Essential Role of the Pre-T Cell Receptor in Allelic Exclusion of the T Cell Receptor β Locus

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Summary

Following the recent realization that TCRβ transgenes can severely inhibit the rearrangement of endogenous Vβ gene segments in the absence of pre-TCRα (pTα) chains, we tested whether the pre-TCR has an essential role in TCRβ allelic exclusion under more physiological conditions by analyzing TCR rearrangement in immature thymocytes by single-cell PCR. Our results in pTα+ mice are consistent with an ordered model of TCRβ rearrangement beginning on one allele and continuing on the other only when the first attempt is unsuccessful. By contrast, a higher proportion of thymocytes from pTα−/− mice exhibited two productive TCRβ alleles. Thus, the pre-TCR–independent suppression of rearrangement by TCRβ transgenes represents a transgene artifact, whereas under physiological conditions the pre-TCR is essential for allelic exclusion.

Introduction

T cell receptor (TCR) genes for antigen are formed by rearrangement of gene segments in developing lymphocytes (Davis and Bjorkman, 1988). The joining first of Dβ to Jβ segments and subsequently of Vβ segments to the DβJβ joint can result in the formation of a productive TCRβ gene that encodes the β chain of the αβ TCR for antigen. Analysis of mature T cell clones has shown that, as a rule, mature T cells contain only one productive TCRβ rearrangement, while the other allele is nonproductively rearranged or the Vβ gene segment remains in germline configuration (Casanova et al., 1991). This constellation ensures that an individual T cell expresses only a limited number of different TCRs. The limited diversity of TCRs expressed by individual T cells is essential for the generation of specific immune responses as well as for effective self–nonself discrimination, which depends on the specific elimination or silencing of self-reactive T cells (von Boehmer et al., 1989; Rocha and von Boehmer, 1991; Watanabe-Fukunaga et al., 1992).

In developing T cells, in general, TCR Vβ gene segments rearrange before TCR Vα gene segments, such that TCRβ rearrangements are easily detectable in CD4−CD8−CD25+CD44− precursor cells of CD4+CD8+ cells, which exhibit TCRα rearrangement (Godfrey et al.,

1993; Petrie et al., 1995). The rearrangement of Vα genes is regulated differently from that of Vβ genes, since about 30% of mature αβ T cells carry two productive TCRα rearrangements (Casanova et al., 1991). Because of this constellation, T cells can express at most two different TCRs that share the same TCRβ chain (Borgulya et al., 1992; Malissen et al., 1992). The ordered rearrangement of receptor gene segments prompted the hypothesis that the first rearranged receptor gene products (i.e., TCRβ and, by analogy, immunoglobulin H [IgH] chains) feed back and prevent further rearrangement at their genetic loci (Alt et al., 1981). In support of this idea, it was shown that in lymphocytes from TCRβ or IgH transgenic mice that express the transgene early in development, endogenous Vβ or V_H gene segments remain in germline configuration (Storb et al., 1986; Uematsu et al., 1988).

Of course, such feedback signals must be generated in the absence of TCRα or IgL chains, and therefore it was of interest that TCRβ chains and IgH chains could form signal-transducing receptor complexes by pairing covalently with pre-TCRα (pTα) chains (Groettrup et al., 1993; Saint-Ruf et al., 1994; von Boehmer and Fehling, 1997) and λ5 chains (Karasuyama et al., 1994; Melchers et al., 1994), respectively. Such receptor complexes were also shown to be noncovalently associated with signal-transducing CD3ε, γ, and ζ (Groettrup et al., 1992; Jacobs et al., 1994) and Igα and Igβ molecules (Brouns et al., 1993), respectively, and cross-linking of these receptors resulted in Ca^{2+} mobilization (Nomura et al., 1991; Groettrup and von Boehmer, 1993). These pre-T and pre-B cell receptors were thus ideal candidates to mediate inhibition of further TCRβ and IgH rearrangements and, thereby, allelic exclusion of the respective loci.

