Allelic Exclusion of the T Cell Receptor β Locus Requires the SH2 Domain-containing Leukocyte Protein (SLP)-76 Adaptor Protein

By Iannis Aifantis,* Vadim I. Pivniouk,† Frank Gärtner,§ Jacqueline Feinberg,* Wojciech Swat,¶ Frederick W. Alt,* Harald von Bohemer,* and R a i f  S. Geha‡

From the *Institut National de la Santé et Recherche Médicale (INSERM) U 373, Hôpital Necker Enfants-Malades, Paris, France; and the †Division of Immunology and §Howard Hughes Medical Institute, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Summary
Signaling via the pre-T cell receptor (TCR) is required for the proliferative expansion and maturation of CD4⁺CD8⁻ double-negative (DN) thymocytes into CD4⁺CD8⁺ double-positive (DP) cells and for TCR-β allelic exclusion. The adaptor protein SH2 domain-containing leukocyte protein (SLP)-76 has been shown to play a crucial role in thymic development, because thymocytes of SLP-76⁻/⁻ mice are arrested at the CD25⁺CD4⁻⁻ DN stage. Here we show that SLP-76⁻/⁻ DN thymocytes express the pre-TCR on their surfaces and that introduction of a TCR-α/β transgene into the SLP-76⁻/⁻ background fails to cause expansion of DN thymocytes or developmental progression to the DP stage. Moreover, analysis of TCR-β rearrangement in SLP-76⁻/⁻ TCR-transgenic mice or in single CD25⁺CD4⁻⁻ DN cells from SLP-76⁻/⁻ mice indicates an essential role of SLP-76 in TCR-β allelic exclusion.

Key words: SLP-76 • allelic exclusion • thymocyte development • pre-TCR • TCR signaling

The pre-TCR controls the survival of CD4⁺CD8⁻ double-negative (DN) thymocytes and their intense proliferation and maturation into CD4⁺CD8⁺ double-positive (DP) thymocytes by mostly unknown mechanisms (1). It is clear that this control requires the pre-TCR-α chain (pTα), the TCR-β chain, signal-transducing molecules of the CD3 complex, Src kinases, and Zap-70 and Syk kinases (2–6). Some experiments suggest that exit from the endoplasmic reticulum is essential for pre-TCR function (7), but other data have been interpreted to indicate that binding to a ligand may not be an essential event (8). In fact, surface expression of the pre-TCR on the CD25⁺CD44⁻ population of thymocytes that require the pre-TCR for further maturation has not been well documented. It is also not clear whether the function of the pre-TCR includes mainly rescue from cell death, allowing an already imprinted program of expansion and maturation to take place, or whether the pre-TCR directly controls cell cycle progression as well as expression of genes encoding various cell surface molecules. Experiments addressing this question have given conflicting results, as in bcl-2-transgenic SCID mice, antiapoptotic bcl-2-dependent signals were insufficient to permit developmental changes, whereas overexpression of bcl-2 in DN thymocytes of recombinant activating gene (RAG)⁻/⁻ mice promoted differentiation to the CD4⁺CD8⁻⁻ DP stage (9, 10).

Another event associated with pre-TCR signaling is the allelic exclusion of the TCR-β locus, i.e., the feedback inhibition of the rearrangement on one allele by the TCR-β protein encoded by the other allele. Effective allelic exclusion as it is observed in α/β T cells requires not only this feedback inhibition but also asynchronous Vβ rearrangement on the two alleles (11). Direct analysis of TCR-β rearrangement in single cells at the CD25⁺CD44⁻ stage of development, i.e., before selection by the pre-TCR, has shown that the pre-TCR has an essential function in this process (12). Although these data were obtained by single-cell PCR analysis of pTα⁻/⁻ mice, experiments in TCR-β-transgenic mouse have shown that TCR-β transgenes can inhibit Vβ rearrangement in the absence of the pTα protein, most likely because of their high and early expression (13). Whatever the mechanism of this suppression, it also appears to depend on signals transduced via CD3, because it is not operative in CD3e-deficient mice (14).

