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# Biased Vβ Usage in Immature Thymocytes Is Independent of DJβ Proximity and pTα Pairing[1] FREE

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# Biased V$\beta$ Usage in Immature Thymocytes Is Independent of DJ$\beta$ Proximity and pT$\alpha$ Pairing[1]

**Anne Wilson, Céline Maréchal, and H. Robson MacDonald[2]**

**During thymus development, the TCR $\beta$ locus rearranges before the TCR $\alpha$ locus. Pairing of productively rearranged TCR $\beta$-chains with an invariant pT$\alpha$ chain leads to the formation of a pre-TCR and subsequent expansion of immature pre-T cells. Essentially nothing is known about the TCR V$\beta$ repertoire in pre-T cells before or after the expression of a pre-TCR. Using intracellular staining, we show here that the TCR V$\beta$ repertoire is significantly biased at the earliest developmental stage in which VDJ$\beta$ rearrangement has occurred. Moreover (and in contrast to the $V_H$ repertoire in immature B cells), V$\beta$ repertoire biases in immature T cells do not reflect proximity of V$\beta$ gene segments to the DJ$\beta$ cluster, nor do they depend upon preferential V$\beta$ pairing with the pT$\alpha$ chain. We conclude that V gene repertoires in developing T and B cells are controlled by partially distinct mechanisms.** ***The Journal of Immunology,*** **2001, 166: 51–57.**

Mature B and T lymphocytes express highly variable heterodimeric Ag receptors at the cell surface. Whereas the B cell receptor (BCR)[3] is composed of Ig H chains and L chains along with the Ig$\alpha$ and Ig$\beta$ signaling complex (1), the TCR is a heterodimer of $\alpha$- and $\beta$-chains in association with the CD3 complex (CD3$\epsilon$, $\gamma$, $\delta$, and $\zeta$) (1). During development the BCR and TCR are assembled from independent rearranging gene segments at the Ig and TCR loci, respectively. In both instances variable (V), diversity (D), and joining (J) gene segments are recombined in a combinatorial fashion to produce a diverse BCR or TCR repertoire (2–4).

There are many striking parallels in B and T cell development. In both cases, one Ag receptor chain (IgH for the BCR and TCR $\beta$ for the TCR) rearranges early in development. Productive rearrangement of this chain leads to the formation of a pre-BCR or a pre-TCR complex in which IgH or TCR $\beta$ is paired with an invariant surrogate L chain or pT$\alpha$ chain, respectively (5, 6). Signaling through the pre-BCR or pre-TCR leads to clonal expansion of immature B or T cells (7, 8), ultimately resulting in rearrangement of the IgL or TCR $\alpha$ locus and subsequently expression of a BCR or TCR. Maturing B and T cells are then positively and negatively selected on the basis of their Ag receptor specificity to yield the peripheral BCR and TCR repertoires (9–13).

Based on these developmental parallels, there are several different ways in which biases in the BCR or TCR repertoires can arise. At early developmental stages, nonrandom VDJ recombination at the IgH or TCR $\beta$ locus may lead to intrinsic biases in $V_H$ or V$\beta$ gene usage in immature B or T cells. Moreover, the obligate formation of a pre-BCR or pre-TCR for subsequent developmental progression may further select for IgH or TCR $\beta$-chains that can pair effectively with the corresponding surrogate L or pT$\alpha$ chains. Finally, positive and negative selection events would be expected to select for mature BCR and TCR based on the appropriate pairing and specificity of the IgH/IgL and TCR $\beta$/TCR $\alpha$ heterodimers.

Although considerable data are available concerning the mature BCR and TCR repertoires, less is known about the factors that shape the pre-BCR and pre-TCR repertoires before positive and negative selection events. Nevertheless, it has been shown that the pre-BCR repertoire is subject to bias in at least two respects (7). First, a preference for rearrangement of $V_H$ gene segments that are proximal to the $DJ_H$ locus results in a nonrandom use of IgH chains in immature B cells. Second, the failure of a large proportion (~50%) of IgH chains to pair effectively with the surrogate L chain introduces further bias in the $V_H$ repertoire that can successfully undergo pre-BCR-mediated selection.

In contrast to B cells, essentially nothing is known about factors that may influence the pre-TCR repertoire during early T cell development. In particular, it is not clear whether the recombination machinery that directs VDJ$\beta$ rearrangements acts at random or alternatively introduces an intrinsic V$\beta$ repertoire bias. Moreover, the possible influence of preferential TCR $\beta$/pT$\alpha$ pairing on the pre-TCR-selected V$\beta$ repertoire has not been directly evaluated.