It therefore came as a surprise that a TCRβ transgene could inhibit rearrangement in thymocytes that were derived from TCRβ transgenic embryonic stem cells deficient in the pTα gene (Xu et al., 1996). When these experiments were repeated in TCRβ transgenic mice that were likewise deficient in the pTα gene (although the deficiency was generated by a different mutation, affecting the transmembrane region that is required for the association with signal-transducing CD3 molecules [Fehling et al., 1995]), it was again noted that the TCRβ transgene could inhibit approximately 90% of the endogenous Vβ rearrangement when compared to wild-type mice. There was, however, also a clear difference between TCRβ transgenic pTα+ and TCRβ transgenic pTα−/− mice: rearrangement of endogenous Vβ segments was more completely inhibited in the former than in the latter mice, indicating that the pre-TCR had some (minor) role in inhibiting rearrangement (Krotkova et al., 1997). It was also noted that the TCRβ transgene could promote some thymic development even in pTα−/− RAG−/− mice, that is, in the absence of other TCR chains (Krotkova et al., 1997). From these studies it was difficult to conclude whether the biological effect of TCRβ transgenes represented some physiologically relevant activity or whether it represented a transgenic artifact

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due to the stronger and earlier expression of transgenes as well as unusual forms, for instance gpi (glycophosphatidylinositol)–linked monomers (Groettrup and von Boehmer, 1993). Such nonphysiological expression patterns may create artificial signals that mimic physiologically generated signals.

On the basis of these results, we considered it necessary to assess the role of the pre-TCR in allelic exclusion of the TCRβ locus without the use of TCRβ transgenes. This can be done by analyzing TCRβ rearrangements in single cells from pTα$^+$ and pTα$^{-/-}$ mice. Since the pTα deficiency is associated with a developmental defect, such experiments should not be conducted on total thymocytes or mature T cells but rather should be conducted in cells that undergo TCRβ rearrangement before being selected by the pre-TCR—specifically, small CD4$^-$CD8$^-$CD25$^+$CD44$^-$ cells (Godfrey et al., 1993). Such cells accumulate in greater numbers in pTα$^{-/-}$ mice and express higher levels of CD25 surface molecules, presumably because of lack of selection by the pre-TCR (Fehling et al., 1995; Krotkova et al., 1997). If the pre-TCR was involved in mediating allelic exclusion of the TCRβ locus, one might expect to see violation of allelic exclusion in these cells before they eventually die as a result of lack of signals that enable them to survive, divide, and develop into mature cells.

Results

Increased Frequency of Cells with Two Rearranged V(D)J TCRβ Alleles in pTα Deficient Mice

In the thymus, TCRβ rearrangement takes place in CD4$^-$CD8$^-$CD3$^-$ cells that express CD25 molecules on the cell surface (Godfrey et al., 1993; Petrie et al., 1995). Since we were aiming at a comparative analysis of cells exhibiting two productive TCRβ rearrangements in pTα$^+$ and pTα$^{-/-}$ mice, we enriched cells that contained at least one productive TCRβ gene by sorting double-positive cells that were stained in the cytoplasm with TCRβ antibodies and on the cell surface with CD25 antibodies. Small CD25$^+$ TCRβ$^+$ cells were gated as shown in Figure 1A and sorted accordingly. As noted in previous reports, the expression of CD25 was higher on cells from pTα$^{-/-}$ mice due to lack of selection of these cells by the pre-TCR (Fehling et al., 1995; Krotkova et al., 1997). A similar phenotype was noted in CD3ε$^{-/-}$ and p56$lck^{-/-}$ mice, which are deficient in pre-TCR signaling (Malissen et al., 1995; van Oers et al., 1996). The staining shown in Figure 1A exaggerates this difference because it was done on different days. Figure 1B shows the difference between pTα$^+$ and pTα$^{-/-}$ mice when the staining is performed on the same day. The intensity of TCRβ staining of CD25$^+$ cells was comparable in pTα$^+$ and pTα$^{-/-}$ mice.