Experiments in mice expressing active p56lck indicate that Src kinases are also involved in TCR-β allelic exclusion, as Vβ rearrangement is inhibited in such mice (15). Recent

I. Aifantis, V. Pivniouk, and F. Gärtner contributed equally to this work.

Abbreviations used in this paper: DN, double-negative; DP, double-positive; RAG, recombination activating gene; SLP, SH2 domain-containing leukocyte protein; WT, wild-type.
data, however, suggest that TCR-β allelic exclusion may require signaling pathways that are distinct from signals controlling proliferation and maturation, indicating that the pre-TCR has functions in addition to those responsible for rescue from programmed cell death. Active Ras, known to pre-TCR has functions in addition to those responsible for quire signaling pathways that are distinct from signals con-

...cured simultaneously by addition to each tube of 35 pmol of each that primed downstream of the J segments 5, 8, and 10, and the downstream primer was located 3' of Jβ2.7. After amplification of the rearrangements by PCR, the products were separated on agarose membranes, and hybridized with a Jβ2.7-specific, 32P-labeled oligonucleotide probe. Hybridizing bands were scanned using a PhosphorImager (Molecular Dynamics). Amplification of a fragment of the nonrearranging Cμ gene served as a loading control.

Single-Cell PCR. TCR-β+/CD25+ small single cells from SLP-76−/− mice were sorted using a FACSVantage™ equipped with an automatic cell deposition unit (Becton Dickinson). DNA from single cells was prepared as previously described (26). Introncellular staining for TCR-β was performed on CD25+ sorted cells as previously described (12). Data on 5 × 10⁶-2.0 × 10⁶ viable, nonerythroid cells (as determined by forward versus side scatter) were collected for each sample. FACSTM analysis was performed on cells from groups of at least three mice aged 2-4 wk.

Generation of SLP-76−/− TCR-α/β-transgenic Mice. The generation of TCR-α−/−, TCR-β−/−, RAG-2−/-, and SLP-76−/− mice was previously described (2, 4, 17, 27). Mice transgenic for the TCRVα13 and TCRVβ8.2 chains of the OVA-specific mouse T cell hybridoma DO 11.10 were a gift of Dr. Dennis Loh (Hoffmann-La Roche, Inc., Nutley, NJ; reference 28). Screening for expression of the transgenic TCR was done by PCR analysis of tail DNA, using primer sequences provided by Dr. K. Murphy (Washington University, St. Louis, MO), and by FACS™ analysis using the antidiotopic mAb KJ126, provided by Dr. K. Murphy (Washington University, St. Louis, MO). Screening for disruption of the SLP-76 gene was done as previously described (17).

PCR Analysis of TCR-β Gene Rearrangements in Thymocytes. Genomic DNA was isolated according to published procedures (29). Vβ to Dβ rearrangements were assessed as described in detail in reference 16. In brief, upstream primers were located on the Vβ segments 5, 8, and 10, and the downstream primer was located 3' of Jβ2.7. After amplification of the rearrangements by PCR, the products were separated on agarose gels, transferred to nylon membranes, and hybridized with a Jβ2.7-specific, 32P-labeled oligonucleotide probe. Hybridizing bands were scanned using a PhosphorImager (Molecular Dynamics). Amplification of a fragment of the nonrearranging Cμ gene served as a loading control.

Single-Cell PCR. TCR-β+, CD25+ small single cells from SLP-76−/− mice were sorted using a FACSVantage™ equipped with an automatic cell deposition unit (Becton Dickinson). DNA from single cells was prepared as previously described (12). TCR-β− gene rearrangements were amplified by a seminested two-step PCR protocol (12, 30). In the first step, both alleles were amplified simultaneously by addition to each tube of 35 pmol of a mixture containing dNTPs, buffer, and Taq polymerase at 0.5 U/sample containing dNTPs, buffer, and Taq polymerase at 0.5 U/sample.

