Onset of TCR-β Gene Rearrangement and Role of TCR-β Expression During $CD3^-CD4^-CD8^-$ Thymocyte Differentiation[1]

Dale I. Godfrey,* Jacqueline Kennedy,* Peter Mombaerts,† Susumu Tonegawa,† and Albert Zlotnik[2]*

*DNAX Research Institute, Palo Alto, CA 94304, and †Howard Hughes Medical Institute at the Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

TCR-β gene rearrangement or expression is necessary and sufficient for the progression of early $\alpha\beta$ thymocyte differentiation from the $CD3^-CD4^-CD8^-$ triple negative (TN)[3] to the $CD4^+CD8^+$ double positive stage. The onset of TCR-β rearrangement is currently thought to occur gradually. Some thymocytes were reported to be rearranged at the earliest ($CD44^+CD25^-$) TN stage, whereas other thymocytes did not initiate TCR-β rearrangement until the latest ($CD44^-CD25^-$) TN stage. Here, we have isolated subsets of TN thymocytes on the basis of surface expression of CD44 and CD25, with c-*kit* as an additional marker. We present a revised model of early T cell development in which TCR-β and TCR-γ rearrangements occur abruptly, at the $CD44^{low}CD25^+$c-*kit*^{low}TN stage. A high level of c-*kit* expression defines pro-T cells which have not yet rearranged their TCR genes. Germ-line TCR-β transcripts, and transcripts of recombination activating genes (RAG)-1 and 2, are detected before TCR-β gene rearrangement. Analyses of TN thymocytes of RAG-1 mutant mice, and of various TCR mutant and TCR transgenic RAG-1 mutant mice, indicate the existence of a control point at the $CD44^-CD25^+$TN stage at which cells expressing a productively rearranged TCR-β chain are selected for further differentiation. *Journal of Immunology,* 1994, 152: 4783.

T cell development occurs mainly in the thymus. The vast majority of T cells in lymphoid organs are $\alpha\beta$ T cells and express a clonally diverse TCR composed of the products of rearranged TCR-α and TCR-β genes. The minor population of $\gamma\delta$ T cells carries a TCR consisting of rearranged TCR-γ and TCR-δ products. These TCR genes are assembled through a process of DNA rearrangement known as V(D)J recombination (reviewed in 1, 2), which is controlled by RAG-1 and -2 (3, 4). Thymic $\alpha\beta$ T cell development entails a series of differentiation stages defined by expression of several surface markers (reviewed in 5, 6). Triple negative thymocytes are among the earliest precursors in the thymus. These cells subsequently become double positives (DP),[3] half of which have detectable levels of CD3/TCR$\alpha\beta$ on the surface. At this stage, thymocytes undergo positive and negative selection, and the surviving cells eventually progress to the mature $CD4^+CD8^-$ or the $CD4^-CD8^+$ single positive stages.

Recent studies with RAG and TCR mutant mice (3, 4, 7) have highlighted the importance of TCR-β in the differentiation of $\alpha\beta$ T cells from the TN to the DP stage. Thymocyte development in RAG-1 or RAG-2 mutant mice is blocked at the TN stage (3, 4). When a rearranged TCR-β transgene was crossed into either the RAG-1 or RAG-2 mutant background, thymocyte development progressed through to the DP stage (7, 8). These data indicate that V(D)J recombination is required for a thymocyte to progress beyond the TN stage, and that expression of a single functional TCR-β product is sufficient to obtain wild-type (WT) numbers of thymocytes. Proof of the essential nature of the role of TCR-β in early thymocyte

Received for publication November 17, 1993. Accepted for publication February 25, 1994.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] DNAX Research Institute is supported by Schering Plough Corporation. P. Mombaerts was a Howard Hughes Medical Institute Predoctoral Fellow in Biological Sciences. S. Tonegawa acknowledges grant support from Howard Hughes Medical Institute and National Institutes of Health.

[2] Address correspondence and reprint requests to Dr. Albert Zlotnik, DNAX Research Institute, 901 California Ave., Palo Alto, CA 94304.

[3] Abbreviations used in this paper: DP, double positives; Lin, lineage markers; RAG; recombination activating genes; TN, triple negative; WT, wild-type; DN, double negative.

Copyright © 1994 by The American Association of Immunologists

0022-1767/94/$02.00

differentiation was delivered by mutating TCR genes (7). The thymus of TCR-β mutant mice contains 10-fold fewer cells than the WT thymus, and 50% of these are DP. When a TCR-δ mutation was crossed into the TCR-β mutant background (7), the total number of thymocytes was further reduced, accompanied by an almost complete absence of DP thymocytes. These data indicate that TCR-β rearrangement or expression is indeed required for the TN to DP transition and expansion of thymocytes, at least in the major αβ T cell lineage.

Given the essential role of TCR-β in early thymocyte differentiation, it is important to define precisely the onset of TCR-β rearrangement and expression in TN thymocytes of WT mice, and to determine whether the block in TN thymocyte development observed in RAG or TCR mutant mice is consistent with this timing. The earliest thymocytes express low levels of CD4 and have not rearranged their TCR genes (9). These cells are the immediate precursors of TN thymocytes, which have been classified into three main subsets on the basis of thymic repopulation potential, TCR-β gene configuration, and expression of CD44 (Pgp-1) and CD25 (IL-2 receptor α-chain; 10, 11; reviewed in Ref. 12). In the first subset ($CD44^+CD25^-$), about 50% of the TCR-β alleles were reported to be rearranged. This increased to approximately 75% by the second TN stage ($CD25^+$) and were almost complete by the last TN stage ($CD44^-CD25^-$; 10). We have recently identified four phenotypically and functionally distinct TN subsets (13) based on the expression of CD44, CD25 and c-*kit* (stem cell factor receptor) (14–17). The maturational sequence of these cells is $CD44^+CD25^-$c-kit^+ → $CD44^+CD25^+$c-kit^+ → $CD44^-CD25^+$c-kit^{low} → CD44 $CD25^-$c-$kit^{very\ low}$. We also made the unexpected observation that $CD44^+CD25^+$TN showed a germ-line configuration at the TCR-β locus. This was difficult to reconcile with the previous model where the onset of TCR-β rearrangement was placed at the earlier $CD44^+CD25^-$TN stage (10), which precedes the $CD44^+CD25^+$ stage in our revised model (13).

In the present study, we purified each TN subset to demonstrate that rearrangement of the TCR-β genes occurs abruptly as the $CD25^+$TN cells down-regulate CD44 and c-*kit* expression. TCR-β rearrangement is preceded by the appearance of TCR-β mRNA (probably germ-line transcripts) and RAG-1 and RAG-2 transcripts, and concurs with the onset of TCR-γ rearrangements. In addition, using RAG-1 and various TCR mutant and TCR transgenic RAG-1 mutant mice, we show that αβ T cell differentiation is arrested at the $CD44^-CD25^+$ stage, unless TCR-β is successfully rearranged. Thus, there is a correlation between the timing of TCR-β rearrangement and expression in WT mice and the differentiation block in RAG-1 or TCR mutant mice. We conclude that TCR-β gene rearrangements are activated at the $CD44^{low}CD25^+$TN stage, and that full length TCR-β transcripts first appear in the $CD44^-CD25^+$TN subset, a stage that is achieved independent of any TCR gene rearrangements. αβ T cell differentiation is arrested at this control point unless a rescue signal is provided by a rearranged TCR-β chain.

