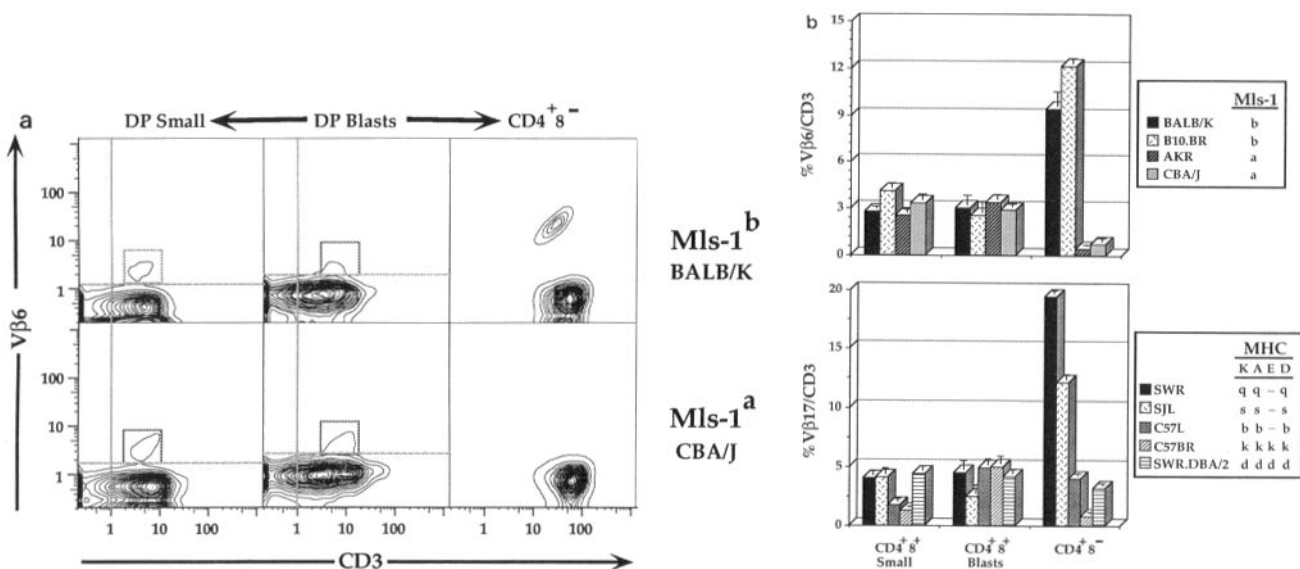


Figure 2. $V\beta6$ and $V\beta17$ frequency among $CD4^+8^+$ and $CD4^+8^-$ thymocytes. (a) $V\beta6$ vs. $CD3$ expression of $CD4^+8^+$ and $CD4^+$ thymocytes in BALB/K and CBA/J mice. Freshly isolated thymocytes were stained with 44-22-1 (anti- $V\beta6$) culture supernatant; goat anti-rat IgG-FITC; 25% normal rat serum to block unbound rat Ig binding sites; PE-GK1.5 (anti- $CD4$), biotin-53-6.7/avidin-allophycocyanin (anti- $CD8$), and 500A2 culture supernatant; and goat anti-hamster IgG-Texas red. After flow cytometric analysis, fluorescence ($CD4$ and $CD8$) and forward scatter (size) signals were computer gated to generate the $V\beta6$ vs. $CD3$ profiles for each subset shown. The dotted lines define the upper limits of background staining, which differ slightly on small vs. blast $CD4^+8^+$ thymocytes, by isotype-matched control antibodies 536 (hamster anti- $V\gamma3$) and R7D4 (rat anti-mouse Ig). The boxes outline $V\beta6^lo$ $CD3^lo$ thymocytes. (b) Thymocytes were stained and analyzed for $V\beta6$ or $V\beta17$ (FITC-KJ23a) vs. $CD3$ expression as described above. BALB/K, B10.BR, AKR, and CBA/J are all H-2^k haplotypes. F23.2 (anti- $V\beta8.2$) served as an isotype-matched control for KJ23a. For both $CD4^+8^+$ subsets, percentages refer to the frequency of $V\beta6^lo$ or $V\beta17^lo$ cells relative to total $CD3^lo$ cells in that subset, whereas percentages indicated for $CD4^+8^-$ cells refer to $V\beta6^hi/CD3^hi$ frequency.