Materials and Methods

Cells, Antibodies, and Flow Cytometric Analysis. The SCB29 cell line, which expresses the pre-TCR on the cell surface, was described previously (23). Single-cell suspensions from thymi were prepared by compression between ground glass slides followed by centrifugation on a density gradient of Lympholyte-M (Cedarlane Labs, Inc.). Streptavidin–Cy-Chrome, streptavidin–PE, and mAbs to the following mouse antigens were purchased from Pharmingen: CD3e (clone 145-2C11), CD4 (L3T4), CD8α (53-6.7), CD25 (7D4), CD44 (IM7), Thy1.2 (53-2.1), MAc-1 (M1/70), Gr-1 (RB6-8C5), and TCR-β (H57-597). Anti-CD25 mAb (3b27) conjugated to PE and anti-CD44 mAb conjugated to FITC were purchased from Sigma Chemical Co. Anti-βTα mAb 2F5 was raised against the extracellular (Ig-like) domain as described previously (24). In the case of pre-TCR surface detection, thymocytes were enriched for the CD4−CD8−/− subset by depletion of CD4+CD8+ positive cells using Dynabeads (Dynal). PhA-α (2F5) and TCR-β (H-57) biotinylated antibodies were revealed with streptavidin–PBX L-3 (Murex Biotec). NK and NK-T cells were gated out with DX-5, NK1.1, or 2B4 antibodies (Pharmingen). Cytoplasmic staining for TCR-β was performed as previously described (25). Cells were surface stained and analyzed on a FACSTM Calibur™ flow cytometer (Becton Dickinson) as previously de-

Results

The Pre-TCR Is Expressed on the Surfaces of SLP-76−/− Thymocytes. Surface expression of the pre-TCR is required...
for TCR-β allelic exclusion (for review see reference 31). In the analysis of allelic exclusion in SLP-76-/- mice, it was therefore important to ascertain expression of the pre-TCR by SLP-76-deficient thymocytes. In previous experiments, we have detected low levels of expression of CD3 and TCR-β on SLP-76-/-CD4-CD8- DN thymocytes, suggesting that the pre-TCR is expressed on the cell surfaces of these immature thymocytes (17). To extend these observations, we repeated these experiments with a fluorochrome reagent, streptavidin-PBXL-3 (Martek Biosciences), that gives much higher signal intensity than conventional reagents (i.e., streptavidin-FITC or -APC) when used in combination with biotinylated antibodies. This can be clearly seen by staining the SCB29 cell line, which expresses the pre-TCR on the cell surface (23), with biotinylated mAb to TCR-β (H57) or pTα (2F5), followed by incubation with either streptavidin-FITC or -PBXL-3 (Fig. 1). Incubation with irrelevant mAbs of the same Ig class produces some slight background that, however, is clearly distinct from the staining obtained with antibodies specific for cell surface-expressed proteins (Fig. 1).

In subsequent experiments, CD4-CD8- DN thymocytes from wild-type (WT), TCR-α-/-, SLP-76-/-, pTα-/-, and RAG-2-/- mice were examined for TCR-β and pTα expression. Thymocyte development in SLP-76-/- mice is blocked at the stage of CD25+CD44- DN cells. CD25-CD44+ cells, which represent a more mature stage of DN thymocytes, are absent in SLP-76-/- mice (17). We therefore focused our analysis on CD25-CD44- DN cells. DN thymocytes were stained with directly labeled antibodies against CD44 and CD25, as well as biotinylated antibodies against either TCR-β or pTα followed by streptavidin-PBXL-3, and gated populations of CD25-CD44- cells were analyzed for TCR-β and pTα expression. The histograms in Fig. 2 show
that, as expected, mAb to TCR-β did not stain thymocytes from RAG-2−/− mice and mAb to pTα did not stain thymocytes from pTα−/− mice when compared with control antibodies. This further confirms the specificity of the staining. There was negligible, if any, staining of RAG-2−/− thymocytes with anti-pTα mAb or of pTα−/− thymocytes with anti-TCR-β mAb. CD25+CD44− DN thymocytes from SLP-76−/− mice stained with both antibodies to the same or slightly higher level as CD25+CD44− DN cells from TCR-α−/− mice (Fig. 2). DN thymocytes from TCR-α−/− mice were used as controls to eliminate the possibility of staining TCR-β proteins that are transported to the cell surface as αβ TCRs. The surface expression of TCR-β and pTα chains on CD25+CD44− as well as CD25−CD44− thymocytes from TCR-α−/− mice and WT controls was identical (data not shown).

We next examined intracellular TCR-β protein expression in SLP-76−/− thymocytes. Fig. 3 shows that SLP-76-deficient CD25+ thymocytes expressed lower levels of intracellular TCR-β protein than WT CD25+ thymocytes (Fig. 3). Decreased levels of expression of intracellular TCR-β protein were also observed in CD25+ thymocytes from pTα−/− mice, which are also blocked at the CD25+CD44− DN stage (2). The CD25+ TCR-β+ cells in SLP-76−/− mice may represent γ/δ cells that express cytoplasmic TCR-β chain, as has been shown recently in pTα−/− mice (32). These results suggest that SLP-76 is not required for assembly and surface expression of TCR-β chains. However, it is required for the generation of CD25+ DN cells that express high levels of TCR-β protein intracellularly.