In this report, we have developed a novel two-color intracellular (ic) immunofluorescence protocol that, when combined with two-color surface staining, allows accurate determination of the TCR V$\beta$ repertoire in immature thymocyte subsets. Our data indicate that significant biases in the V$\beta$ repertoire are already apparent at the earliest developmental stage in which VDJ$\beta$ rearrangement has occurred. However, in contrast to the $V_H$ repertoire in immature B cells, V$\beta$ repertoire biases in immature T cells do not reflect proximity of V$\beta$ gene segments to the DJ$\beta$ cluster, nor do they depend upon preferential pairing with pT$\alpha$.

## Materials and Methods

*Mice and cell suspensions*

C57BL/6 female mice (V$\beta^b$ allotype) were purchased from Harlan Olac (Bicester, U.K.). Congenic V$\beta^a$ allotype mice (backcrossed 15 generations to the C57BL/6 background) were a kind gift from Alexandra Livingstone (Basel Institute for Immunology, Basel, Switzerland). They were bred and maintained at the Institut Suisse de Recherche Experimentale sur le Cancer animal facility and have been described in more detail elsewhere (14). pT$\alpha$-deficient mice on a C57BL/6 background (15) were a kind gift from

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[3] Abbreviations used in this paper: BCR, B cell receptor; RSS, recombination signal sequence; ic, intracellular; DN, double negative; ISP, immature single positive.



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Hans Jorg Fehling (Basel Institute for Immunology). All mice were used at 6–8 wk of age. $CD4^-CD8^-$ (double-negative (DN)) thymocytes were prepared as previously described (16, 17). Contaminating mature αβ or γδ T cells and immature $CD44^+$ thymocytes were eliminated during subsequent FACS analysis by gating out all cells stained with a mixture of FITC-conjugated Abs to CD4, CD8, CD44, CD3ε, TCR αβ, and TCR γδ (either prepared in this laboratory or purchased from PharMingen, San Diego, CA). For the analysis of immature single positive (ISP) ($CD4^-CD8^+CD3^-$) thymocytes, depletion was performed using mAbs to CD4, CD25, and CD44.

### *ic staining, FACS analysis, and Abs*

Thymocyte subsets were analyzed by four-color simultaneous surface and ic flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) as previously described (17–19). Briefly, purified DN thymocytes were surface stained with the FITC mixture described above and CD25-Cy5 (prepared in this laboratory) and fixed in 2% paraformaldehyde before permeabilization with 0.5% saponin (Sigma, St. Louis, MO). Two-color ic staining of TCR Vβ vs TCR Cβ was performed by staining with PE-conjugated anti-Vβ mAbs followed by anti-TCR Cβ-CyChrome (PharMingen). mAbs to TCR Vβs 2, 6, 8.2, 8 total (8.1/8.2/8.3), 9, and $17^a$ were prepared in this laboratory by conjugation of the purified proteins with the Phycolink PE conjugation kit (Prozyme, San Leandro, CA); mAbs to TCR Vβs 4, 8.3, $10^b$, 11, and 12 were purchased from Caltag (Burlingame, CA); and those to TCR Vβs 3, 5.1/5.2, 7, 8.1/8.2, and 13 were purchased from PharMingen. The percentage ic TCR $V\beta^+$ of total ic TCR $C\beta^+$ for DN3 ($CD25^+CD44^-$) or DN4 ($CD25^-CD44^-$) thymocytes was calculated using the CellQuest (Becton Dickinson) program. For ISP thymocytes, three-color surface staining with anti-CD8-CyChrome, anti-TCR β-PE (PharMingen), and anti-CD24-FITC (prepared in this laboratory) was followed by fixation and ic staining with anti-TCR Vβ-PE conjugates. ISP thymocytes were defined as $CD8^+CD24^+$TCR $\beta^{low}$. Since ISP thymocytes have been previously shown to be 100% ic TCR $C\beta^+$ (17), the percentage of ic $V\beta^+$ ISP was calculated directly.

### *Statistical analysis*

The percentages of ic $V\beta^+$ DN3 thymocytes as well as the ratios of ic $V\beta^+$ DN4:DN3 or ISP:DN3 thymocytes were compared pairwise for all Vβ domains using the Student *t* test. Values of $p < 0.001$ were considered to be significant.