The sorted cells were diluted and single cells picked by micromanipulation. DNA was extracted and TCRβ rearrangement was assessed by single-cell polymerase chain reaction (PCR) using the primers depicted in Table 1, which amplify many but not all Vβ gene segments and were likewise designed to distinguish rearrangements of V or D gene segments joining to either Jβ1 or Jβ2. PCR products were identified and reamplified by a semi-nested PCR as described in Experimental Procedures.

Single cells from pTα$^+$ and pTα$^{-/-}$ mice (240 and 165, respectively) were studied by analyzing their PCR fragments. Of these cells, 24% and 25%, respectively, yielded no PCR product, possibly as a result of inappropriate DNA extraction, use of nonsuitable primers, or failure of the PCR reaction for unknown reasons. For similar reasons, 33% and 31% of the respective cells yielded only one PCR fragment. Two PCR fragments were found for 36% of cells from pTα$^+$ and 33% of cells from pTα$^{-/-}$ mice, and these cells were further analyzed. A small percentage of the cells exhibited three or four PCR fragments. Whenever the latter cells were analyzed further they contained either one or two rearrangements to the Jβ1 locus. Since this type of rearrangement was highly enriched in cells with more than two PCR products, these results indicate that DNA excision loops were amplified in addition to rearrangements to Jβ2 with proper chromosomal location (Table 2).

To analyze cells with rearrangements on both alleles we considered in our further analysis only cells that yielded two PCR products and exhibited rearrangements to the J2 region. As shown in Table 3, for pTα$^+$ mice 55 cells fell into this category whereas in pTα$^{-/-}$ mice we detected 36 cells. Further analysis showed a significant difference (Fisher's exact probability, P = 0.046) between pTα$^+$ and pTα$^{-/-}$ mice: in pTα$^+$ mice 34 cells, or 62% of all cells with rearrangements involving Jβ2, were of the V(D)J/DJ type, while 38% exhibited two Vβ rearrangements (V(D)J/V(D)J) with involvement of the indicated Vβ gene segments (Table 3). On the other hand, in pTα$^{-/-}$ mice, 16 of 36 cells (44%) were of the V(D)J/DJ type and 20 (56%) were of the V(D)J/V(D)J type. This indicates that among CD25$^+$ cells of pTα$^{-/-}$ mice, Vβ rearrangement can more often proceed on both alleles, perhaps because of lack of inhibitory feedback from a first productive TCRβ rearrangement.

The Proportion of Cells with Two Inframe TCRβ Rearrangements among Cells Exhibiting Two Distinct Vβ Rearrangements Is Higher in pTα$^{-/-}$ Than in pTα$^+$ Mice

The ultimate test for lack of inhibitory feedback from a productive TCRβ gene in pTα$^{-/-}$ mice required sequencing of both alleles from cells exhibiting two V(D)J rearrangements from both pTα$^+$ and pTα$^{-/-}$ mice. We had obtained PCR products from 21 such cells from pTα$^+$ and 20 cells from pTα$^{-/-}$ mice and were able to obtain sequences of both PCR products from 19 and 20 of these cells, respectively. Whereas only 1 of 19 cells from pTα$^+$ mice exhibited V$^+$/V$^+$ rearrangements, 7 of 20 from pTα$^{-/-}$ mice were of the V$^+$/V$^+$ type, that is, contained two productive TCRβ genes (Tables 4 and 5). This finding clearly indicates that in CD25$^+$ cells from pTα$^{-/-}$ mice, because of lack of the pre-TCR, rearrangement can proceed despite the presence of an already productive TCRβ gene; in pTα$^+$ mice, in contrast, a productive TCRβ gene inhibits further rearrangement. The sequences and the Vβ gene segment use shown in Table 5 indicate that there is no preferential use of certain Vβ genes in cells that express two productive alleles.