Materials and Methods

Mice

Four- to six-wk-old male BALB/c mice were used, unless otherwise stated. RAG-1 and TCR mutant mice or TCR transgenic RAG-1 mutant mice were in a (129 × C57BL/6) mixed background.

Isolation of TN thymocyte subsets

Triple negative thymocytes were enriched using complement killing of CD4- and CD8-expressing cells as previously described (18). Enriched TN cells were subsequently labeled with anti-c-*kit* (clone ACK2, a gift of Dr. S.-I. Nishikawa, Institute for Medical Immunology, Kumamoto, Japan), followed by goat anti-rat IgG-Texas red (mouse adsorbed; Caltag Laboratories, South San Francisco, CA). Unoccupied binding sites of the latter reagent were blocked with rat serum, followed (without washing) by anti-CD3 (clone 145–2C11, PharMingen, San Diego, CA), anti-CD4 (clone RM4–4, PharMingen), anti-CD8 (clone 53–6.7, Becton Dickinson, San Jose, CA), anti-B220 (clone RA3–6B2, PharMingen), anti-Mac-1 (clone M1/70, Caltag Laboratories), anti-Gr-1 (clone RB6–8C5, PharMingen), (all labeled with biotin), anti-CD44 phycoerythrin (clone IM7, PharMingen) and anti-CD25-FITC (clone 7D4, A. Miyajima, DNAX, Palo Alto, CA). Biotinylated reagents were subsequently labeled with streptavidin-allophycocyanin (Caltag) and sorted using a $FACStar^{Plus}$ (Becton Dickinson, Mountain View, CA). In some cases, when c-*kit* expression was not one of the criteria for separation of these cells, the first three steps of this staining procedure were omitted. The purity of sorted TN populations was always greater than 97%.

Southern blot analysis

High m.w. DNA was prepared from 5×10^5 sorted TN thymocytes as previously described (10). The DNA was digested to completion with either *Hin*dIII or *Eco*RI. Digested DNA was electrophoresed in a 0.8% agarose gel and then transferred to a GeneScreen membrane, according to manufacturer's recommendation (DuPont, Wilmington, DE). The filters were hybridized as described (13) with the appropriate ^{32}P-labeled probes. The *Eco*RI fragment of the 86T5 murine Cβ2 plasmid (a gift from Y. Chien, Stanford University Medical School, CA, and described by Born et al., 19) and the *Eco*RI/*Cla*I fragment of Jβ2 were used to probe *Hin*dIII-digested DNA. The Cγ4 plasmid, pMGC513 (a gift of Dr. David Ferrick, Dept. Immunology, U.C. Davis), was used to probe *Eco*RI-digested DNA. The filters were subsequently washed in 0.2 × SSC/0.1% SDS at 50°C and exposed to Kodak XAR x-ray film (Eastman Kodak, Rochester, NY) at −70°C for 1 to 5 days.

Repopulation of fetal thymic lobes in the presence of anti-c-kit

$CD44^+CD25^-$TN were sorted independently of c-*kit* expression, and 5×10^3 of these cells were combined with 2-deoxyguanosine-treated fetal thymic lobes (prepared as previously described, 18, 20, 21) in a hanging drop culture for 2 days in the presence or absence of anti-c-*kit* or control IgG2a (both at 50 μg/ml). Lobes were subsequently cultured in fetal thymic organ culture for a further 26 days in media alone (no mAb added), and the thymocytes were recovered and counted using a hemocytometer.

CD4 expression by $CD44^+CD25^-$c-kit^+"TN"

$CD44^+CD25^-$c-kit^+TN cells were sorted and labeled with a different anti-CD4 mAb (anti-CD4-FITC; clone RM4–5, PharMingen) from that used in previous steps, or control rat IgG-FITC (PharMingen), and analyzed using a FACScan (Becton Dickinson) utilizing LYSYS II software (Becton Dickinson).

Northern blot analysis

Total RNA from 2 × 10^6 sorted $CD44^+CD25^+$, $CD44^-CD25^+$, and $CD44^-CD25^-$TN was prepared using the RNAzol method (TM Cinna Scientific Inc., Friendswood, TX) and mRNA was prepared using the Micro-Fast Track *kit* (Invitrogen Corp., San Diego, CA). Both total RNA and mRNA were separated on 1% agarose/formaldehyde gels and transferred to GeneScreen membranes according to the manufacturer's recommendations (Dupont). Filters were hybridized using the preceding conditions (13) with the following probes: the *Eco*RI fragment of the 86T5 mouse Cβ plasmid; the cDNA for G3PDH; and PCR fragments of RAG-1 and RAG-2. The primers for RAG-1 were 5′-CCAAGCTG CAGACATTCTAGCACTC-3′ and 5′-CAACATCTGCCTTCACGTC GATCC-3′. The primers for RAG-2 were 5′-CACATCCACAAGCAG GAAGTACAC-3′ and 5′-GGTTCAGGGACATCTCCTACTAAG-3′. The filters were washed as described for Southern Blot analysis and exposed to Kodak XAR x-ray film at −70°C for 4 h to 5 days.

RAG-1 and TCR mutant/transgenic mice

The RAG-1 and TCR mutant mice have been described before (3, 7). The TCR transgenes incorporated into the RAG-$1^{-/-}$ background were derived from 2C TCR-α transgenic mice (22; a gift from Dennis Loh) and anti-H-Y TCR-β transgenic mice (23, 24; a gift from Anton Berns).

Results

TCR-β gene configuration within individual TN thymocyte subsets

In previous work, we showed that $CD44^+CD25^+$TN thymocytes are germ-line at their TCR-β locus (13). This result was inconsistent with a previous pathway of TN thymocyte development (10), where the onset of TCR-β rearrangement was mapped to the earlier $CD44^+$ $CD25^-$TN subset. Also in our previous work, we showed that the $CD44^+CD25^-$TN contain c-kit^+ and c-kit^- subsets, an unexpected result inasmuch as their precursors ($CD4^{lo}CD44^+CD25^-CD3^-CD8^-$, the first cells to arrive in the thymus from the bone marrow; 9) and their progeny ($CD44^+CD25^+$TN thymocytes) are c-kit^+ (13, 18). Therefore, an important experiment to resolve these conflicting results was to divide $CD44^+CD25^-$TN into $CD44^+CD25^-$c-kit^+TN and $CD44^+CD25^-$c-kit^-TN subsets. DNA from these and other TN populations ($CD44^+CD25^+$, $CD44^-$ $CD25^+$, and $CD44^-CD25^-$, isolated as described previously; 13) was analyzed using a Southern blot of *Hin*dIII digests, which were hybridized using a ^{32}P-labeled Cβ2 probe (Fig. 1*A*) to detect TCR-β locus rearrangements. Whole $CD44^+$ $CD25^-$TN showed partial gene rearrangement, as reported previously (10). However, $CD44^+CD25^-$c-kit^+TN showed a germ-line TCR-β locus, as indicated by the sharp and undisrupted 9-kb band, whereas the $CD44^+CD25^-$c-kit^-TN cells showed a disrupted 9-kb band, indicating rearranged TCR-β loci in this population. The next TN stage ($CD44^+CD25^+$) displayed a germ-line TCR-β configuration, whereas the downstream TN subsets ($CD44^-CD25^+$ and $CD44^-CD25^-$) were extensively rearranged, as indicated by the disrupted 9-kb fragment, and resembled the whole thymus positive control. To confirm these observations, a separate Southern blot of *Hin*dIII digests of the $CD44^+CD25^+$TN and $CD44^-CD25^+$TN subsets was screened with a ^{32}P-labeled Jβ2 probe (Fig. 1*B*), which detects a 5-kb fragment if the TCR-β genes are germ-line (*liver lane*) or a disrupted pattern of smaller fragments if rearranged (*thymus lane*). Again, TCR-β rearrangements were not detected in the $CD44^+CD25^+$TN, but were in the $CD44^-CD25^+$TN subset. Collectively, these data indicate that pro-T cells that have not rearranged their TCR-β loci can be defined by high levels of c-*kit* expression, and that TCR-β rearrangement begins as $CD25^+$TN down-regulate CD44 and c-*kit* expression.