Introduction of TCR-α/β T transgenes does not overcome thymic development in SLP-76−/− littermates, and there were no detectable DP nor single-positive thymocytes (17). Introduction of the transgenic TCR into the SLP-76−/− background did not increase thymic cellularity (1.30 ± 0.18 × 10^6 cells in SLP-76−/−-tg mice versus 1.62 ± 0.75 × 10^6 cells in SLP-76−/− mice; n = 4 in each group), but resulted in an increase in CD3+ TCR-β+ thymocytes, consistent with surface expression of the transgene (Fig. 4 A). The transition from DN to DP cells remained severely impaired, as we could detect no or very few DP thymocytes in SLP-76−/−-tg mice (Fig. 4 B). Analysis of the B220+Mac-1−Gr-1−CD3−CD4−CD8− DN compartment for the expression of CD25 and CD44 revealed that introduction of the transgenic TCR failed to overcome the block in thymic development at the CD25+CD44− stage present in SLP-76−/− mice (Fig. 4 C). These results suggest that the failure of DN cells from SLP-76−/− mice to progress to the DP stage is due to deficient SLP-76-mediated signaling and cannot be overcome by surface expression of the transgenic TCR.

Introduction of a TCR-α/β T transgene does not inhibit endogenous Rearrangement at the TCR-β Locus in SLP-76−/− Mice. Having established that the pre-TCR is expressed on DN thymocytes from SLP-76−/− mice, we proceeded to address the issue of whether TCR-β allelic exclusion takes place in the absence of SLP-76. There are two ways in which feedback inhibition by the pre-TCR resulting in TCR-β allelic exclusion has been analyzed: one is the inhib-
Inhibition of endogenous Vβ rearrangements by TCR-β transgenes, and the other is the analysis of both TCR-β alleles in single cells. We initially examined inhibition of Vβ rearrangements by TCR transgenes.

In normal thymocytes, introduction of functionally rearranged TCR-β transgenes leads to inhibition of rearrangements in the endogenous TCR-β locus at the V to DJ step (35). Furthermore, introduction of a TCR-β transgene in pTα2/2 and p56lck2/2 also leads to inhibition of endogenous TCR-β rearrangements (13, 36). To examine if introduction of the transgenic TCR into SLP-76–deficient thymocytes could also inhibit endogenous Vβ to DJβ rearrangements, we used a semiquantitative PCR assay as previously described (16, 37). In these experiments, Vβ to DJβ rearrangements involving representative Vβ segments (Vβ5, Vβ8, and Vβ10) were amplified from genomic DNA samples isolated from DN thymocytes of TCR-transgenic or nontransgenic SLP-76-/- mice. The identity of the resulting DNA fragments corresponding to rearrangements between a particular Vβ and one of the Jβ segments was confirmed by Southern blot analysis with a Jβ2.7-specific probe. In agreement with our previously published observations (17), Vβ to DJβ rearrangements were readily detectable in both SLP-76-/- and SLP-76+/+ thymocytes (Fig. 5, lanes 1, 3, 5, 7, 9, and 11). As expected, introduction of the TCR-β transgene into SLP-76-/- mice almost completely blocked Vβ to DJβ rearrangements of the endogenous TCR-β genes (Fig. 5, lanes 1, 2, 5, 6, 9, and 10). In contrast, rearrangements of the endogenous TCR-β genes occurred at comparable levels in both TCR-transgenic and nontransgenic SLP-76+/+ mice (Fig. 5, lanes 3, 4, 7, 8, 11, and 12). These results suggest that SLP-76 is essential for transgene-mediated inhibition of TCR-β locus rearrangement.

The SLP-76 Adaptor Protein Is Required for TCR-β Allelic Exclusion.

Introduction of a TCR-β transgene into pTα-/- mice (13) inhibits rearrangements of endogenous Vβ segments, although single-cell PCR analysis of Vβ rearrangements reveals a failure of TCR-β allelic exclusion in pTα-/- mice (12). This suggests that expression of the transgene provides signals for inhibition of TCR-β rearrangement that differ quantitatively and/or qualitatively from physiologic signals delivered by the pre-TCR and may not reflect authentic allelic exclusion. To assess the status of TCR-β allelic exclusion in unmanipulated SLP-76-/- mice, we ex-
amined Vβ rearrangements in single CD25⁺CD44⁻ thymocytes from these mice.