Discussion

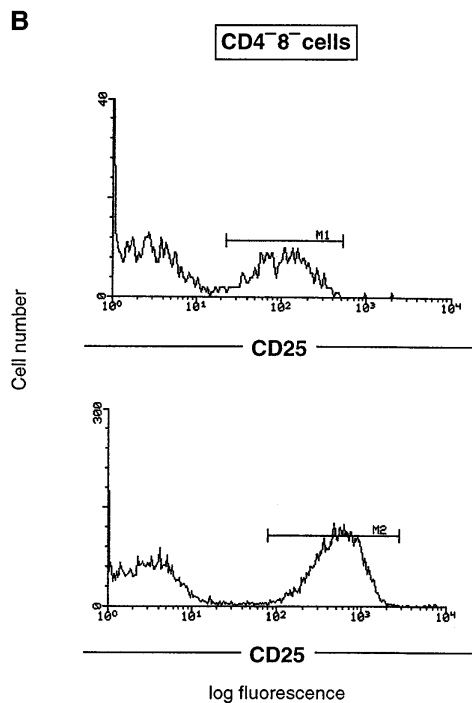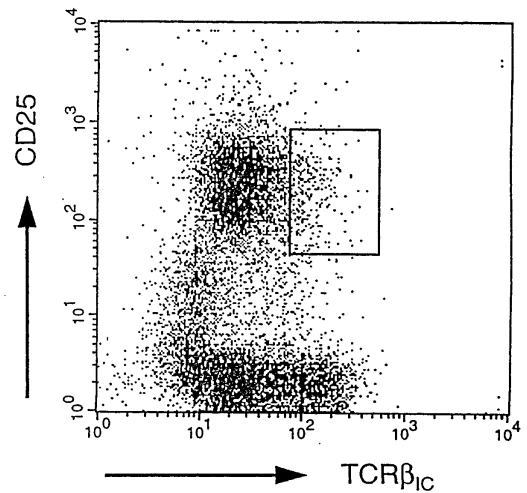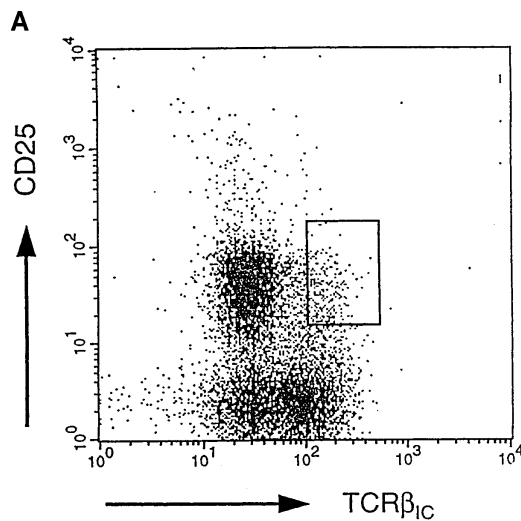Results with regard to allelic exclusion in TCRβ transgenic pTα$^{-/-}$ mice (Xu et al., 1996; Krotkova et al., 1997)

A

B

CD4⁻8⁻cells

Figure 1. Flow Cytometric Analysis of Thymocytes from pTα⁺ and pTα⁻/⁻ Mice

(A) Total thymocytes from (left) pTα⁺ and (right) pTα⁻/⁻ mice were surface-stained with phycoerythrin-conjugated CD25 antibodies, and cytoplasmic staining was performed with panTCRβ antibodies. Cells from pTα⁺ mice were enriched for the CD4⁻CD8⁻ subset by negative depletion of CD4/CD8 positive cells using Dynabeads prior to staining. Small TCRβ⁺ CD25⁺ cells were sorted according to the indicated gates using a FACS Vantage cell sorter. Since the staining was performed on different days, the difference in the CD25 staining between pTα⁺ and pTα⁻/⁻ cells is exaggerated.