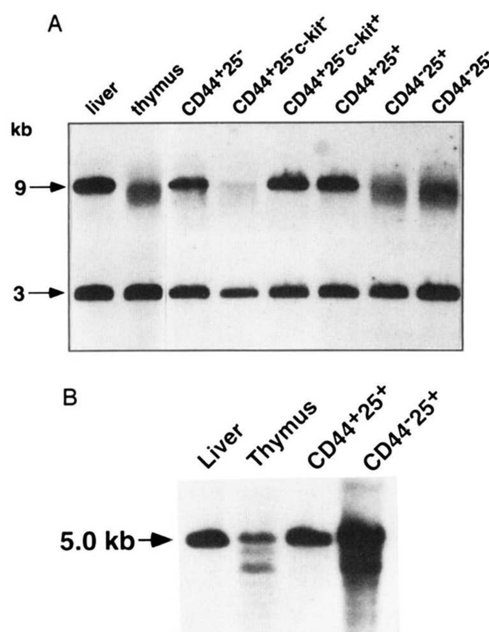

FIGURE 1. TCR-β gene configuration of TN subsets. *A*: DNA from BALB/c liver (TCR-β germ-line control), whole thymocytes (TCR-β rearranged control), and sorted $CD44^+$ $CD25^-$, $CD44^+CD25^-$c-kit^-, $CD44^+CD25^-$c-kit^+, $CD44^+$ $CD25^+$, $CD44^-CD25^+$, and $CD44^-CD25^-$TN thymocytes was digested with the restriction enzyme *Hin*dIII and examined by agarose gel electrophoresis and Southern blot analysis using a Cβ2 probe. *B*: DNA from BALB/c liver (TCR-β germ-line control), whole thymocytes (TCR-β rearranged control) and sorted $CD44^+CD25^+$ and $CD44^-CD25^+$TN was digested and similarly analyzed using a Jβ2 probe.

The $CD44^+CD25^-$c-kit^-TN do not represent pro-T cells; when cultured overnight, these cells spontaneously express cell surface TCR-$\alpha\beta$, adopting a TCR-$\alpha\beta^+$ $CD4^-CD8^-$ (TCR-$\alpha\beta^+$DN) phenotype (not shown). Furthermore, unlike the other TN subsets, these cells express CD38 (not shown), a marker that is mainly associated with the TCR-$\alpha\beta^+$DN cells.[4] Taken together, $CD44^+$ $CD25^-$c-kit^-TN thymocytes probably represent more mature T cells which are associated with the TCR-$\alpha\beta^+$DN lineage. In support of this conclusion, a neutralizing anti-c-*kit*

[4] A. G. D. Bean, D. I. Godfrey, L. Santos-Argumedo, M. Howard, and A. Zlotnik. 1994. Expression of CD38 on mouse T cells: CD38 defines functionally distinct subsets of αB TCR^+ $CD4^-$ $CD8^-$ T cells. *Submitted for publication.*

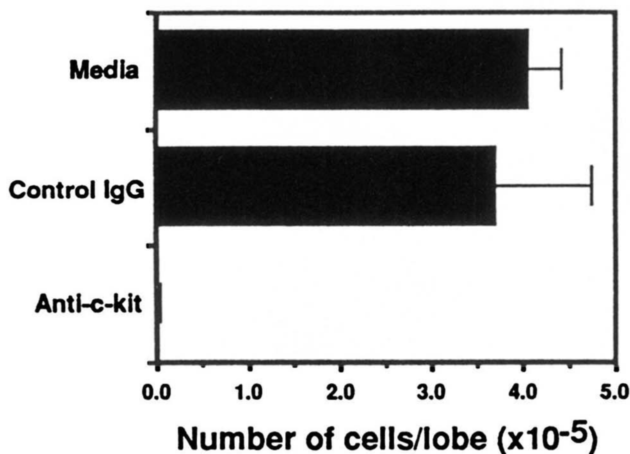

FIGURE 2. Anti-c-*kit* mAb treatment completely abrogates the reconstitution potential of CD44$^+$CD25$^-$TN thymocytes. 2-deoxyguanosine-treated fetal thymic lobes were combined with sorted CD44$^+$CD25$^-$TN thymocytes in the presence of anti-c-*kit* or control IgG, cultured for 28 days, and cell yields per lobe were determined. The graph shows the mean and SD of four separate tests.

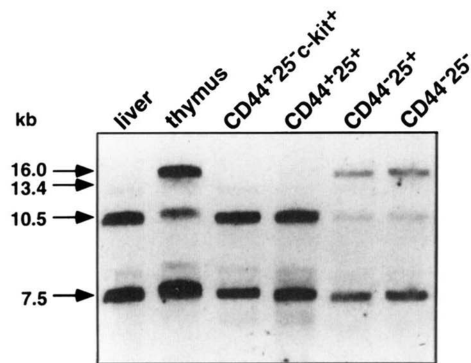

FIGURE 3. DNA from BALB/c liver (TCR-γ germ-line control), whole thymocytes (TCR-γ gene rearranged control), and sorted CD44$^+$CD25$^-$c-kit$^+$, CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, and CD44$^-$CD25$^-$TN thymocytes was digested with the restriction enzyme *Eco*RI and examined by agarose gel electrophoresis and Southern blot analysis using a Cγ4 probe.

reagent was able to inhibit thymic repopulation by whole CD44$^+$CD25$^-$TN (which included c-*kit*$^+$ and c-*kit*$^-$ fractions), indicating that only the CD44$^+$CD25$^-$c-*kit*$^+$TN subset contained thymic precursors (Fig. 2).

TCR-γ gene configuration within individual TN thymocyte subsets

Because TCR-γ gene rearrangements also occur during the earliest stages of thymocyte development, in both $\gamma\delta$ and $\alpha\beta$ T cell precursors (25), we probed *Eco*RI digests of DNA from the same TN populations (except for the CD44$^+$CD25$^-$c-*kit*$^-$ TN) for rearrangements of the TCR-γ locus (Fig. 3). The germ-line control tissue shows two intense *Eco*RI bands at 7.5 and 10.5 kb, plus a faint band at 13.4 kb, as predicted when probing with a Cγ4 fragment (26). The whole thymus positive control shows three *Eco*RI bands at 7.5, 10.5, and 16.0 to 17.0 kb, representative of a rearranged TCR-γ configuration in adult thymocytes (26). Analysis of the TN subsets indicated that rearrangement at the TCR-γ locus also occurs between the CD44$^+$CD25$^+$TN and the CD44$^-$CD25$^+$TN.