## Results

### *ic TCR Vβ staining in immature thymocytes*

Although several groups have identified immature fetal or adult thymocytes expressing TCR β protein based on ic staining with anti-TCR Cβ mAb (17, 20–23), very little is known about the relative expression of TCR Vβ domains before positive and negative selection. To address this issue, we prepared a panel of PE-conjugated anti-Vβ mAbs and used them in conjunction with Cy-Chrome-conjugated anti-Cβ mAb in two-color ic staining of immature $CD4^-CD8^-$ (DN) adult thymocytes. Surface staining with a Cy5-conjugated mAb to CD25 and a FITC-conjugated mAb mixture to CD44 and a panel of mature thymocyte markers (see *Materials and Methods*) was used in the other two colors to define $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4) subsets (Fig. 1). As reported previously (17), a clearly defined subset of ic TCR $\beta^+$ cells was distinguishable in both DN3 and DN4 subsets (25 and 75%, respectively). In addition, a small proportion of double-staining ic TCR $V\beta5^+$ ic $C\beta^+$ cells (corresponding to 11 ± 2% of ic TCR $C\beta^+$ cells) was detectable in both the DN3 and DN4 subsets (Fig. 1). This staining was shown to be specific, since immature thymocytes from C57BL/6 ($V\beta^b$) mice stained positively for Vβ8.2 but negatively for $V\beta17^a$ (Fig. 2). In contrast, immature thymocytes from congenic C57BL/6 mice of the $V\beta^a$ haplotype stained positively for $V\beta17^a$ but negatively for Vβ8.2 (Fig. 2). These results are compatible with the $V\beta^a$ genotype, which harbors a large deletion including Vβ8.2, as well as a polymorphism that allows expression of $V\beta17^a$ (24, 25).

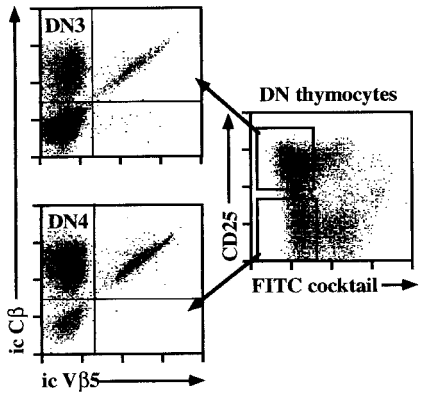


**FIGURE 1.** ic TCR Vβ proteins are expressed in immature thymocytes. Purified DN thymocytes were surface stained with a mixture of FITC-conjugated mAbs including CD4, CD8, CD3, TCR β, TCR γδ, and CD44 along with CD25-Cy5. After fixation and permeabilization, ic staining was performed with mAb to Cβ (CyChrome) to distinguish all ic $C\beta^+$ cells, followed by anti-Vβ5 (PE). Cytograms of ic Vβ5 vs ic Cβ staining are gated on $CD44^-CD25^+$ (DN3) or $CD44^-CD25^-$ (DN4) thymocytes.

### *Biased Vβ repertoire in DN3 thymocytes*

The DN3 subset is the earliest stage at which full-length VDJβ transcripts and TCR Cβ protein are expressed (16, 26–31). It was therefore of interest to determine whether biases in Vβ usage, which are pronounced after positive and negative thymic selection (32–36), could already be detected at the DN3 stage. Fig. 3 represents a summary of ic Vβ staining in DN3 thymocytes of

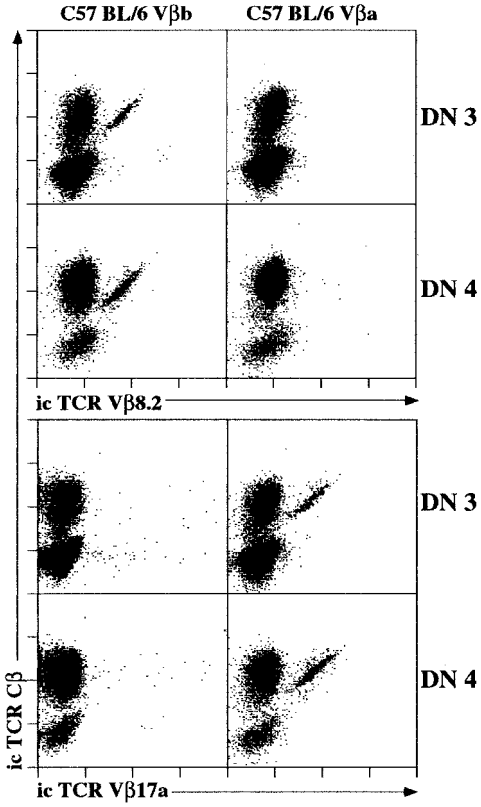


**FIGURE 2.** ic Vβ staining is specific; ic Cβ vs ic Vβ staining on immature DN3 and DN4 subsets (purified, stained, and gated as described in Fig. 1) in C57BL/6 mice of the $V\beta^b$ and $V\beta^a$ allotypes. In $V\beta^a$ mice, a large portion of the Vβ locus including the Vβ8.2 gene segment is deleted, while the polymorphic $V\beta17^a$ gene segment is expressed.