(B) Differences in CD25 expression levels in CD4⁻CD8⁻ thymocytes when cells were stained and analyzed in the same experiment.

have indicated that TCRβ transgenes can mediate efficient inhibition of TCRβ rearrangement in the absence of the pre-TCR. This may indicate that the pre-TCR is not needed for allelic exclusion or that abnormal expression of TCRβ proteins encoded by TCRβ transgenes can somehow artificially mimic the function of the pre-TCR in TCRβ transgenic mice but not in normal mice. To distinguish between these two possibilities we studied allelic exclusion of the TCRβ locus in the absence of TCRβ transgenes in pTα⁺ and pTα⁻/⁻ mice by analyzing immature T cells that undergo TCRβ rearrangement rather than by analyzing mature T cells that may be inefficiently rescued by a mechanism independent of the pre-TCR (Buer et al., 1997).

The single-cell PCR method works with similar efficiency for TCRβ⁺ cells from pTα⁺ and pTα⁻/⁻ mice but of course does not detect rearrangements in all cells subjected to it. This may be due in part to the selection

of primers used in the PCR and in part to the limiting amount of appropriately extracted DNA. Nevertheless, approximately 40% of cells gave results that might be expected from a TCRβ⁺ CD25⁺ cell, namely, two or more PCR fragments of which at least one represented a V(D)J fragment. Cells with two DJ fragments might be considered contaminants of the sorted population. Cells with three or four fragments may contain DNA excision loops involving rearrangements to Jβ1 in addition to rearrangements to Jβ2. We did not include cells with two DJ rearrangements or cells exhibiting more or fewer than two PCR fragments in our further analysis but rather concentrated on cells with two rearrangements involving Jβ2, since these can be expected to involve both alleles and not to represent amplifications of excision loops. Nevertheless, it is already clear from Table 2 that among cells with two PCR fragments the proportion of cells with two V(D)J fragments is significantly higher in

Table 1. Nucleotide Sequences of Primers Used in Single-Cell PCR

Primers	5'	3'
5' Vβ family primers		
Vβ1	GTTGATTCGAAATGAGACGGTGCCC	
Vβ2	GGAGTCCTGGGGACAAAAGAGGTCA	
Vβ4	CCTGATATGCGAACAGTATCTAGGC	
Vβ5	CCCAGCAGATTCTCAGTCCAACAG	
Vβ6	GCGATCTATCTGAAGGCTATGATGC	
Vβ8	GCATGGGCTGAGGCTGATCCATTA	
Vβ10	TCCAAGGCGCTTCTCACCTCAGTC	
Vβ11	TGCTGGTGTCATCCAAACACCTAG	
Vβ12	AGTTACCCAGACACCCAGACATGA	
Vβ13	CTGCTGTGAGGCCTAAAGGAACTA	
Vβ14	AGAGTCGGTGGTGCAACTGAACCT	
Vβ15	CCCATCAGTCATCCCAACTTATCC	
Vβ16	TAGGACAGCAGATGGAGTTTCTGG	
5' Dβ primers		
Dβ1	GCTTATCTGGTGGTTTCTTCCAGC	
Dβ2	GTAGGCACCTGTGGGGAAGAAACT	
3' Jβ primers		
Jβ1	GCAGAGTTCCATTTCAGAACCTAGC	
Jβ2	TGAGAGCTGTCTCCTACTATCGATT	
Jβ1 nested	CTAAATTTCCCAGTCCCTTCCACC	
Jβ2 nested	TTTCCCTCCCGGAGATTCCCTAA	

The size of the amplified PCR product (VJ or DJ) was 250 bp to 1.5 kb, depending on the respective position of the different V- or D-specific primers in the nucleotide sequences.

pTα$^{-/-}$ than in pTα$^+$ mice. This becomes even more evident if one considers only rearrangements to Jβ2: Here the proportion was 38% in pTα$^+$ mice and 56% in pTα$^{-/-}$ mice. The use of various Vβ gene segments (Table 3) does not reveal any strong bias for particular Vβ gene segments. Finally, the sequencing of PCR products from 19 cells with two V(D)Jβ2 rearrangements from pTα$^+$ mice and 20 cells with the same type of rearrangements from pTα$^{-/-}$ mice revealed one cell with two inframe TCRβ rearrangements (5%) in the former and 7 cells with two inframe rearrangements (35%) in the latter mice. This clearly indicates that TCRβ protein–mediated feedback inhibition fails in CD25$^+$ cells in pTα$^{-/-}$ mice.