Both RAG-1 and RAG-2 are expressed at the CD44$^+$CD25$^+$ TN stage

Northern blots of TN thymocyte subsets were probed for expression of RAG-1 and RAG-2 (Fig. 4*A*). Transcripts of both genes are present at the CD44$^+$CD25$^+$TN stage, before the onset of TCR-β and TCR-γ gene rearrangement. RAG-1 expression was further up-regulated between the CD44$^+$CD25$^+$TN and the CD44$^-$CD25$^+$TN stages. The earliest CD44$^+$CD25$^-$c-*kit*$^+$TN subset was not included in these Northern blots because of the difficulty in accumulating enough purified cells for RNA preparation.

Full length TCR-β transcription begins at the CD44$^-$CD25$^+$ TN stage

To assess how transcription of the TCR-β chain was regulated in relation to the rearrangement of this gene locus, Northern blots of the last three TN subsets were screened with the Cβ2 probe (Fig. 4*B*). It has been reported that truncated 1-kb transcripts are produced before the full length 1.3-kb TCR-β transcripts during development of the $\alpha\beta$ T cell lineage (10). In line with this, we observed primarily 1-kb transcripts and a small amount of 1.3-kb transcripts in the CD44$^-$CD25$^+$TN. By the CD44$^-$CD25$^-$TN stage, the proportion of 1-kb to 1.3-kb transcripts resembled that of more mature thymocytes. In addition, the CD44$^+$CD25$^+$TN subset was producing 1-kb TCR-β transcripts, and larger (approximately 1.6 kb) transcripts. Because these cells have not yet rearranged their TCR-β locus (Figs. 1, *A* and *B*) and because full length transcripts of rearranged TCR-β genes should not be larger than 1.3 kb, the observed 1-kb and 1.6-kb bands probably represent germ-line transcripts.

CD44$^+$CD25$^-$c-kit$^+$"TN" express low levels of CD4

Because CD44$^+$CD25$^-$c-*kit*$^+$TN are germline at both the TCR-β and TCR-γ gene loci (Fig. 1*A*), there was little left to distinguish these cells from what have been described to be their immediate precursors, the CD4^{low} pre-TN thymocytes (9), which are also CD44$^+$CD25$^-$c-*kit*$^+$. When sorted CD44$^+$CD25$^-$c-*kit*$^+$TN cells were relabeled with anti-CD4 (a different mAb from those used to isolate the cells), it was clear that these cells express this marker (Fig. 5), apparently at levels low enough to avoid being killed by the complement treatment or excluded during sorting. These cells showed no detectable surface CD3 or CD8 expression. We therefore propose that these thymocyte

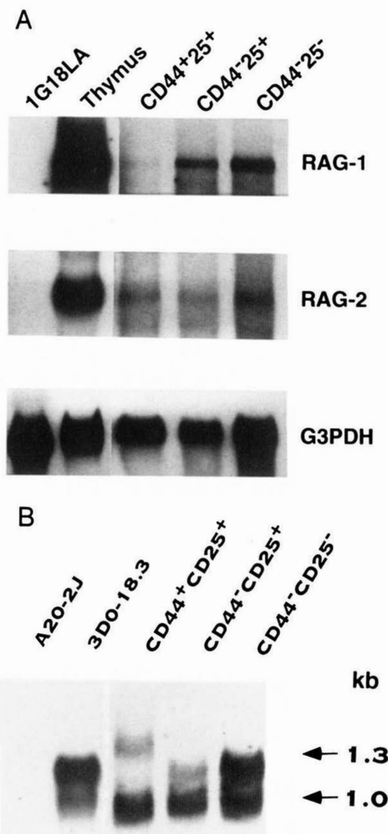

FIGURE 4. *A*: RAG-1 and RAG-2 are expressed at the CD44$^+$ CD25$^+$TN stage. Total RNA from sorted CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, and CD44$^-$CD25$^-$TN subsets was screened for RAG-1 and RAG-2 expression by Northern blot analysis. 1G18LA, a thymic macrophage line, was used as a negative control and whole thymus was used as a positive control. *B*: Full length TCR-β transcription begins at the CD44$^-$CD25$^+$TN stage. mRNA from CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, and CD44$^-$CD25$^-$TN subsets was analyzed for TCR-β expression by Northern blot analysis. A20–2J, a B cell line which does not transcribe TCR-β mRNA, was used as a negative control sample, and 3D0–18.3, a mature T cell hybridoma, was the positive control. The amount of RNA is equivalent in all lanes (not shown).

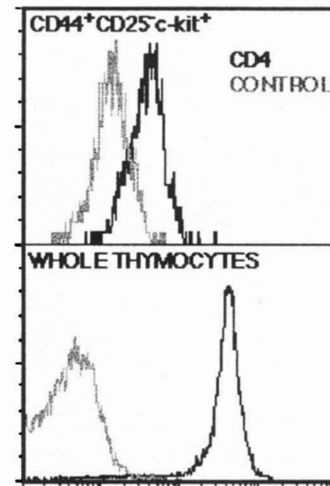

FIGURE 5. CD44$^+$CD25$^-$c-*kit*$^+$"TN" cells express low levels of CD4. Sorted CD44$^+$CD25$^-$c-*kit*$^+$TN cells were labeled with either anti-CD4 (*black histogram*) or control IgG2a (*grey histogram*). Whole thymocytes labeled with the anti-CD4 or control IgG are also shown.

subsets (CD44$^+$CD25$^-$c-*kit*$^+$ CD4^{low}CD3$^-$CD8$^-$ and CD4^{low} pre-TN) represent the same population, which contains the earliest pro-T cells present in the thymus. The downstream CD44$^+$CD25$^+$ and CD44$^-$CD25$^+$ "TN" subsets were devoid of CD3, CD4, and CD8 surface expression (not shown). The CD44$^-$CD25$^-$TN subset has previously been shown to express low levels of CD3, CD4, and CD8 (6).

Analysis of TN thymocyte development in genetically manipulated mice

The blockages at different stages of T cell development were recently described for RAG-1, RAG-2, or TCR-mutant mice (3, 4, 7). T cell differentiation in RAG mutant mice could be partially restored by the introduction of a rearranged TCR-β transgene (7, 8). These studies however, have not investigated the effect of these mutations on the TN subsets, an important aspect because this is the stage at which TCR rearrangement and expression begins. Here, we have examined the subsets of TN, lineage marker (B220, Mac-1, and Gr-1) negative (Lin$^-$), thymocytes from a range of these mice, including RAG-1$^{-/-}$, TCR-$\alpha^{-/-}$, TCR-$\delta^{-/-}$, TCR-$\beta^{-/-}$, TCR-αx$\beta^{-/-}$, and TCR-βx$\delta^{-/-}$ mice. We have also looked at RAG-1$^{-/-}$ mice that express a fully rearranged TCR-α or TCR-β transgene. Although there were large differences in total thymocyte numbers in each type of mouse, the numbers of TN, Lin$^-$ thymocytes were similar (Fig. 6). An example of the gate used to sort TN, Lin$^-$ thymocytes is shown in Figure 7*A*. This gate was the same for each type of mouse tested. The CD44 vs CD25 profiles on TN, Lin$^-$ thymocytes from each type of mouse are shown in Figure 7, *B* through *J*. The WT littermates showed a normal distribution of CD44 vs CD25 staining (Fig. 7*B*; 13). In the RAG-1$^{-/-}$ mice (Fig. 7*C*), the thymocytes were unable to progress beyond the CD44$^-$ CD25$^+$TN stage, corresponding to the first population that exhibits rearranged TCR-β and TCR-γ genes in the WT mouse.