Table 1. Influence of Thymic Microenvironment on *Vβ6* Expression by Mature Progeny of CD4⁺8⁺ Blasts

Donor	Host	Phenotype of donor-derived thymocytes	
		CD3 ^{hi}	<i>Vβ6</i> ^{hi} /CD3 ^{hi}
			%
AKR/J	B10.BR	18	6.1
		14	8.3
		30	9.3
BALB/K	AKR/J	21	<0.5
		19	<0.5
		10	<0.5
AKR control			0.9 ± 0.3
B10.BR control			8.2 ± 1.1
BALB/K control			10.3 ± 1.6

CD4⁺8⁺ blasts (98–99% pure) were isolated by cell sorting and injected intrathymically (3–5 × 10⁵/lobe) into unirradiated 3–5-wk-old hosts. 4.5 d later, thymocyte cell suspensions were enriched for donor-derived cells by immunomagnetic bead selection and analyzed for CD3, CD4, CD8, and *Vβ6* expression as previously described (12).

phenotypically before the maturation process is complete. Because thymocyte maturation occurs continuously and non-synchronously in neonatal and young adult animals, freshly isolated young adult thymocytes were analyzed for the presence of cells displaying transitional phenotypes. CD4^{hi} thymocytes that are CD8^{med} or CD8^{lo} were considered to be likely candidates for cells undergoing a transition from CD4⁺8⁺ precursors to CD4⁺8⁻ mature cells, and they are not observed in secondary lymphoid organs (Fig. 3 *a*). The data in Fig. 3 *b* show that 54% of CD8^{med} and 79% of CD8^{lo} transitional thymocytes bear approximately sevenfold higher TCR levels than TCR^{lo} CD4⁺8⁺ blasts. These cells are designated TCR^{med}, because average TCR density is still two- to threefold less than that of mature CD4⁺8⁻ cells. Cells bearing medium levels of a particular TCR-*Vβ* can be readily identified among transitional CD4⁺8^{med} and CD4⁺8^{lo} thymocytes (Fig. 3 *c*, see legend).

Complementary analysis of CD8^{hi} thymocytes bearing transitional levels of CD4 is complicated by the fact that CD4⁻8⁺ thymocytes include TCR^{-/lo} precursors that give rise to TCR^{lo} CD4⁺8⁺ blasts, as well as TCR^{hi} mature cells that develop from TCR^{lo} CD4⁺8⁺ blasts (12). Thus, additional phenotypic criteria must be used in order to distinguish CD8⁺ thymocytes that are acquiring CD4 from those in the process of losing it. Only the latter maturational event is marked by increases in surface TCR and CD5 density, and *Vβ6*^{med} CD3^{med} cells are evident among CD4^{med}8⁺ and CD4^{lo}8⁺ thymocytes (Fig. 3 *c*, bottom; Table 2). The majority of TCR^{med} cells among these transitional populations were also CD5^{hi} (data not shown), providing further evi-

Table 2. TCR Expression During T Cell Maturation from CD4⁺8⁺ Precursors

Thymocyte subset	Relative CD3 level	TCR designation
CD4 ⁺ 8 ⁺ blasts	1	lo
CD4 ⁺ 8 ^{lo}	5–7	med
CD4 ⁺ 8 ⁻	15–20	hi
CD4 ^{lo} 8 ⁺	2–3	med
CD4 ⁻ 8 ⁺	12–15	hi

TCR expression by transitional and mature thymocytes was evaluated relative to that of TCR^{lo} CD4⁺8⁺ blasts. The mean CD3-specific fluorescence intensity of each subset was divided by that of the TCR^{lo} CD4⁺8⁺ blast subset, which was arbitrarily set to 1. CD4⁺8^{lo} thymocytes include both the CD8^{med} and CD8^{lo} populations defined in Fig. 3, and CD4^{lo}8⁺ include CD4^{med} and CD4^{lo} cells. These maturational stage-specific differences in relative TCR level were always observed, regardless of mouse strain or anti-TCR antibody used.

dence that they are in transition from CD4⁺8⁺ blasts to TCR^{hi} (and CD5^{hi}) CD4⁻8⁺ mature thymocytes. The TCR^{lo} cells within the CD4⁻8⁺ subset (Fig. 3 *c*, bottom right) are CD5^{lo} precursors of CD4⁺8⁺ thymocytes (12, and data not shown). Note that the relative level of TCR differs among cells committed to the CD4 vs. CD8 lineage (Table 2). For example, CD4⁺8^{lo} transitional thymocytes express sevenfold more surface TCR than TCR^{lo} CD4⁺8⁺ blasts, whereas CD4^{lo}8⁺ cells express only threefold more. In addition, maturation from the CD4^{lo}8⁺ to CD4⁻8⁺ stage involves a greater increase in surface TCR level than the analogous maturation step in the CD4 lineage.