The data observed in pTα$^+$ mice, with more than 60% of cells yielding two PCR fragments of the V(D)J/DJ type, are well in line with earlier observations by Casanova et

Table 2. Efficiency of Amplification of TCRβ Rearrangement in CD25$^+$ TCRβ$^+$ Single Cells of pTα$^+$ and pTα$^{-/-}$ Mice

PCR Products	pTα$^+$ (n = 240)	pTα$^{-/-}$ (n = 165)
No amplification	24%	25%
One fragment	33%	31%
Two fragments, DJ/DJ	2%	3%
Two fragments, V(D)J/DJ	22%	15%
Two fragments, V(D)J/VDJ	12%	15%
Three or four fragments	6%	10%

TCRβ rearrangements were amplified by a two-step single-cell PCR method as described in Experimental Procedures. One fragment seen on an ethidium bromide–stained agarose gel corresponded to a DJ or V(D)J rearrangement. Single cells carrying three fragments were interpreted as amplification of two alleles and an additional excision loop. The amplified fragments in cells with two or more rearrangements were determined to represent either V(D)J or DJ rearrangements, and the particular Jβ element used was determined by fragment size and sequence analysis.

al. in mature T cell clones (Casanova et al., 1991). They are consistent with an ordered model of TCRβ rearrangement that begins on one chromosome and continues on the other only if the first attempt yields a nonproductive gene, whereas a productive rearrangement inhibits further rearrangement and thus the Vβ gene on the other chromosome remains in germline configuration. Therefore, CD25$^+$ cells with one productive TCRβ rearrangement on the first rearranging allele proceed in their maturation without further alterations at the second allele.

From our data it appears that in normal mice only the pre-TCR is responsible for allelic exclusion of the TCRβ locus since in pTα$^{-/-}$ mice a highly significant fraction of CD25$^+$ cells with two Vβ rearrangements contained two productive TCRβ genes. The theoretical expectation for the frequency of such cells depends on several assumptions, such as consecutive rearrangement on the two alleles, supported by the data in pTα$^+$ mice, as well as the availability of time for consecutive rearrangements when inhibitory feedback fails because of lack of formation of the pre-TCR. The expectation of "allelicaly included" cells among cells that exhibit at least one productive rearrangement is 20% if there is no feedback by products of productive TCRβ genes and if there is sufficient time for rearrangement, that is, if the cells live long enough to complete rearrangements on both alleles. Our observed value of 35% is clearly above this expectation.

One trivial reason for this larger than expected value is our relatively small sample size: the observed frequency may represent a chance deviation. It also is conceivable that the sorting procedure induced a bias against cells that produce a TCRβ chain only late in their lifespan, after initial nonproductive rearrangements. If such cells could not accumulate sufficient levels of TCRβ protein to be detected before they die, the sorting procedure would preferentially sample cells that began with a productive rearrangement on the initially rearranging allele and then continue rearranging at the other allele. Such an experimentally induced bias would increase the proportion of V$^+$/V$^+$ cells among V$^+$ cells to more than 30% if there were no inhibitory feedback by the first produced TCRβ chain in pTα$^{-/-}$ mice. The same would not be true in pTα$^+$ mice because in this case cells could be rescued from programmed cell death by the pre-TCR, and therefore even cells with a productive rearrangement on the second allele would survive long enough to produce sufficient TCRβ proteins to be detected by cytoplasmic staining.