Analysis of mice with mutations in individual TCR genes allowed the determination of which of them are involved in TN development. The TCR-$\delta^{-/-}$ mice have normal $\alpha\beta$ T cell development, but completely lack $\gamma\delta$ T cells (27). These mice showed no apparent abnormalities in their TN subsets (Fig. 7*D*), suggesting that either $\gamma\delta$-committed cells represent only a very minor component of TN thymocytes, that TCR-δ expression is not essential for the earliest stages of $\gamma\delta$ T cell development, or that $\gamma\delta$ T cells

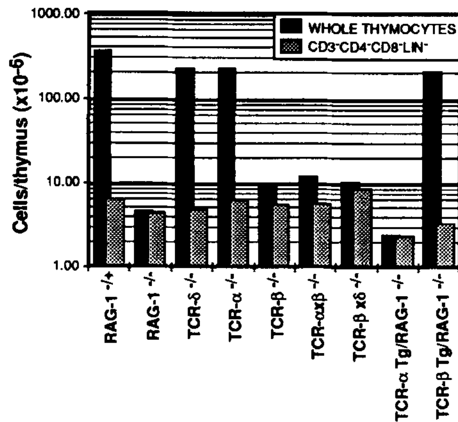

FIGURE 6. Comparison of TN thymocyte numbers from RAG-1$^{-/-}$, TCR$^{-/-}$, and TCR transgenic × RAG-1$^{-/-}$ mice. Thymocytes were isolated from a range of genetically manipulated mice: WT mice (RAG-1$^{-/+}$ heterozygous), RAG-1$^{-/-}$, TCR-$\delta^{-/-}$, TCR-$\alpha^{-/-}$, TCR-$\beta^{-/-}$, TCR-αx$\beta^{-/-}$, TCR-βx$\delta^{-/-}$, TCR-α transgenic × RAG-1$^{-/-}$, and TCR-β transgenic × RAG-1$^{-/-}$. These cells were counted using a Coulter counter and labeled with CD3, CD4, CD8, Mac-1, Gr-1 and B220 (all conjugated to phycoerythrin), CD44-biotin, and CD25-FITC. The proportions of phycoerythrin$^-$ cells (TN, Lin$^-$) were determined using a FACScan (Becton Dickinson), and the total number of these cells per thymus was calculated and presented on a logarithmic graph in relation to the total number of whole thymocytes. Each column represents the mean result from three to nine separate mice.

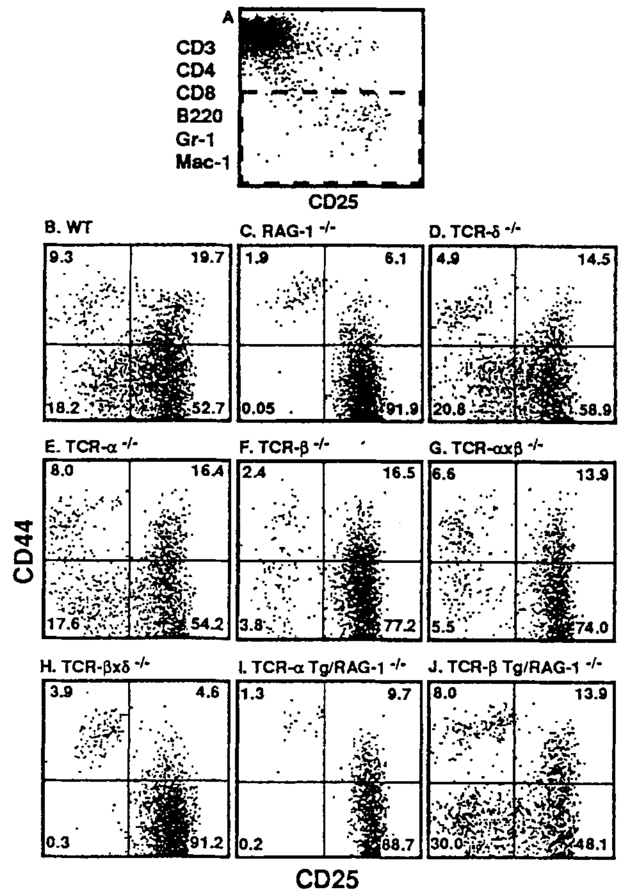

FIGURE 7. Comparison of TN thymocyte subsets from RAG-1$^{-/-}$, TCR$^{-/-}$, and TCR transgenic × RAG-1$^{-/-}$. Thymocytes were isolated from the mice and labeled as described in Figure 6. *A* shows the gate used to identify TN, Lin$^-$ cells. *B* through *J* show representative CD44 vs CD25 profiles on TN, Lin$^-$ cells from each type of mouse. All acquisition and analysis was done using a FACScan (Becton Dickinson) running LYSYS II software. The percentages within each quadrant are listed in the corner of that quadrant.

branch off late in the CD44$^-$CD25$^-$TN stage. As expected, TN development was unaffected in the TCR-$\alpha^{-/-}$ mice (Fig. 7*E*), inasmuch as differentiation proceeds apparently unimpaired until the DP stage in these mice (7, 28). In contrast, TCR-$\beta^{-/-}$ mice showed impaired development beyond the CD44$^-$CD25$^+$TN stage (Fig. 7*F*). Some CD44$^-$CD25$^-$TN were present, but they were approximately five times fewer in number than in the WT mice. An identical phenotype was seen in the TN thymocytes from TCR-αx$\beta^{-/-}$ double mutant mice (Fig. 7*G*). This is expected, because the overall phenotype of these double mutant mice did not seem to differ from the TCR-$\beta^{-/-}$ mice (7). In TCR-βx$\delta^{-/-}$ double mutant mice (Fig. 7*H*), there were almost no CD44$^-$CD25$^-$TN. The incorporation of a TCR-α transgene into the RAG-1$^{-/-}$ background did not influence the blockage at the CD44$^-$CD25$^+$TN stage (Fig. 7*I*). However, TN thymocytes from RAG-1$^{-/-}$ mice expressing a TCR-β transgene (Fig. 7*J*) showed a normal distribution of TN subsets, in line with the restoration of T cell development to the DP stage in these mice.

Discussion

The importance of TCR-β in early T cell development

The importance of TCR-β gene rearrangement and expression in early T cell development is becoming increasingly apparent, particularly in light of recent studies on RAG or TCR mutant mice (3, 4, 7, 8). The overall implication from these studies is that TCR-β gene expression is both necessary and sufficient for the progression from the TN to the DP stage. Consistent with this notion, TCR-α mutant mice have a WT number of thymocytes, mostly DP (7, 27). TCR-β rearrangement and transcription in TCR-α mutant thymocytes were found to be as extensive as in WT thymocytes (7). Sequencing of V(D)J-rearranged TCR-β genes from TCR-α mutant thymocytes revealed that the majority of these rearrangements were productive in that they could give rise to a functional TCR-β protein, suggesting that they had undergone a selection step (29). In the latter study, however, it was not addressed as to whether such functionally rearranged TCR-β genes were present in the majority of TCR-α mutant thymocytes rather than in a minor subset. TCR-β surface expression

may occur in the form of TCR-β containing dimers (30, 31), either as a homodimer or paired with an as yet undefined surrogate TCR-α chain. Investigation of early T cell development in normal mice has been hampered by the cellular heterogeneity, and by the lack of unequivocal evidence regarding the timing of TCR gene rearrangement and expression within early T cell subsets.