*Mls-1 and I-E-specific Differences in the Frequency of *Vβ6* and *Vβ17* Expression among TCR^{med} Transitional Thymocytes.* Numerous studies in both normal and TCR- α/β transgenic mice have suggested that maturation of CD4⁺8⁺ precursors into CD4⁺8⁻ and CD4⁻8⁺ T cells occurs as a result of TCR-mediated positive selection. Accordingly, there should be a direct correlation between the frequency of a particular *Vβ* among lineage-committed TCR^{med} descendants of CD4⁺8⁺ blasts, and the presence of a positive selecting element for that *Vβ*. A hierarchy of positive selection efficiency for *Vβ17*⁺ TCR has been demonstrated among I-E⁻ haplotypes: H-2^a > H-2^s > H-2^b for the CD4 lineage, whereas H-2^s > H-2^a for the CD8 lineage (22–24). We therefore compared *Vβ17* frequencies among TCR^{lo} CD4⁺8⁺ blasts, TCR^{med} transitional cells, and TCR^{hi} CD4 or CD8 T cells in these and other H-2 haplotypes. The data in Fig. 4 *a*, top show that among TCR^{med} CD4⁺8^{lo} transitional cells in I-E⁻ haplotypes, the *Vβ17* frequency is highest in H-2^a and lowest in H-2^b mice. Among TCR^{med} transitional thymocytes committed to the CD8 lineage (CD4^{lo}8⁺), the *Vβ17* frequency is higher in H-2^s than in H-2^a mice (Fig. 4 *b*, top). Thus, *Vβ17* frequency at the TCR^{med} transitional stage reflects the previously described efficiency of positive selection for each strain. Note that for both *Vβ6* and *Vβ17*, the frequency among