Whatever the reason for the high frequency of allelically included cells in pTα$^+$ mice—whether a statistical anomaly or a bias induced by the sorting procedure—it is clear that the results strongly indicate that there is no feedback inhibition by productive TCRβ rearrangements in pTα$^{-/-}$ mice, and thus it is likely that under physiological conditions the TCRβ/pTα complex is the only relevant TCRβ-containing protein complex that mediates allelic exclusion. This conclusion concurs with recent data on the role of the λ5 gene in allelic exclusion in B cells, in which 31% of cells with two V$_H$ rearrangements contained two productive immunoglobulin heavy chain alleles in λ5-deficient mice (Löffert et al., 1996). It is thus difficult to determine whether results obtained in TCRβ

Table 3. Characterization of TCRβ Rearrangements on Both Alleles Obtained from pTα+ and pTα−/− Mice

pTα+				pTα−/−			
Code[a]	VJ2/DJ2	Code	VJ2/VJ2	Code	VJ2/DJ2	Code	VJ2/VJ2
2	V6/D1	14	V12/V1	K1	V1/D1	K30	V4/V10
5	V2/D2	16	V10/V14	K2	V10/D1	K32	V8/V10
8	V6/D1	40	V8/V10	K35	V6/D2	K23	V6/V10
32	V6/D1	44	V1/V12	K6	V5/D1	K53	V6/V6
41	V5/D2	165	V8/V10	K11	V10/D2	K43	V2/V4
71	V8/D1	170	V1/V15	K45	V16/D2	K44	V4/V10
101	V11/D2	171	V6/V10	K61	V5/D2	K5	V8/V10
87	V5/D2	142	V12/V16	K72	V12/D1	K60	V1/V12
88	V8/D1	86	V5/V10	K76	V16/D2	K120	V10/V12
93	V10/D1	103	V1/V10	K143	V6/D1	K105	V1/V8
97	V16/D2	108	V10/V14	K112	V8/D1	K148	V4/V6
117	V10/D2	127	V2/V12	K116	V1/D1	K151	V1/V12
120	V5/D1	114	V5/V8	K128	V4/D2	K130	V5/V8
113	V1/D2	99	V8/V15	K134	V11/D2	K170	V1/V4
141	V12/D1	228	V5/V8	K167	V5/D2	K117	V8/V16
163	V16/D1	232	V4/V8	K180	V12/D1	K171	V4/V16
151	V12/D1	207	V6/V8			K169	V5/V8
172	V8/D2	219	V1/V2			K139	V8/V8
210	V14/D2	222	V6/V8			K164	V5/V12
213	V5/D1	237	V2/V8			K132	V8/V10
225	V6/D2	285	V4/V6				
290	V12/D1						
211	V4/D2						
252	V1/D2						
244	V1/D2						
226	V2/D2						
233	V8/D2						
231	V4/D2						
239	V2/D2						
259	V13/D1						
262	V1/D1						
266	V16/D2						
282	V5/D1						
286	V16/D1						

[a] A number code was assigned to the cells isolated from pTα+ mice, and the letter K plus a number code to cells from pTα−/− mice. Data shown represent rearrangements of VD or D with the Jβ2 cluster genes.

transgenic mice deficient in signal transduction (Wallace et al., 1995) and exhibiting some allelic exclusion of the TCRβ locus are due to signals transduced by the pre-TCR or signals transduced by TCRβ proteins that generate these signals in a nonphysiological manner, as observed in TCRβ transgenic pTα−/− mice (Xu et al., 1996; Krotkova et al., 1997).

Finally, the results obtained in pTα−/− mice indicate, at least by the criteria of cell surface staining with Vβ antibodies, that mature T cells exhibit allelic exclusion (Krotkova et al., 1997), in contrast to CD25+ cells that cannot mature further because they lack the pre-TCR (Fehling et al., 1995). This can be explained by the assumption that in a few TCRβ rearranging cells, TCRα proteins are expressed so early that they mimic the pre-TCRα chain with regard to induction of cell maturation as well as allelic exclusion (Buer et al., 1997). It will be useful to identify these cells at an early stage of development and to determine whether they represent a lineage of αβ T cells distinct from the lineage that uses the pre-TCRα chain.

Experimental Procedures

Mice

The pTα−/− mice have been described elsewhere (Fehling et al., 1995). The genotype of mice with respect to endogenous pTα was determined by PCR. C57BL/6 mice were purchased from IFFA CREDO (France). Animals were analyzed at 6–8 weeks of age. Animal care followed institutional guidelines.