Timing of TCR-β rearrangement

In our study, we have examined early thymocyte subsets from the adult thymus on the basis of CD44, CD25, and c-*kit* surface expression. The earliest $CD4^{low}$ pre-TN subset has previously been shown to be unrearranged at both the TCR-β and TCR-γ loci (9). Rearrangement was reported to begin at the $CD44^+CD25^-TN$ stage (10). In this study, we also saw partial rearrangement within the $CD44^+CD25^-TN$, but this was caused by the c-kit^- subset which was extensively rearranged at the TCR-β locus. These cells acquired a TCR-$\alpha\beta^+$DN phenotype after overnight culture, indicating that they are not part of the immature development sequence. They probably belong to a separate lineage because they also express CD38, a marker that was not on any of the other TN subsets, and is preferentially expressed by mature TCR-$\alpha\beta^+$DN but absent from most other T cells.[4] Therefore, the $CD44^+CD25^-$c-kit^-TN may represent late precursors to TCR-$\alpha\beta^+$DN, or alternatively, consist of mature TCR-$\alpha\beta^+$DN that had previously down-regulated their TCR in vivo, accounting for their apparently immature TN phenotype. The presence of these cells explains the previous observation that TCR-β rearrangement was already detectable in the $CD44^+CD25^-TN$ population (10) and indicates that the c-kit^+ fraction contains the real, unrearranged precursor cells within the $CD44^+CD25^-TN$. In support of this notion, addition of anti-c-*kit* Ab to fetal thymus repopulation cultures completely abrogated the reconstitution potential of whole $CD44^+CD25^-TN$, suggesting that the $CD44^+CD25^-$c-kit^+ cells contain all of the thymic precursor activity. Furthermore, the expression of low levels of CD4 by $CD44^+CD25^-$c-kit^+ cells leads us to propose that these are the same as the $CD4^{low}$ pre-TN (9). Correspondingly, a distinct $CD44^+CD25^-$"TN" stage does not exist, and $CD44^+CD25^+TN$ cells therefore represent the first TN precursor population. These results also imply that expression of high levels of c-*kit* defines pro-T cells that have not yet rearranged their TCR genes.

The existence of both $CD44^+$ and $CD44^-$ cells within the $CD25^+TN$ group has been reported (13, 32–34), but most subsequent studies have treated these as a single population, including the only study addressing TCR-β rearrangement on mouse TN subsets (10). In light of our present results, showing that the minor (approximately 25%) subset of $CD44^+CD25^+TN$ exhibit germ-line configuration, whereas the major (approximately 75%) $CD44^-CD25^+TN$ are fully rearranged, we now understand why in the previous study (10) the whole $CD25^+TN$ seemed to be 75% rearranged.

This implies that the $CD44^+CD25^+TN$ cells are probably the target for the signal(s) that induce TCR-β and TCR-γ gene rearrangement. Despite the fact that both RAG-1 and RAG-2 transcripts are expressed at the $CD44^+CD25^+TN$ stage, we have so far been unable to induce the rearrangement of their TCR-β genes in vitro, even after culture for 5 days (not shown). We therefore propose that the transition from a germ-line to a rearranged TCR-β configuration represents a control point in thymocyte development that, in addition to RAG-1 and RAG-2 expression, is regulated by as yet unidentified factors. The induction of rearrangement may also be linked to germ-line transcription of TCR-β genes. The $CD44^+CD25^+TN$ express TCR-β transcripts (most likely germline) before rearrangement, and germ-line transcription of TCR-β (35), TCR-α (36), and TCR-γ (37) have been reported during embryogenesis. This may be a means for targeting different TCR loci for rearrangement, by transcription from their specific promoters, which may make the DNA accessible for the recombination machinery (38). Alternatively, the presence of these germ-line transcripts may be a consequence of the opening of the chromatin at the TCR loci targeted for rearrangement, an event that is likely to be one of the first molecular consequences of the commitment of these cells to the T cell lineage.

The $\gamma\delta$ T lineage branch point

The rearrangement of TCR-γ genes between the $CD44^+CD25^+TN$ and the $CD44^-CD25^+TN$ stages further emphasizes the distinct nature of these subsets. It is known that both TCR-β and TCR-γ genes are at least partially rearranged in both $\alpha\beta$ and $\gamma\delta$ T cell lineages (reviewed in 26). Therefore, TCR-γ rearrangement is not necessarily related to the branching of the $\gamma\delta$ T cell lineage, but probably represents the onset of TCR-γ rearrangement that occurs in virtually all T cell precursors. It is possible that a minor subset of $CD44^+CD25^+TN$ or earlier cells have already rearranged their TCR-γ genes and are in the process of becoming $\gamma\delta$ T cells but are so under-represented that we can not detect them by Southern blot analysis. We previously showed (13) that $CD44^+CD25^+TN$ and $CD44^-CD25^+TN$, but not $CD44^-CD25^-TN$ can repopulate the $\gamma\delta$ T cell component in fetal thymic repopulation cultures. Considering our present observation that TCR-β rearrangement has occurred by the $CD44^-CD25^+TN$ stage and that these cells have started producing full length TCR-β transcripts, we propose that this represents the last possible branch point of the $\gamma\delta$ lineage. This hypothesis contrasts with a recent study that suggested that the $\gamma\delta$ T cell branch point may occur as late as the $CD44^-CD25^-TN$ stage (11). Petrie et al. pointed out, however, that the branch point may occur at an earlier stage, and that

αβ and γδ T cell precursors may undergo parallel development before TCR expression. As previously discussed (13), this is the most likely scenario and would simply entail down-regulation of CD25 before expression of the TCR-γδ.

An early control point in αβ T cell development

The analysis of thymocytes from the genetically manipulated mice has demonstrated the tight relationship between successful TCR-β rearrangement and the progression of normal TN T cell development. In support of our TCR rearrangement model, RAG-$1^{-/-}$ mice do not develop beyond the $CD44^{-}CD25^{+}$TN stage. Since the completion of this study, a similar blockage was reported in the SCID mouse thymus (39). This probably results from the inability of thymocytes to rearrange and express TCR-β genes, as the TCR-$\beta^{-/-}$ mice are severely retarded beyond this stage of development, with only a very small subset of the $CD44^{-}CD25^{-}$TN cells still detectable. These few cells may be early γδ T cells, which develop normally within the TCR-$\beta^{-/-}$ mice (7). In line with this, TCR-β×δ double mutant mice had almost no $CD44^{-}CD25^{-}$TN. As shown in a previous study, the incorporation of a TCR-β transgene into the RAG-$1^{-/-}$ mutant background (7) was sufficient to restore thymocyte development up to the DP stage. A rearranged TCR-α transgene did not produce this effect (8), a predictable result, because in normal mice TCR-α is not rearranged and expressed until the next $CD44^{-}CD25^{-}$TN stage (10, 40). Taken together, these observations imply that expression of rearranged TCR-β genes regulates development beyond the $CD44^{-}CD25^{+}$ TN stage.