In WT mice, only ~40% of CD25⁺TCR-β⁺ thymocytes rearrange Vβ on both alleles due to the feedback inhibition by the first productively rearranged allele that prevents further rearrangement on the other allele (12). In pTα⁻/⁻ mice, this feedback inhibition fails, and hence a greater population of cells (60%) exhibits two completely rearranged VDJβ alleles (12). When single CD25⁺CD44⁻ DN cells from SLP-76⁻/⁻ mice that expressed cytoplasmic TCR-β chains were sorted and analyzed, a similar propor-

tion of the cells (60%) was found to contain rearranged VDJβ on both alleles (Table I).

T cells with two productive Vβ rearrangements are a rare event in WT mice; <5% of normal cells with two Vβ rearrangements contain two productive alleles (12). When the alleles from SLP-76⁻/⁻ thymocytes with two Vβ rearrangements were sequenced, it was found that 31% of the cells with two Vβ rearrangements had two productive alleles (Table I). The Vβ usage and the sequences of the two in-frame alleles are shown in Tables II and III. They provide no indication for the selection of certain Vβ alleles in allelically included cells, even though it is difficult to entirely rule out this possibility due to the relatively small sample size. Taken together, the data obtained by the single-cell PCR analysis indicates that allelic exclusion at the TCR-β locus requires the SLP-76 adapter protein.

**Table I.** Number of CD25⁺CD44⁻ T thymocytes from SLP-76⁻/⁻ Mice According to the Type of Rearrangements on Both TCR-β Alleles

| Type of rearrangement | V(D)J/V(D)J⁺ | V(D)J⁺/V(D)J⁺ | V(D)J/V(D)J⁺⁺ | V(D)J⁺⁺/V(D)J⁺⁺ |
|-----------------------|--------------|---------------|---------------|-----------------|
| V(D)J/V(D)J⁺         | 18 (40%)     | 27 (60%)      | 8 (31%)       | 18 (69%)        |

*Total cells = 45.
†TCR-β rearrangements were analyzed by PCR and subsequent migration of the products on an ethidium bromide-stained agarose gel. Each V(D)J joint was determined by sequence analysis of V(D)J⁺ and V(D)J⁺⁺. The presence of a very small population of CD25⁺CD44⁻ DN thymocytes (5%) of normal cells with two V(D)J joints from single CD25⁺CD44⁻ DN thymocytes may be accounted for by the developmental arrest in thymic development could be overcome by introduction of a functional TCR-α/β transgene.

We have ascertained that CD25⁺CD44⁻ DN thymocytes from SLP-76⁻/⁻ as well as WT mice express the pTα and TCR-β chains of the pre-TCR complex (Fig. 2). As the 2F5 antibody we used is directed against the extracellular Ig-like domain of pTα, it is also clear from our studies that it is the form of pTα that expresses the extracellular Ig-like domain that reaches the cell surface. DN thymocytes from SLP-76⁻/⁻ mice expressed equal or slightly higher amounts of TCR-β chains on their surfaces compared with TCR-α⁻/⁻ mice (Fig. 2) or WT mice (data not shown). The fact that DN thymocytes from both SLP-76⁻/⁻ and pTα⁻/⁻ mice express lesser amounts of intracellular TCR-β chains than WT mice raises the possibility that signals through the pre-TCR mediated by SLP-76 may be important in upregulating TCR-β chain expression. The presence of a very small population of CD25⁺TCR-β⁺ DN thymocytes in SLP-76⁻/⁻ thymocytes (~2%) is consistent with the presence of a very small population of CD25⁺CD44⁻ triple-negative cells in these mice (1.2%; Fig. 4C). The equal or slightly higher surface expression of TCR-β and pTα in SLP-76⁻/⁻CD25⁺CD44⁻ DN thymocytes may then be accounted for by the developmental arrest that results in the accumulation of small, noncycling CD25⁺CD44⁻ DN cells, allowing these cells to bring a relatively higher amount of the pre-TCR to the cell surface. Alternatively, in the absence of SLP-76, internalization and subsequent degradation of the pre-TCR, which is dependent on activation of serine/threonine and tyrosine kinases (38), may be retarded.
Table II. Junctional Sequences* of SLP-76^{−/−} CD25^{+44} Thymocytes Bearing Two Productive TCR-β Alleles