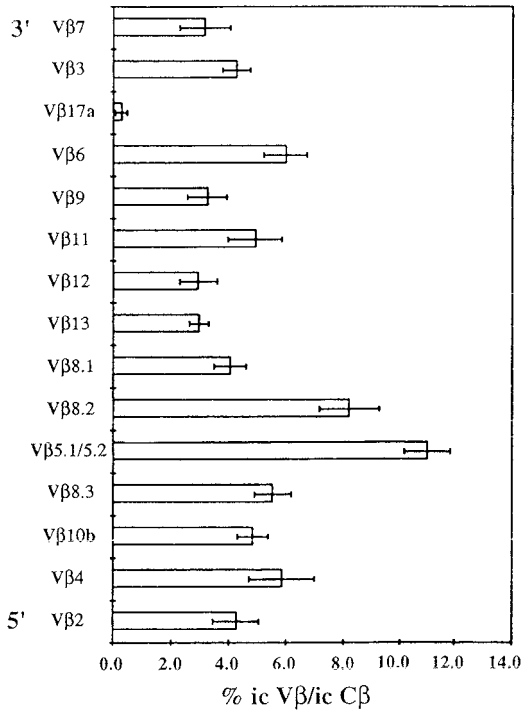


**FIGURE 3.** Biased V$\beta$ repertoire in DN3 thymocytes; a summary of ic V$\beta$ staining in DN3 thymocytes of C57BL/6 mice. Results are the mean ± SD of 5–10 separate experiments. Staining and gating were as described in Fig. 1. The different V$\beta$ segments are arranged in the same order as found on the chromosome with the most 5′ segments at the bottom and the most 3′ segments at the top.

C57BL/6 mice for the 16 V$\beta$ domains analyzed in this study. With the exception of V$\beta$5.1/5.2 (which cannot be distinguished by the mAb used) and V$\beta$17$^a$ (which is not expressed in the V$\beta^b$ haplotype), the other 13 individual V$\beta$ domains were used at frequencies varying between 2.9% (for V$\beta$12) and 8.6% (for V$\beta$8.2) in the DN3 subset. A pairwise statistical comparison of these 13 V$\beta$ domains indicated that they could be subdivided into four groups based on their level of utilization in the DN3 subset: V$\beta$8.2 > V$\beta$4, V$\beta$6, V$\beta$8.3 > V$\beta$2, V$\beta$3, V$\beta$8.1, V$\beta$10, V$\beta$11 > V$\beta$7, V$\beta$9, V$\beta$12, V$\beta$13. Thus there is a considerable (up to 3-fold) variation in the probability of utilization of a particular V$\beta$ domain at this early developmental stage.

### *Biased Vβ repertoire in DN3 thymocytes is independent of proximity to DJβ segments*

At the IgH locus, there is a large body of evidence indicating that proximal $V_H$ segments rearrange preferentially to $DJ_H$ segments during ontogeny (37–44). Thus it is possible that the variations observed in V$\beta$ usage among DN3 thymocytes reflect proximity to the DJ$\beta$ segments. However, as shown in Fig. 3 (in which the V$\beta$ segments are ordered according to their positions on the chromosome), there is no correlation between frequency of V$\beta$ usage and chromosomal localization. Indeed, the most DJ$\beta$-proximal V$\beta$ segments (V$\beta$7 and V$\beta$3) are used at a relatively low frequency, whereas the most frequently used segment (V$\beta$8.2) is relatively distal to DJ$\beta$. Moreover, within each group of V$\beta$ domains defined by statistically similar usage in DN3 thymocytes (see above), the individual members appear to be located randomly within the V$\beta$ locus.

### *Vβ repertoire in DN3 thymocytes is independent of β-selection*

As mentioned previously, VDJ$\beta$ rearrangement and TCR $\beta$ protein expression first occur at the DN3 stage of adult thymus development. Since DN3 thymocytes also express pT$\alpha$ and CD3 components (45, 46), de novo synthesized TCR $\beta$ protein can presumably be rapidly incorporated into a pre-TCR complex. Formation of a pre-TCR will in turn signal the cell to enter cell cycle. As a result, DN3 thymocytes are heterogeneous in size (as measured by forward light scatter), with ~70% small (i.e., resting) and 30% large (i.e., cycling) cells (Fig. 4*A*). As expected, the majority of large DN3 cells (65 ± 7%) but very few small DN3 cells (12 ± 2%) express ic TCR $\beta$ protein (Fig. 4*A*). Most large DN3 cells are thus