This early differentiation step is likely to involve the protein tyrosine kinase $p56^{lck}$. A severe block in αβ thymocyte development occurs in $p56^{lck-/-}$ mutant mice (41), and a complete block has been reported in transgenic mice overexpressing a catalytically inactive form of $p56^{lck}$ (42). In the latter mice, the block is at the $CD44^{-}CD25^{+}$TN stage and their thymocytes show complete V(D)J rearrangements at the TCR-β locus, but the TCR-α locus remains in germ-line configuration (42). Conversely, transgenic mice overexpressing WT $p56^{lck}$ show specific inhibition of Vβ-Dβ rearrangement, but not of Vα-Jα rearrangement (43). The notion that the TCR-β chain transmits intracellular signals via $p56^{lck}$ has recently been confirmed with mice that simultaneously express a catalytically inactive $p56^{lck}$ transgene and a TCR-β transgene (44): In these double transgenic mice, but not in TCR-β single transgenic mice, there was no allelic exclusion at the endogenous TCR-β locus. Taken together, these observations support a model where $p56^{lck}$ regulates the early differentiation step, independent of CD4 and CD8 expression (45), by perceiving the presence of a productively rearranged TCR-β chain. This $p56^{lck}$ mediated signal prevents further TCR-β rearrangements (i.e., induces allelic exclusion), and promotes the differentiation of TN cells to the

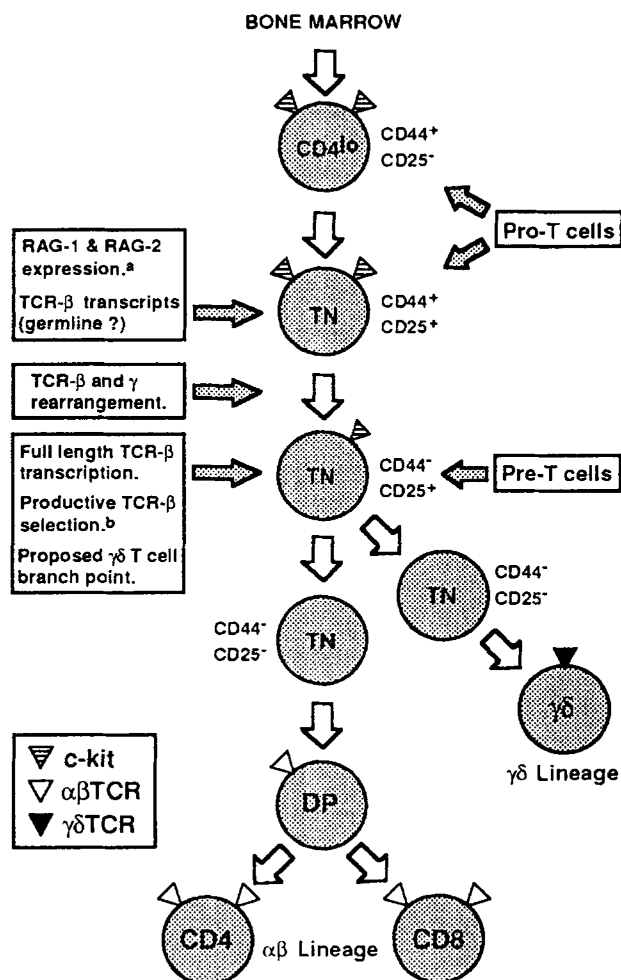

FIGURE 8. A new model for early thymocyte development. (*top*) RAG-1 and RAG-2 are expressed at least as early as the $CD44^{+}CD25^{+}$TN stage. (*bottom*) αβ T cell development is arrested at the $CD44^{-}CD25^{+}$TN stage in RAG-$1^{-/-}$ and TCR-$\beta^{-/-}$ mutant mice. This blockage can be overcome by the incorporation of a productive TCR-β transgene.

DP stage. Two important issues are unclear at the moment. First, it is not known whether TN to DP differentiation and expansion of DP thymocytes are linked in the same process. Second, it remains to be shown whether functionally rearranged TCR-β chains need to be expressed at the cell surface to mediate this early selection step. Although we have been unable to detect them by flow cytometric analysis (not shown), it is possible that low levels of surface TCR-β are present and are sufficient to transduce a signal. If surface expression is required, interaction with an extracellular ligand may take place.

It is noteworthy that our model mirrors the recently published, refined analysis of early B cell development in the bone marrow (46). Through analysis of B cell progenitor subsets of WT and three different types of mutant mice, expression of the pre-B cell receptor complex (Ig Heavy Chain) was shown to be necessary for the transition from

the large $CD43^+$ to the small $CD43^-$ pre-B cell stage. By comparison with B cell developmental stages, $CD44^+$ $CD25^-CD4^{low}$ and $CD44^+CD25^+$TN can be defined as pro-T cells, and $CD44^-CD25^+$TN as pre-T cells.

Conclusion

These results have allowed us to revise the model of early thymocyte maturation (Fig. 8). TCR-β rearrangement, which was previously believed to be a gradual process spanning the entire course of TN thymocyte development, occurs as a significantly later event in T cell differentiation than previously reported. TCR-β rearrangement seem to be tightly regulated, occurring in a single wave as $CD44^+CD25^+$TN pro-T cells down-regulate CD44 and c-*kit* expression, and is preceded by transcription of RAG-1, RAG-2, and TCR-β transcripts (probably germ-line). Full length TCR-β transcripts are first expressed by the $CD44^-CD25^+$TN pre-T cells before TCR-α rearrangement and expression. It follows that the proposed early TCR-β selection step also occurs at this stage, and that thymocytes are unable to mature further in the absence of productive TCR-β expression.

Acknowledgments

The authors thank Rachel Freiberg and Melissa Fischer for technical assistance and Dr. James Cupp, Josephine Polakoff, and Eleni Callas for their dedicated assistance with FACS sorting. We thank Dr. Shin-Ichi Nishikawa for the gift of the anti-c-*kit* mAb, Dr. Dennis Loh for the 2C TCR-α transgenic mice, and Dr. Anton Berns for the anti-H-Y TCR-β transgenic mice. We also thank Dr. Martin Pearse for helpful discussions.