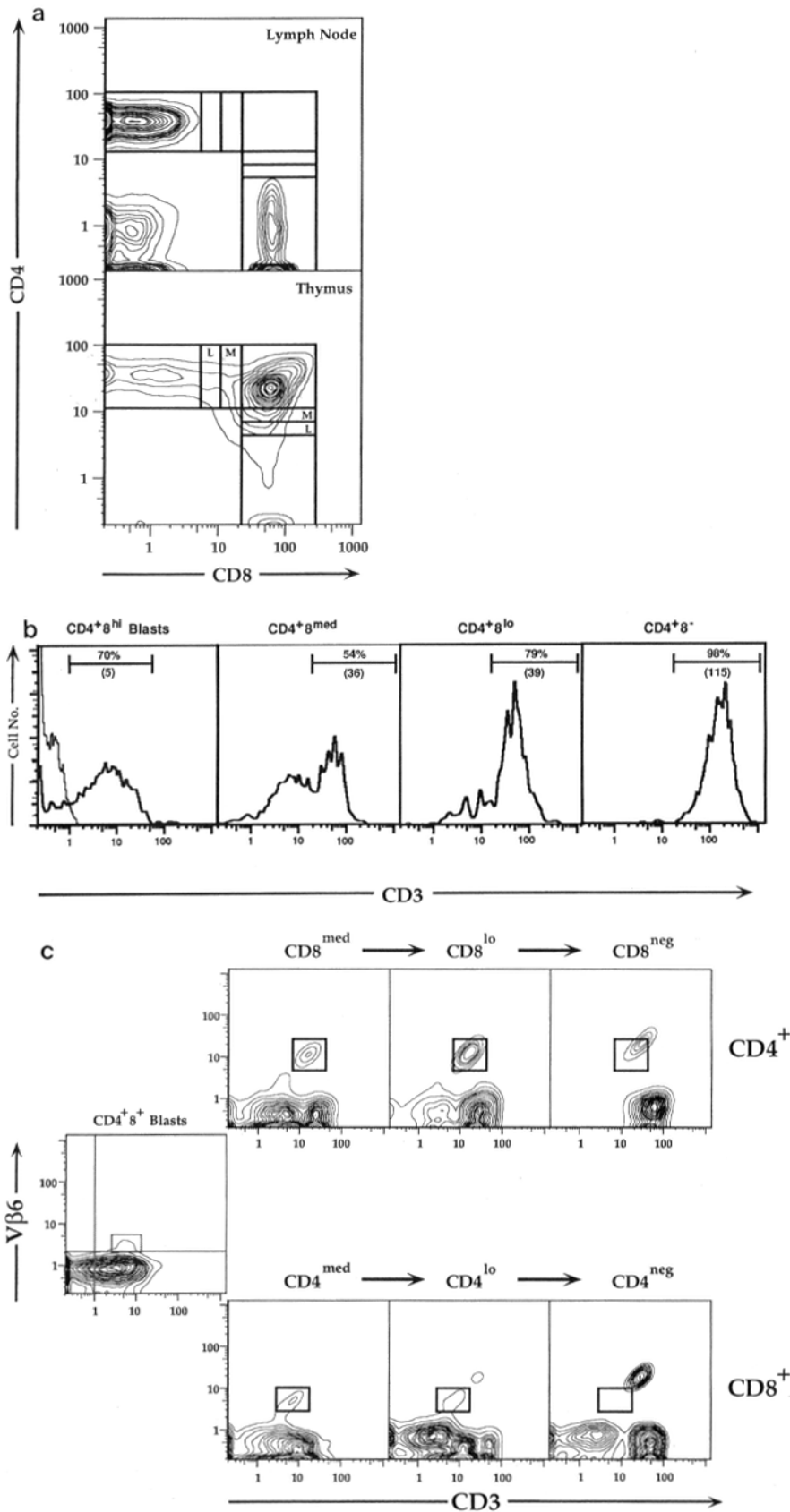


Figure 3. (a) Comparison of CD4 and CD8 expression by lymph node cells and thymocytes as used to define transitional subsets. AKR lymph node cells and thymocytes were stained with 500A2 (or 536)/biotin anti-hamster IgG/Texas red-avidin, FITC-CD8, and allophycocyanin-CD4, and the fluorescence signals of 500,000 cells were analyzed by flow cytometry. CD4⁺ AKR thymocytes were divided into CD8^{hi} blasts, CD8^{med} (M), CD8^{lo} (L), and CD8⁻ subsets. The CD3 fluorescence profile of each subset is shown in *b*. (b) Percentages of cells with fluorescence intensities falling within the range indicated by the bar are shown, with the mean fluorescence intensity of that population noted in parentheses below the bars. CD4⁺8^{med} thymocytes (3–5% of total) expressed an average of twofold less CD8 than the peak CD8 fluorescence of CD4⁺8^{hi} blasts. CD4⁺8^{lo} (1–3% of total) expressed an average of twofold more CD8 than the highest background level found on CD4⁺ lymph node T cells. CD8^{hi} thymocytes were similarly divided into CD4^{med} and CD4^{lo} subsets that constituted 5–7% and 2–4% of total thymocytes, respectively. The CD3 vs. Vβ6 fluorescence profile of each thymocyte subset is shown in *c*. (c) BALB/K thymocytes were stained as described in Fig. 2 *a*. The dotted box defines Vβ6^{lo}/CD3^{lo} thymocytes, and solid boxes outline Vβ6^{med} cells. Note the higher Vβ6 and CD3 density among CD4⁺8⁻ and CD4⁻8⁺ thymocytes. Within the CD8⁺ subset, TCR^{med} and TCR^{hi} cells were all CD5^{hi} (data not shown). In subsequent analyses, the transitional designations CD4⁺8^{lo} and CD4^{lo}8⁺ include CD8^{med} plus CD8^{lo} cells and CD4^{med} plus CD4^{lo} cells, respectively, since the only apparent difference between the medium and low transitional phases is the frequency of TCR^{med} cells.

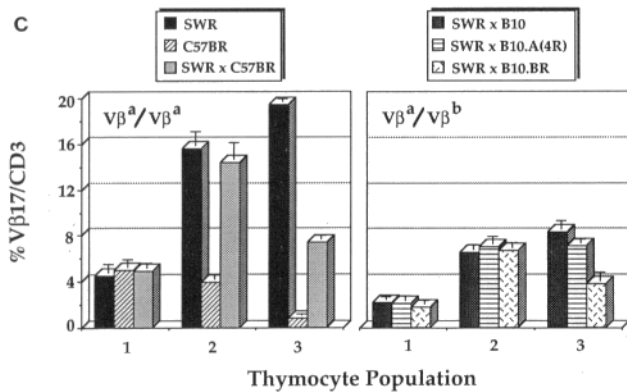
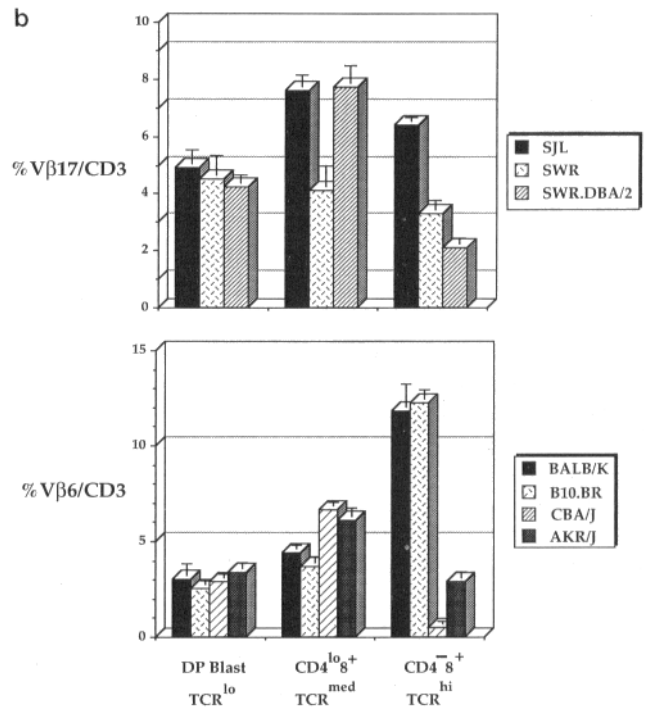
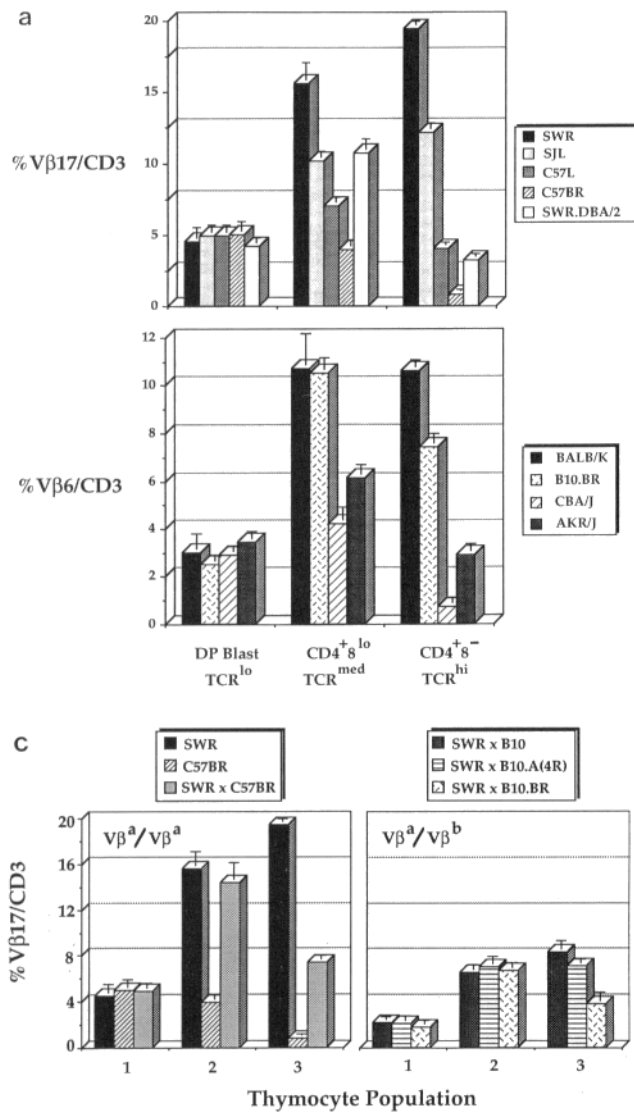