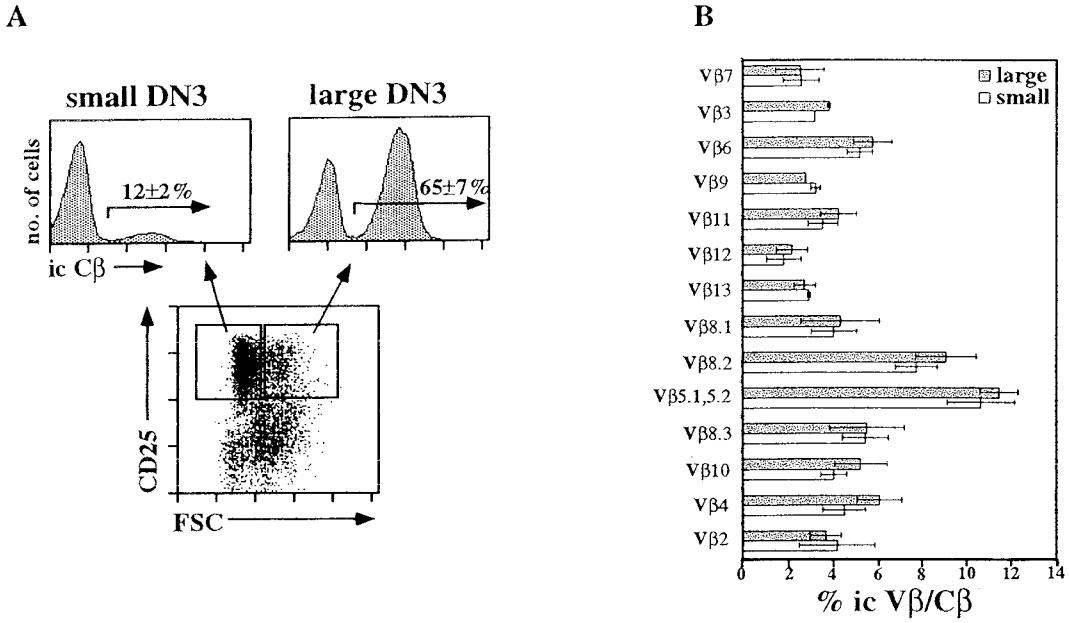


**FIGURE 4.** The V$\beta$ repertoire is not affected by $\beta$ selection. *A*, ic C$\beta$ staining of DN3 thymocytes gated on small (70%) and large (30%) cells by forward light scatter. Percentages of ic C$\beta^+$ cells are the means ± SD of eight independent experiments. *B*, Percentage of ic V$\beta^+$ among ic C$\beta^+$ cells in small (open bars) and large (filled bars) DN3 thymocytes. Data are the means ± SD of four to eight independent experiments.

"β -selected," while the minor subset of small ic TCR $\beta^+$ DN3 cells presumably represents cells that either have failed to assemble a pre-TCR complex (due to the inability of their TCR β-chains to pair with pTα) or have not yet entered the cell cycle despite the presence of a functional pre-TCR (47). In either case, these small DN3 cells would be considered to be not β selected.

To determine whether β selection significantly alters the Vβ repertoire of DN3 thymocytes, we compared ic Vβ staining in ic $C\beta^+$ DN3 cells gated according to size. As shown in Fig. 4*B*, the hierarchy of ic Vβ usage observed previously in total ic $C\beta^+$ DN3 thymocytes was preserved in the small and large DN3 subsets. Moreover, analysis of the TCR Vβ repertoire of DN3 thymocytes from pTα-deficient mice (15), which are genetically incapable of forming a pre-TCR, indicated no significant differences in ic Vβ usage as compared with wild-type mice (Table I). Taken together, these data demonstrate that the biased Vβ repertoire of DN3 thymocytes does not result from the preferential ability of certain Vβ domains to form a functional pre-TCR.

### *Vβ repertoire is not significantly affected by pre-TCR-mediated expansion of immature thymocytes*

Following productive VDJβ rearrangements at the DN3 stage, the TCR β-chain pairs with the invariant pTα-chain and CD3 components to form the pre-TCR complex (8). As a consequence of pre-TCR signaling, DN3 thymocytes shut off further VDJβ recombination, down-regulate the expression of CD25, and enter a phase of rapid proliferation (DN4). Proliferating DN4 thymocytes subsequently express CD8 at the ISP stage before becoming $CD4^+CD8^+$ cells and initiating VJα rearrangement. To determine whether the Vβ repertoire is selected during this pre-TCR-mediated expansion phase of thymocyte development, we measured ic Vβ expression in DN4 and ISP thymocyte subsets and compared these values with those obtained in the preexpansion (DN3) stage. As shown in Fig. 5, only very slight changes in the Vβ repertoire were observed in DN4 and ISP subsets, since the ratio of DN4:DN3 or ISP:DN3 ic TCR $V\beta^+$ cells was not significantly different from 1 in virtually all cases. These data indicate that the dramatic expansion of immature thymocytes that occurs between the DN3 and ISP stages of development does not depend upon the Vβ domain utilized by the pre-TCR.