| 3' Vβ | N region | 5' Jβ | Vβ/Jβ |
|-------|----------|-------|-------|
| 97    | TGT GCC AGC AG.................................................................CT GGA AAT ACG CTC TAT TTT GGA | V1J1.3 |
|       | TGT GCC AGC AGC CA.............................................................A AAC ACA GAA GTC TCT TTT GGG | V1J1.1 |
| 118   | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.5 |
|       | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.5 |
| 120   | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.5 |
|       | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.5 |
| 131   | TGT GCC AGC TC.....................................................................G CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
|       | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
| 143   | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
|       | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
| 114   | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
|       | TGT GCC AGC AGC CA.............................................................A G..........................AC CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
| 11    | TGC ACC TGC AGT G...............................................................G CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
|       | TGC ACC TGC AGT G...............................................................G CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
| 39    | TGT GCC AGC AGC CA.............................................................G CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |
|       | TGT GCC AGC AGC CA.............................................................G CAA GAC ACC CAG TAC TTT GGG | V1J2.6 |

*The two productive V(D)J joints are arbitrarily numbered 1 and 2 for each cell. N regions (italics) are determined by alignment of the sequences with the known germline sequences of Vβ (42), Dβ (43), and Jβ (43, 44) genes. Sequences are aligned starting with the codon of the invariant cysteine 109.
endogenous TCR-β rearrangement by TCR-β transgenes in pT α−/− mice might be artifactual and might reflect activation of downstream pathways not ordinarily engaged by the endogenous pre-TCR (12). Indeed, whereas introduction of a TCR-β transgene into the pT α−/− background shuts off endogenous TCR-β rearrangement, single-cell analysis in pT α−/− mice reveals failure of TCR-β allelic exclusion (12, 13). As introduction of TCR-β transgenes into pT α−/− mice also drives the maturation of DN cells into DP cells and their proliferation, premature transgene-generated signals might accelerate development of normal cells through stages at which the endogenous TCR-β genes are assembled, leading to a block in rearrangement (40). The failure of TCR-α/β transgenes to suppress endogenous TCR-β rearrangement and induce maturation and expansion of DN cells in SLP-76−/− mice suggests that transgene-generated signals that lead to maturation and expansion of DN cells and to feedback inhibition of endogenous TCR-β rearrangement are strictly dependent on SLP-76.

Normally, 60% of surviving thymocytes carry only a single Vβ to DJβ rearrangement, whereas the remaining 40% carry one nonproductive and one productive Vβ to DJβ rearrangement (35). The presence of >40% of cells with Vβ to DJβ rearrangement on both alleles indicates a violation of TCR-β allelic exclusion. Single-cell PCR analysis revealed that 60% of CD25−CD44− TCR-β+ DN thymocytes from SLP-76−/− mice have undergone Vβ to DJβ rearrangement on both TCR-β alleles (Table I). This indicates that TCR-β allelic exclusion is defective in the absence of SLP-76 and suggests that SLP-76 is essential for the transduction of the physiologic pre-TCR signal that inhibits V to DJ rearrangement on the second TCR-β allele.

Due to feedback inhibition of TCR-β locus rearrangement by a productively rearranged allele (41), <3% of normal T cells carry two productive Vβ gene rearrangements (12). In the absence of feedback inhibition, it is expected that in 20% of cells that rearrange two alleles, both alleles are rearranged productively. When the alleles from SLP-76−/− thymocytes with two Vβ rearrangements were sequenced, it was found that in 31% of the cells both rearrangements were productive, i.e., in frame (Table I). This is almost exactly the same percentage that was found in pT α−/− mice (12). The discrepancy between the observed and the predicted fraction of cells with two productive Vβ rearrangements may be due to selection against cells that make a productive rearrangement on the second allele only, due to lower TCR-β staining intensity, when cells are sorted for expression of cytoplasmic TCR-β chains. There was no indication for the selection of specific Vβ alleles in allelically included cells in either SLP-76−/− mice (Tables II and III) or pT α−/− mice (12). The presence of a significant proportion of SLP-76−/− thymocytes with two productively rearranged TCR-β alleles documents an essential role of SLP-76 in allelic exclusion at the TCR-β locus. The fact that similar percentages of cells with two in-frame alleles were found in SLP-76−/− and pT α−/− mice suggests that the pre-TCR is the most important receptor that mediates feedback inhibition of Vβ rearrangement under physiological conditions.