Figure 4. Frequency of $V\beta 6$ and $V\beta 17$ among $TCR^{lo} CD4^{+}8^{+}$ blasts, TCR^{med} transitional cells, and TCR^{hi} mature thymocytes in different mouse strains. Staining, flow cytometric analyses, and transitional phenotypic designations were carried out as described in Fig. 3. Thymocyte population 1, $TCR^{lo} CD4^{+}8^{+}$; population 2, $TCR^{med} CD4^{+}8^{lo}$; population 3, $TCR^{hi} CD4^{+}8^{-}$.

both TCR^{med} transitional lineages does not correlate with the presence of a deleting element. For example, SJL ($I-E^{-}$) and SWR.DBA/2 ($I-E^{+}$) mice have virtually identical $V\beta 17$ frequencies among transitional $TCR^{med} CD4^{+}8^{lo}$ thymocytes (Fig. 4 a, top). With respect to the CD8 lineage, $V\beta 6$ and $V\beta 17$ frequencies among TCR^{med} transitional thymocytes were often higher in the deleting strains (Fig. 4 b, bottom). These observations support the idea that maturation from $TCR^{lo} CD4^{+}8^{+}$ blasts to the $TCR^{med} CD4^{+}8^{lo}$ or $CD4^{lo}8^{+}$ transitional stages occurs as a result of positive selection, and that negative selection occurs after acquisition of these transitional phenotypes.

One prediction of this hypothesis would be that the outcome of positive selection should be demonstrable in TCR^{med} transitional cells even in strains that coexpress a negative selecting element. We therefore compared $V\beta 17$ frequency at different maturational stages in F_1 mouse strains that all express the $H-2^q$ -positive selecting element, but differ in expression of two $H-2^k$ -deleting elements ($I-E^k$ and

K^k/A^k) (23). Because $V\beta^a/V\beta^b$ haplotypes have only one functional copy of the $V\beta 17$ structural gene, $V\beta 17$ frequency at all maturational stages is only $\sim 50\%$ of that seen in $V\beta^a$ homozygous mice with comparable selecting elements. Among $V\beta^a$ homozygous strains, $V\beta 17$ frequency among $TCR^{med} CD4^{+}8^{lo}$ transitional thymocytes was high and nearly identical in both $H-2^q$ haplotypes: SWR and (SWR \times C57BR) F_1 (Fig. 4 c). Similarly, the $H-2^q$ -positive $V\beta^a/V\beta^b$ haplotypes had very similar frequencies of $V\beta 17$ among transitional $CD4^{+}8^{lo}$ thymocytes. Thus, the influence of the $H-2^q$ -positive selecting element on $V\beta 17$ frequency is observable at the $TCR^{med} CD4^{+}8^{lo}$ transitional stage even in the presence of the $H-2^k$ -negative selecting elements. In $V\beta^a/V\beta^b$ mice, $V\beta 17$ frequency among $TCR^{hi} CD4^{+}8^{-}$ thymocytes was highest in (SWR \times B10) F_1 mice (no $H-2^k$ -deleting elements) and lowest in (SWR \times B10.BR) F_1 mice (both $H-2^k$ -deleting elements) (Fig. 4 c). (SWR \times B10.A [4R]) F_1 mice (K^k/A^k -deleting element only) had only slightly fewer $V\beta 17^{+} CD4^{+}8^{-}$ thymocytes than (SWR \times B10) F_1 mice,