## Discussion

The data presented here represent (to our knowledge) the first attempt to analyze the TCR Vβ repertoire early in development before positive and negative selection events. Although it is known that the expressed TCR Vβ repertoire is modified during both positive (32, 35) and negative (33, 34, 36) thymic selection, the contribution of other factors (such as rearrangement frequency and probability of pairing with pTα) to TCR Vβ repertoire formation has not been previously evaluated. Our data indicate that the TCR Vβ repertoire is already significantly skewed at the earliest stage of adult thymus development in which VDJβ rearrangements can be detected. Moreover, and in marked contrast to the $V_H$ repertoire in immature B cells, the bias in Vβ repertoire observed in immature T cells does not reflect any obvious preference for rearrangement of proximal Vβ segments or for pairing with the pTα-chain. Thus, although the general parallels in B and T cell development are striking, the mechanisms responsible for controlling Vβ and $V_H$ usage at early developmental stages are quite distinct.

Table I. *TCR Vβ repertoire in DN3 thymocytes of pTα-deficient mice*[a]

| Vβ Domain | % of Total ic Cβ | |
|---|---|---|
| | Wild type | $pT\alpha^{-/-}$ |
| ic Vβ4 | 6.1 ± 1.0 | 4.9 ± 1.6 |
| ic Vβ5.1,5.2 | 10.8 ± 1.5 | 10.0 ± 0.5 |
| ic Vβ6 | 6.1 ± 0.7 | 5.5 ± 1.5 |
| ic Vβ8.2 | 8.8 ± 1.6 | 9.2 ± 1.4 |
| ic Vβ9 | 3.4 ± 0.5 | 2.6 ± 1.0 |
| ic Vβ13 | 2.7 ± 0.4 | 2.5[b] |

[a] DN3 thymocytes of wild-type or $pT\alpha^{-/-}$ mice were stained and gated as in Fig. 1. For each Vβ domain tested results are expressed as mean percentage (±SD) of ic $V\beta^+$ among total ic $C\beta^+$ cells. The percentage of ic $C\beta^+$ cells among DN3 thymocytes was 25 ± 4% and 18 ± 3% for wild-type and $pT\alpha^{-/-}$ mice, respectively.
[b] Data from a single experiment.

### *Technical considerations*

Previous studies of $V_H$ repertoire formation in immature B cells have mainly relied upon PCR amplification and sequencing at the population level. Such techniques have the advantage of detecting both nonproductive and productive IgH rearrangements. However, they are subject to biases due to the variable efficiency of amplification by different PCR primers and do not evaluate rearrangements at the single-cell level. The latter problem can be overcome using single-cell PCR (42, 48), but this method introduces sampling errors due to the relatively small number of cells that can be analyzed.

To assess the TCR Vβ repertoire in immature thymocytes, we have developed a two-color ic staining procedure using a panel of PE-conjugated anti-Vβ mAbs in conjunction with CyChrome-conjugated anti-Cβ mAb. Anti-Vβ mAbs represent a powerful tool to analyze the TCR Vβ repertoire, since (at least in the mouse) mAbs are available for the majority of Vβ-chains. Indeed, the panel of anti-Vβ mAbs used here detected ~70% of the total Vβ repertoire in both DN3 and DN4 subsets as assessed by ic staining. ic staining is, however, necessary to reliably detect TCR β protein expression at early stages of thymus development, since the pre-TCR is expressed at very low levels on the cell surface and can only be detected using elaborate multistage staining protocols (49). Moreover, simultaneous assessment of ic Vβ and ic Cβ protein expression in individual cells allows the proportion of ic $V\beta^+$ cells to be measured with greater precision, particularly in immature subsets (such as DN3) where only a minority of cells express TCR β protein.

### *Intracellular Vβ staining detects only productive VDJβ rearrangements*

Imprecise joining of V, D, and J gene segments during VDJβ recombination leads to premature stop codons and/or an incorrect translational reading frame for the Cβ domain in two of three cases. Nevertheless, the Vβ domain could still in theory be translated correctly in cases of nonproductive rearrangement, leading to a truncated TCR β protein. Two-color ic staining for Vβ and Cβ domains argues against the presence of stable truncated TCR β proteins, since (for all 16 Vβ domains analyzed) ic Vβ staining was only detected in cells that stained positively for ic Cβ. Moreover, the clear quantitative correlation between ic Vβ and ic Cβ staining in individual cells (manifested as a diagonal staining pattern in two-color plots) demonstrates that the two mAbs are binding to the same molecule and hence (by implication) that there are no detectable truncated TCR β proteins expressing Vβ but not Cβ.