References

1. Davis, M. M. and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature 334:395.*
2. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature 302:575.*
3. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaloannou. 1992. RAG-1 deficient mice have no mature B and T lymphocytes. *Cell 68:869.*
4. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, and F. W. Alt. 1992. RAG-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell 68:855.*
5. Boyd, R. L., and P. Hugo. 1991. Towards an integrated view of thymopoiesis. *Immunol. Today 12:71.*
6. Nikolic Zugic, J. 1991. Phenotypic and functional stages in the intrathymic development of $\alpha\beta$ T cells. *Immunol. Today 12:65.*
7. Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itohara, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, and S. Tonegawa. 1992. Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature 360:225.*
8. Shinkai, Y., S. Koyasu, K.-I. Nakayama, K. M. Murphy, and F. W. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science 259:822.*
9. Wu, L., R. Scollay, M. Egerton, M. Pearse, G. J. Spangrude, and K. Shortman. 1991. CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. *Nature 349:71.*
10. Pearse, M., L. Wu, M. Egerton, A. Wilson, K. Shortman, and R. Scollay. 1989. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA 86:1614.*
11. Petrie, H. T., R. Scollay, and K. Shortman. 1992. Commitment to the T cell receptor-$\alpha\beta$ or $\gamma\delta$ lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur. J. Immunol. 22:2185.*
12. Shortman, K. 1992. Cellular aspects of early T-cell development. *Curr. Opin. Immunol. 4:140.*
13. Godfrey, D. I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple negative adult mouse thymocytes defined by CD44 and CD25 expression. *J. Immunol. 150:4244.*
14. Flanagan, J. G., and P. Leder. 1990. The *kit* ligand: A cell surface molecule altered in steel mutant fibroblasts. *Cell 63:185.*
15. Huang, E., K. Nocka, D. R. Beier, T. Y. Chu, J. Buck, H. W. Lahm, D. Wellner, P. Leder, and P. Besmer. 1990. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-*kit* receptor, the gene product of the W locus. *Cell 63:225.*
16. Williams, D. E., J. Eisenman, A. Baird, C. Rauch, K. Van Ness, C. J. March, L. S. Park, U. Martin, D. Y. Mochizuki, H. S. Boswell, G. S. Burgess, D. Cosman, and S. D. Lyman. 1990. Identification of a ligand for the c-*kit* proto-oncogene. *Cell 63:167.*
17. Zsebo, K. M., D. A. Williams, E. N. Geissler, V. C. Broudy, F. H. Martin, H. L. Atkins, R. Y. Hsu, N. C. Birkett, K. H. Okino, D. C. Murdock, F. W. Jacobson, K. E. Langely, K. A. Smith, T. Takeishi, B. M. Cattanach, S. J. Galli, and S. V. Suggs. 1990. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-*kit* tyrosine kinase receptor. *Cell 63:213.*
18. Godfrey, D. I., A. Zlotnik, and T. Suda. 1992. Phenotypic and functional characterization of c-*kit* expression during intrathymic T cell development. *J. Immunol. 149:2281.*
19. Born, W., J. Yagüe, E. Palmer, J. Kappler, and P. Marrack. 1985. Rearrangement of T cell receptor β chain genes during T cell development. *Proc. Natl. Acad. Sci. USA 82:2925.*
20. Jenkinson, E. J., L. L. Franchi, R. Kingston, and J. J. T. Owen. 1982. Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment in vitro: application in the production of chimeric thymus rudiments. *Eur. J. Immunol. 12:583.*
21. Kingston, R., E. J. Jenkinson, and J. J. T. Owen. 1985. A single stem cell can recolonize an embryonic thymus, producing phenotypically distinct T-cell populations. *Nature 317:811.*
22. Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh. 1988. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature 335:271.*
23. Krimpenfort, P., F. Ossendorp, J. Borst, C. Melief, and A. Berns. 1989. T cell depletion in transgenic mice carrying a mutant gene for TCR-β. *Nature 341:742.*
24. Uematsu, Y., S. Ryser, Z. Dembic, P. Borgulya, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. In transgenic mice the introduced functional T cell receptor β gene prevents expression of endogenous β genes. *Cell 52:831.*
25. Haas, W., and S. Tonegawa. 1992. Development and selection of $\gamma\delta$ T cells. *Curr. Opin. Immunol. 4:147.*
26. Garman, R. D., P. J. Doherty, and D. H. Raulet. 1986. Diversity, rearrangement, and expression of murine T cell γ genes. *Cell 45:733.*
27. Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A. R. Clarke, M. L. Hooper, A. Farr, and S. Tonegawa. 1993. T cell receptor δ gene mutant mice: Independent generation of $\alpha\beta$ T cells and programmed rearrangements of $\gamma\delta$ TCR genes. *Cell 72:337.*
28. Philpott, K. L., J. L. Viney, G. Kay, S. Rastan, E. M. Gardiner, S. Chae, A. C. Hayday, and M. J. Owen. 1992. Lymphoid development in mice congenitally lacking T cell receptor-α-expressing cells. *Science 256:1448.*
29. Mallick, C. A., E. C. Dudley, J. L. Viney, M. J. Owen, and A. C. Hayday. 1993. Rearrangement and diversity of T cell receptor β chain genes in thymocytes: A critical role for the β chain in development. *Cell 73:513.*
30. von Boehmer, H. 1992. Thymic selection: A matter of life and death. *Immunol. Today 13:454.*

31. Groettrup, M., and H. von Boehmer. 1993. T cell receptor β chain dimers on immature thymocytes from normal mice. *Eur. J. Immunol. 23:1393.*
32. Husmann, L. A., R. P. Shimonkevitz, I. N. Crispe, and M. J. Bevan. 1988. Thymocyte subpopulations during early fetal development in the BALB/c mouse. *J. Immunol. 141:736.*
33. Lesley, J., R. Schulte, J. Trotter, and R. Hyman. 1988. Qualitative and quantitative heterogeneity in Pgp-1 expression among murine thymocytes. *Cell Immunol. 112:40.*
34. Lesley, J., R. Schulte, and R. Hyman. 1988. Kinetics of thymus repopulation by intrathymic progenitors after intravenous injection: Evidence for successive repopulation by an IL2R+, Pgp1+ progenitor. *Cell. Immunol. 117:378.*
35. Pardoll, D. M., B. J. Fowlkes, R. I. Lechler, R. N. Germain, and R. H. Schwartz. 1987. Early genetic events in T cell development analyzed by in situ hybridization. *J. Exp. Med. 165:1624.*
36. Shimizu, T., S. Takeshita, M. Muto, E. Kubo, T. Sado, and H. Yamagishi. 1993. Mouse germline transcript of TCR-α joining region and temporal expression in ontogeny. *Int. Immunol. 5:155.*
37. Goldman, J. P., D. M. Spencer, and D. H. Raulet. 1993. Ordered rearrangement of variable region genes of the T cell receptor-γ locus correlates with transcription of the unrearranged genes. *J. Exp. Med. 177:729.*
38. Alt, F. W., E. M. Oltz, F. Young, J. Gorman, G. Taccioli, and J. Chen. 1992. VDJ recombination. *Immunol. Today 13:306.*
39. Rothenberg, E. V., D. Chen, and R. A. Diamond. 1993. Functional and phenotypic analysis of thymocytes in SCID mice: evidence for functional response transitions before and after the SCID arrest point. *J. Immunol. 151:3530.*
40. Nikolic Zugic, J., and M. W. Moore. 1989. T cell receptor expression on immature thymocytes with in vivo and in vitro precursor potential. *Eur. J. Immunol. 19:1957.*
41. Molina, T. J., K. Kishihara, D. P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C. J. Paige, K. U. Hartmann, A. Veillette, D. Davidson, and T. Mak. 1992. Profound block in thymocyte development in mice lacking $p56^{lck}$. *Nature 357:161.*
42. Levin, S. D., S. J. Anderson, K. A. Forbush, and R. J. Perlmutter. 1993. A dominant negative transgene defines a role for $p56^{lck}$ in thymopoiesis. *EMBO J. 12:1671.*
43. Anderson, S. J., K. M. Abraham, T. Nakayama, A. Singer, and R. M. Perlmutter. 1992. Inhibition of T-cell receptor β-chain gene rearrangement by overexpression of the nonreceptor protein tyrosine kinase $p56^{lck}$. *EMBO J. 11:4877.*
44. Anderson, S. J., S. D. Levin, and R. M. Perlmutter. 1993. Protein tyrosine kinase $p56^{lck}$ controls allelic exclusion of T-cell receptor β-chain genes. *Nature 365:552.*
45. Levin, S. D., K. M. Abraham, S. J. Anderson, K. A. Forbush, and R. M. Perlmutter. 1993. The protein tyrosine $p56^{lck}$ regulates thymocyte development independently of its interaction with CD4 and CD8 coreceptors. *J. Exp. Med. 178:245.*
46. Ehlich, A., S. Schaal, H. Gu, D. Kitamura, W. Muller, and K. Rajewsky. 1993. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell 72:695.*