suggesting that K^k/A^k causes only minor deletion of $V\beta 17^+$ TCR in these mice. These results suggest that genetic influences of the $H-2^q$ -positive selecting element and the $H-2^k$ -negative selecting elements on $V\beta 17$ frequency are manifested at distinct thymocyte maturational stages.

Discussion

During the intrathymic maturation of T lymphocytes, immature precursors receive positive and/or negative TCR-mediated signals that specify cell fates, but the precursor cells and the mechanisms involved have remained obscure. Using the IT transfer system to determine directly the developmental potentials of candidate precursor populations, we recently showed that $CD4^+8^+$ blast cells, which arise in the outer thymic cortex from $TCR^- CD4^-8^-$ precursors via $CD8^+$ intermediates, have three possible developmental fates; the majority retain the $CD4^+8^+$ phenotype, become small, and die in situ, whereas a minority (20–40% at most) mature into $CD4^+8^-$ or $CD4^-8^+$ thymocytes (12). In this study, we have shown that most $CD4^+8^+$ blasts express low levels of functional surface TCR, and that they mature into $TCR^{hi} CD4^+8^-$ or $CD4^-8^+$ thymocytes via a TCR^{med} transitional intermediate stage. The influence of different positive and negative selecting elements on the developmental fate of $TCR^{lo} CD4^+8^+$ blasts and their $TCR^{med} CD4^+8^{lo}$ and $CD4^{lo}8^+$ progeny was examined, and the results are most consistent with the idea that negative selection occurs after $TCR^{lo} CD4^+8^+$ blasts have been positively selected into the CD4 or CD8 lineages.

Because positive selection is thought to involve interactions between MHC proteins on epithelial cells in the thymic cortex and the TCR complex on immature thymocytes (2, 32, 43, 44), the TCR^{lo} phenotype and outer cortical location of $CD4^+8^+$ blasts make them good candidates for targets of TCR-mediated positive selection. However, the frequencies of $V\beta 6^{lo}$ and $V\beta 17^{lo} CD4^+8^+$ blasts in different mouse strains were very similar, and did not vary in a way that correlated with the presence of known positive (or negative) selecting elements for these receptors (Fig. 2 *b*). Thus, the outcome of positive selection is not demonstrable at this maturational stage, and has only been observed previously in mature thymocytes (43, 44). Here, we present two observations that suggest that maturation of $TCR^{lo} CD4^+8^+$ blasts to the TCR^{med} transitional stage occurs as a result of positive selection. First, in the absence of a strong deleting element, the frequency of $V\beta 6$ and $V\beta 17$ among TCR^{med} transitional thymocytes was usually very similar to the mature frequency (Fig. 4). Moreover, the transitional frequency was generally higher than that among $TCR^{lo} CD4^+8^+$ blasts, even in deleting strains. Second, the frequency of $V\beta 17^+ TCR^{med}$ transitional thymocytes varied in a strain-specific way that correlates perfectly with the known positive selection efficiency of each strain for this receptor, even when $H-2^k$ -deleting elements were coexpressed in the same thymus (Fig. 4, *a* and *b*, *top*; *c*). In contrast, there was no universal correlation between $V\beta 6$ and $V\beta 17$ transitional fre-

quency among TCR^{med} thymocytes and the presence of deleting elements for these receptors. For example, $V\beta 17$ frequency among TCR^{med} transitional cells was very similar in SJL ($I-E^-$) and SWR.DBA/2 ($I-E^+$) mice, suggesting that like $H-2^s$, $H-2^d$ may provide efficient positive selection for $V\beta 17$ into both the CD4 and CD8 lineage, whereas positive selection of $V\beta 17$ in $H-2^q$ (SWR) mice is significantly skewed towards the CD4 lineage (22, 23). Previous studies have not determined positive selection efficiency in deleting strains, because negative selection is genetically dominant (7), so the low $V\beta 6$ and $V\beta 17$ frequencies we observed among $TCR^{med} CD4^+8^{lo}$ thymocytes in some deleting strains (C57BR for $V\beta 17$, and CBA/J and AKR/J for $V\beta 6$; Fig. 4 *a*) could be due to poor positive selection.