There are several possible explanations for the absence of detectable truncated ic TCR β proteins arising from nonproductive VDJβ rearrangements. First, although out-of-frame TCR and BCR rearrangements are clearly transcribed, several studies indicate that the resulting mRNAs are considerably less stable than mRNAs arising from in-frame rearrangements (50–52). Second, even if they are translated, truncated ic TCR β proteins may be degraded

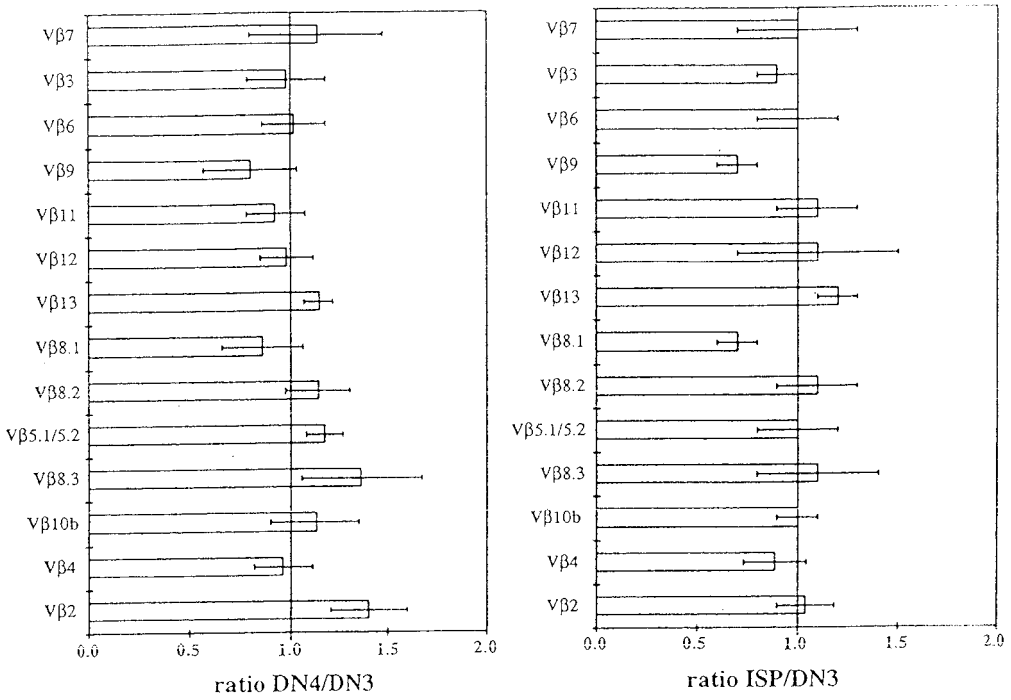


**FIGURE 5.** The V$\beta$ repertoire is unaffected by pre-TCR-mediated expansion of immature thymocytes. Data are a summary of the ratios between DN4 and DN3 (*left*) or ISP and DN3 (*right*) of the percentage of ic C$\beta^+$ cells expressing a particular ic V$\beta$ protein. Ratios are the means $\pm$ SD calculated from four to eight individual experiments in which DN3 and DN4 or DN3 and ISP subsets were compared in the same sample. The vertical lines correspond to a ratio of 1.

much more rapidly than full-length proteins. Finally, truncated ic TCR $\beta$ proteins (even if relatively stable) may not fold correctly and thus may not be recognized by anti-V$\beta$ mAbs. Whatever the explanation, the fact that none of the 15 independent anti-V$\beta$ mAbs used in our study recognizes truncated ic TCR $\beta$ proteins rules out the possibility that the ic V$\beta$ staining technique itself introduces a repertoire bias by selectively detecting certain nonproductive VDJ$\beta$ rearrangements.

### *Biases in VDJβ rearrangement at the TCR β locus*

VDJ$\beta$ rearrangements and subsequent expression of a TCR $\beta$ protein first occur during the DN3 stage of adult thymus development. Thus DN3 thymocytes would be expected to express a TCR V$\beta$ repertoire representative of the frequency at which individual V$\beta$ gene segments recombine during development. As shown here by ic V$\beta$ staining, the frequency of expressed V$\beta$ segments varies by a factor of 3 among DN3 thymocytes (8.6% for V$\beta$8.2 vs 2.9% for V$\beta$12). This variation in V$\beta$ usage is not due to preferential association of certain V$\beta$-chains into a pre-TCR complex, since similar V$\beta$ repertoires were observed in small and large DN3 thymocyte subsets, which represent stages before and after pre-TCR selection events (47). Moreover, V$\beta$ repertoires were not significantly different among DN3 thymocytes from pT$\alpha$-deficient and wild-type mice, formally ruling out any major influence of pre-TCR-mediated selection on the observed V$\beta$ bias. Subject to the caveat that ic V$\beta$ staining measures only the frequency of productively rearranged VDJ$\beta$ alleles (see above), we conclude that there is a significant developmental bias inherent in VDJ$\beta$ recombination in adult thymocytes before pre-TCR- or TCR-mediated selection. Whether a similar bias also applies to fetal VDJ$\beta$ rearrangement remains to be investigated.