### Table III.

| Code | R arrangement       | Code | R arrangement       |
|------|---------------------|------|---------------------|
| 10   | V15/2/D1J1          | 34   | V11J2/V4J2          |
| 5    | V16/1/D2J2          | 39   | V8/1/V1J2           |
| 15   | V11/1/D2J2          | 40   | V4/2/V10J           |
| 29   | V5/1J2              | 49   | V10/2/V15J1         |
| 41   | V4/2/D1J1           | 54   | V12J2/V4J2          |
| 43   | V1/1J2              | 62   | V11J2/V1J1          |
| 45   | V8/2/D1J1           | 64   | V12/2/V10J2         |
| 47   | V14/1J1/D1J1        | 65   | V8/1/V12J1          |
| 52   | V1/2J2              | 69   | V5/2/V17J2          |
| 58   | V3/1J2              | 74   | V1/2/V5J2           |
| 68   | V14/1J2/D1J2        | 76   | V12/2/V15J2         |
| 73   | V8/2/D1J1           | 93   | V15/1/V14J2         |
| 79   | V8/2/D1J1           | 97   | V1/1J/V10J1         |
| 87   | V4/1J1/D2J2         | 104  | V8/1/V5J2           |
| 100  | V6/1J2/D2J2         | 114  | V1/2/V8J2           |
| 103  | V8/2J2              | 118  | V1/2/V6J2           |
| 119  | V5/2/D1J1           | 120  | V5/2/V10J2          |
| 134  | V11/2J2/D1J1        | 131  | V8/1/V5J2           |
| 94   | V5/2/V15J2          | 138  | V10/2/V15J1         |
| 7    | V3/1J/V17J2         | 141  | V7/2/V11J1          |
| 11   | V8/2/V2J1           | 143  | V8/2/V11J2          |
| 19   | V8/2J4/2            | 2     | V2/2/V9J2           |
| 32   | V7/1/V12J2          |      |                     |

* A number code was assigned to the CD25−CD44− TCR-β+ cells isolated from SLP-76−/− mice. Data shown represent rearrangements of Vβ(Dβ) or Dβ with the Jβ cluster of genes.

Introduction of TCR-β transgenes into rearrangement-deficient mice drives proliferative expansion and differentiation of DN to DP cells (33, 34). In contrast, introduction of functional TCR-β transgenes into SLP-76−/− mice failed to increase thymic cellularity and failed to overcome the block in the development of CD25−CD44− DN cells and in the transition from DN to DP cells (Fig. 4). We had previously shown that treatment of SLP-76−/− mice with anti-CD3ε mAb induced the appearance of only a few DP thymocytes (17), suggesting that an SLP-76−/− independent pathway for DN to DP transition may exist. Such a pathway could involve linker for activation of T cells (LAT)-Grb2-Sos-mediated activation of R as, as a transgene encoding an active form of R as (R as12) has been shown to drive the development of DP cells in the RAG−/− background (39). It appears from our data that expression of a transgenic TCR may not be sufficient to activate an SLP-76−/− independent pathway of thymocyte maturation.

Introduction of TCR-β transgenes into the SLP-76 background failed to suppress endogenous TCR-β rearrangement (Fig. 5). It has been suggested that inhibition of...
Recent data suggest that activation of Ras results in differentiation and expansion of DN thymocytes but not in TCR-β allelic exclusion (16). Thus, suppression of TCR-β gene rearrangements appears to require the activity of an additional and/or complementary pathway. As SLP-76-/- thymocytes are arrested at the DN stage and also fail to suppress endogenous Vβ to DJβ rearrangements, even after introduction of TCR-αβ transgenes, SLP-76 is likely to be critical for TCR-mediated activation of both Ras-dependent and Ras-independent pathways.

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Address correspondence to Ralf S. Geha, Enders B, Div. of Immunology, 300 Longwood Ave., Children's Hospital, Boston, MA 02115. Phone: 617-355-7603; Fax: 617-355-8205; E-mail: geha@a1.tch.harvard.edu

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