Several observations lead us to suggest that negative selection occurs only after thymocytes have matured to the TCR^{med} transitional stage and show evidence of commitment to the CD4 or CD8 lineage. First, in deleting strains, maturation from the TCR^{med} to TCR^{hi} stage was marked by a decrease in frequency of $V\beta 6^+$ or $V\beta 17^+$ cells, without exception. Second, in F_1 mice that coexpress the $H-2^q$ -positive selecting element and $H-2^k$ -deleting elements, $V\beta 17$ frequency was high among $TCR^{med} CD4^+8^{lo}$ transitional thymocytes but low among $TCR^{hi} CD4^+8^-$ cells (Fig. 4 *c*). Finally, $TCR^{lo} CD4^+8^+$ blasts from $Mls-1^a$ mice showed no irreversible commitment to delete $V\beta 6^+$ cells when transferred to an $Mls-1^a$ thymus (Table 1). These observations strongly suggest that positive and negative selection occur sequentially during thymocyte maturation.

$CD4^+8^+$ thymocytes have been implicated as the targets of clonal deletion based on the severe depletion of $CD4^+8^+$ thymocytes observed in transgenic mice expressing self-reactive TCR α and β transgenes (15, 18, 19, 21). However, the developmentally precocious and higher levels of TCR expression observed in TCR- α/β and TCR- α transgenic mice (20) could cause depletion of the precursor pool, rather than $CD4^+8^+$ thymocytes themselves. For example, $CD4^-8^+$ (and perhaps also $CD4^-8^-$) progenitors could be subjected to TCR-mediated differentiation signals that lead to premature clonal deletion or accelerated maturation and export from the thymus. In support of this idea, our preliminary data show that transgenic expression of a TCR α chain with no known anti-self reactivity significantly reduces the frequency of $CD4^+8^+$ thymocytes (and increases the frequency of $TCR^{hi} CD4^-8^-$) that develop within 1–3 d from IT-injected $TCR^- CD4^-8^-$ precursors (Guidos, C., B. Fazekas de St. Groth, I. Weissman, and M. Davis, manuscript in preparation).

Phenotypic comparisons of the frequency of particular $V\beta$ segments among $CD4^+8^+$ thymocytes in deleting and non-deleting strains have implicated $CD4^+8^+$ thymocytes as targets of clonal deletion in normal mice. $CD4^+8^+$ thymocytes were reported to have 1.3–2-fold lower frequencies of $V\beta 3$ ($Mls-2^a$ -reactive) and $V\beta 6$ ($Mls-1^a$ -reactive) in nonpermissive $Mls-2^a$ and $Mls-1^a$ strains relative to permissive $Mls-2^b$ and $Mls-1^b$ strains (31). However, similar studies of $V\beta 17$ frequency have yielded conflicting results (7, 31, 32), and no $Mls-1$ -specific differences in $V\beta 8.1$ frequency were observed

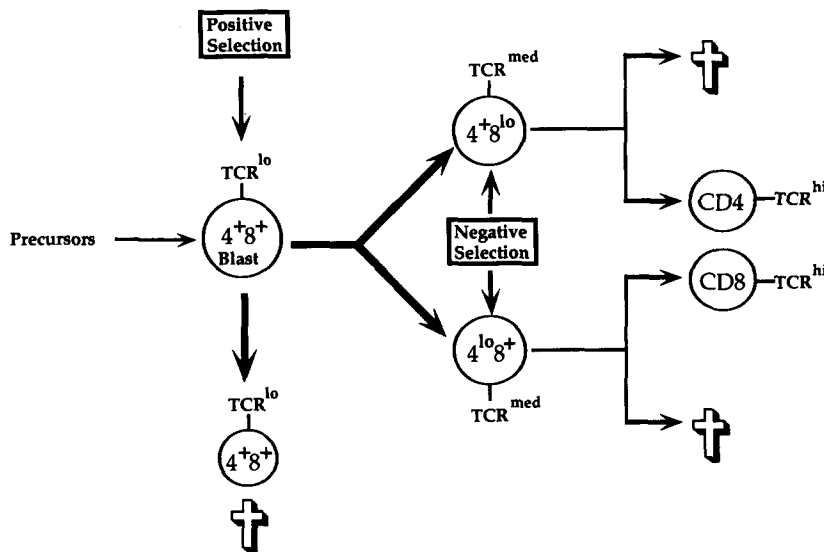


Figure 5. Sequential view of TCR-mediated signaling events during maturation of the CD4⁺ and CD8⁺ T cell lineages. In this model, the response of immature thymocytes to TCR-mediated signals is developmentally programmed. Signals received by TCR^{lo} CD4⁺8⁺ blasts are postulated to result in positive selection, manifested by maturation to the TCR^{med} CD4⁺8^{lo} or TCR^{med} CD4^{lo}8⁺ transitional stage and susceptibility to negative selection. Signals received during this transitional phase are postulated to result in clonal deletion. Complete maturation would require first a TCR-mediated signal at the TCR^{lo} CD4⁺8⁺ blast stage, followed by no signal during the transitional phase.

among CD4⁺8⁺ thymocytes (25, 31). Because the majority of CD4⁺8⁺ thymocytes being detected in these studies are not immature precursor cells, the significance of very small strain-specific differences in Vβ frequency among CD4⁺8⁺ thymocytes is unclear. We found no phenotypic evidence for clonal deletion of Vβ6, Vβ17 (Fig. 2), or Vβ3, Vβ7, and Vβ11 (data not shown) among precursor (blast) or small CD4⁺8⁺ thymocytes in nonpermissive strains.