### *Possible origin of biased VDJβ recombination in immature T cells*

There are a number of factors that could influence the frequency of rearrangement of individual V$\beta$ gene segments in DN3 thymocytes. By analogy with studies of $VDJ_H$ recombination in immature B cells, one of the most obvious possibilities would be the relative proximity of a given V$\beta$ segment to the DJ$\beta$ cluster. Indeed, in both fetal and adult mouse pre-B cells, it has been shown that $DJ_H$-proximal $V_H$ segments (such as $V_H$ 81X) are preferentially rearranged (37–40, 42–44). In marked contrast to pre-B cells, the most DJ$\beta$-proximal V$\beta$ segments in DN3 thymocytes (V$\beta$7 and V$\beta$3) are apparently rearranged at relatively low frequency, whereas the relatively distal V$\beta$8.2 segment is rearranged most frequently. These data indicate that physical proximity on the chromosome does not play an equivalent role in directing VDJ rearrangement in immature mouse T and B cells.

Several other molecular mechanisms could influence the frequency of rearrangement of individual V$\beta$ gene segments. For example, subtle differences in the conserved heptamer/nonamer recombination signal sequences (RSS) or slightly differing lengths of the conserved 23-aa spacers could favor recognition or cleavage of certain V$\beta$ segments by the recombinase machinery (53–56). In this respect, the recent availability of the complete nucleotide sequence of the TCR $\beta$ locus has allowed us to compare RSS and spacer lengths for all V$\beta$ genes examined in this study (see GenBank data sequences under AE000663, AE000664, and AE000665). This analysis indicates that there is no obvious difference in either parameter that correlates with the observed frequency of V$\beta$ recombination in DN3 thymocytes (A. Wilson and C. V. Jongeneel, data not shown). Clearly, quantitative functional analysis of the efficiency of cleavage of these V$\beta$ recombination substrates in vitro will be required to formally address this issue.

Finally, it is possible that differences in the frequency of rearrangement of particular Vβ genes simply reflect differences in their accessibility to recombinase (53, 56, 57). In this regard, recent studies have demonstrated that the efficiency of VDJ recombination is influenced by nucleosomal structure, histone acetylation, methylation status, and transcriptional activity (58–61). Obviously any (or all) of these factors may contribute to the biased Vβ repertoire of immature T cells.

### *No evidence for pre-TCR-mediated Vβ-selection*

Another factor influencing the $V_H$ repertoire in immature B cells is the differential ability of individual IgH chains to form a functional pre-BCR (62, 63). It has been estimated that only 50% of IgH chains in pre-B cells have the capacity to pair with the surrogate L chain and hence to form a pre-BCR (7, 42). This failure to form a functional pre-BCR is at least in part due to structural constraints imposed by the $V_H$ domain, since certain $V_H$ families (such as $V_H$ 7183 and $V_H$ Q52) are significantly less represented in the expressed $V_H$ repertoire following pre-BCR-mediated selection (42).

In contrast to these findings for immature B cells, we find no evidence for a role of the Vβ domain in pre-TCR-mediated selection. In particular, large DN3 thymocytes (which are already cycling as a consequence of pre-TCR signaling) expressed a Vβ repertoire similar to that of their small, noncycling DN3 counterparts. Moreover, the Vβ repertoire in DN3 thymocytes of pTα-deficient mice, which are genetically incapable of assembling a pre-TCR, was not significantly different from that observed in wild-type mice. Even more strikingly, the Vβ repertoire in DN4 and ISP thymocyte subsets, which have undergone extensive proliferation as a consequence of pre-TCR signaling, could not be distinguished from the DN3 Vβ repertoire. Clearly, these data do not formally exclude the possibility that the pre-TCR may exert a selective role in forming the TCR β-chain repertoire, perhaps by favoring certain CDR3β lengths or sequence motifs. Nevertheless, they argue strongly that Vβ domains (unlike $V_H$ domains) do not play an important structural role in the assembly of the pre-TCR complex. This result might have been anticipated in view of the fact that a genetically engineered pre-TCR complex lacking most of the extracellular portion of the TCR β-chain (including the Vβ domain) appears to signal normally in transgenic mice (64, 65).

Differing requirements for the $V_H$ and Vβ domains in pre-BCR vs pre-TCR selection may have structural implications. Whereas the pre-BCR contains (in addition to the surrogate L chain and signaling components) a VpreB component, the existence of a comparable VpreT element in the pre-TCR has not been demonstrated. Based on our data, one could speculate that the hypothetical VpreT chain does not exist and consequently that fewer molecular constraints are imposed upon Vβ (as opposed to $V_H$) pairing during pre-TCR and pre-BCR assembly.

### *Concluding remarks*

In conclusion, we have shown that the overall striking parallels in T and B cell development extend to biases in V gene segment recombination at the TCR β and IgH loci. However, in contrast to $V_H$ gene biases in immature B cells, Vβ gene biases in immature T cells do not reflect proximity to D segments and are not significantly influenced by pairing with the surrogate (pTα) chain. Thus, inherent developmental biases in Vβ gene rearrangement represent one of the major elements shaping the mature TCR Vβ repertoire.

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