Genetic studies showing that class II MHC-restricted deleting elements for the CD4 lineage often result in symmetrical deletion of the CD8 lineage have provided circumstantial evidence for deletion during a CD4⁺8⁺ maturational phase. More direct support for this idea was obtained from studies showing that anti-CD4 treatment of bone marrow radiation chimeras during the thymic regenerative phase (29, 30) allowed Vβ6⁺ or Vβ17⁺ CD4⁻8⁻ thymocytes to develop in a normally nonpermissive host. The authors suggested that CD4⁺8⁺ precursors received negative signals before they became committed to the CD4 or CD8 lineage, but this experimental approach can only address the functional involvement of CD4 during clonal deletion. In contrast, we present direct evidence that Vβ6^{lo} CD4⁺8⁺ blasts from an Mls-1^a donor have not received negative signals sufficient to cause deletion when transferred to an Mls-1^b host (Table 1). Furthermore, clonal deletion of Mls-1^a and I-E-autoreactive thymocytes in normal mice was evident only after thymocytes have attained a TCR^{med} CD4⁺8^{lo} or CD4^{lo}8⁺ transitional phenotype that is indicative of commitment to the CD4 or CD8 lineage, respectively (Fig. 4). Although we have evidence from Mls-1 congenic mice and I-E transgenic mice that Mls-1^a and I-E can function as deleting elements for Vβ6⁺ or Vβ17⁺ cells of the CD8 lineage in these strains (data not shown), we have no evidence of the functional involvement of CD4 in this process. However, that is the implication of studies showing that anti-CD4 or anti-I-E (45) can block deletion of Vβ6⁺ or Vβ17⁺ CD4⁻8⁺

thymocytes. Taking all of these observations into account, we suggest that TCR^{med} CD4⁺8^{lo} and CD4^{lo}8⁺ transitional thymocytes are the targets of negative selection, at least for these particular Vβ/self antigen combinations. Confirmation of this hypothesis will require cell transfer experiments to assess the developmental fate of TCR^{med} transitional thymocytes in the presence and absence of various deleting elements.

Models of T lymphocyte maturation must ultimately explain how TCR-mediated signals delivered to immature precursors can result in at least two distinct cell fates: rescue from programmed cell death and maturation (positive selection), or death by apoptosis (negative selection). Others have suggested that the different outcomes of positive and negative selection are determined by qualitative differences in the TCR signal, because many studies have shown that positive selection takes place primarily in the thymic cortex, whereas negative selection occurs primarily in the thymic medulla (2, 32, 43, 44). Thus, cortical and medullary thymic stromal cells may provide different non-TCR accessory signals, or different self-peptide/self-MHC complexes. Another possibility is that the developmental outcome (positive or negative selection) is determined by quantitative differences in the TCR signal. Low avidity interactions would generate few signals and result exclusively in positive selection, whereas high avidity interactions would invariably lead to clonal deletion (2). Because our results are most consistent with the idea that positive and negative selection occur sequentially during distinct thymocyte maturational stages, we propose a third model, which posits that the response of TCR⁺ immature thymocytes to TCR-mediated signals is developmentally programmed, as originally suggested for B cells (46). Thus, signaling through the TCR would result in positive selection for TCR^{lo} CD4⁺8⁺ blasts, negative selection for TCR^{med} transitional cells, and proliferation and differentiation into effector cells for TCR^{hi} mature cells, as outlined schematically in Fig. 5. A concomitant increase in response threshold

as thymocytes progress from the TCR^{lo} to TCR^{med} stages would ensure that at least some positively selected cells escape clonal deletion. Selective localization of these events would result if changes in developmental programming are temporally coincident with cell migration from the cortex to the medulla. Although the genetic dominance of negative selection is explained equally well by our model and the quantitative model, the latter is hard to reconcile with our observation that the outcome of positive selection of V β 17⁺ precursors is ap-

parent even when a strong deleting element is present in the same thymus (Fig. 4 c). The in vivo studies of thymocyte maturation described here provide a framework for devising in vitro strategies, using purified normal thymocytes of defined maturational stages, to determine which model of repertoire selection is correct, and ultimately to investigate the molecular and genetic basis of TCR-mediated specification of thymocyte fates.

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