Table 4. Number of Cells According to the Type of Rearrangements on Both TCRβ Alleles in Single Cells of pTα+ and pTα−/− Mice

	Rearrangements[a]	
	V(D)J/DJ	V(D)J/V(D)J
pTα+	34 (62%)	21 (38%)
pTα−/−	16 (44%)	20 (56%)

	Sequences[b]	
	V(D)J+/V(D)J−	V(D)J+/V(D)J+
pTα+	18 (95%)	1 (5%)
pTα−/−	13 (65%)	7 (35%)

[a] A number of cells carrying two V(D)J rearrangements on both alleles or a V(D)J rearrangement on one allele and a DJ rearrangement on the other (differences between pTα+ and pTα−/− mice by Fisher's exact test, p = 0.046). TCRβ rearrangements were analyzed by PCR on an ethidium bromide–stained agarose gel (reading frames were not determined).
[b] Comparison of cells with two productive V(D)J rearrangements between pTα+ and pTα−/− mice. Each V(D)J joint was determined by sequence analysis. VDJ+ and VDJ− correspond to productive and nonproductive rearrangements, respectively.

Table 5. Junctional Sequences of the Two Productive TCRβ Genes in CD25⁺ Single Cells of pTα⁺ and pTα⁻/⁻ Mice

3′ Vβ	N-Dβ-N	5′ Jβ	Vβ	Dβ	Jβ
287*					
1-TGTGCCAGCTCTCTC	GAGACTGGGGGT	AGTCAAAACACCTTGTACTTTGGTGCG	V5	D2	J2.4
2-TGTGCCAGCAG	TGGACTGGGGGA	TGAACAGTACTTCGGTCCC	V8	D2	J2.6
K23					
1-TGTGCCAGCAGCC	CTGGACAG	GAACAGTACTTCGGTCCC	V10	D1	J2.6
2-TGTGCCAGCAGTAT	TGGGACAA	CAAACACCGGGCAGCTCTACTTTGGTGAA	V6	D1	J2.2
K30					
1-TGTGCCAGCAGCC	CCCCGGGACTGGG	CTATGAACAGTACTTCGGTCCC	V4	D2	J2.6
2-TGTGCCAGCAGCTA	CCCGGGACACCA	CTCCTATGAACAGTACTTCGGTCCC	V10	D1	J2.6
K44					
1-TGTGCCAGCAGCTA	TAACAGT	AGTGCAGAAACGCTGTATTTTGGCTCA	V10	D1	J2.3
2-TGTGCCAGCAGCC	TCGATGGATGGGGG	CTTCGGTCCC	V4	D2	J2.6
K117					
1-TGTGCCAGCAGCC	CTCGGACTGGGAGG	TGCTGAGCAGTTCTTCGGACCA	V16	D2	J2.1
2-TGTGCCAGCGGTGATG	CGACAGGCGAAGA	TGCAGAAACGCTGTATTTTGACTAA	V8	D1	J2.3
K139					
1-TGTGCCAGCAGTG	GGACTGGGGGGG	ATGAACAGTACTTCGGTCCC	V8	D2	J2.6
2-TGTGCCAG	GGTGGTTCTCCTGGG	CCAGACACCCAGTACTTTGGGCCA	V8	D2	J2.5
K148					
1-TGTGCCAGCAGCC	GAGACTGGGT	CTCCTATGAACAGTACTTCGGTCCC	V4	D2	J2.6
2-TGTCCCAGCAGTAT	TACGGGAGA	GCAGAAACGCTGTATTTTGGCTCA	V6	?	J2.3
K164					
1-TGTGCCAGCTC	CGGGACAGGGCCG	TATGAACAGTACTTCGGTCCC	V5	D1	J2.6
2-TGTGTGCCAGCAGT	CCGGGACAGGGG	TATGAACAGTACTTCGGTCCC	V12	D1	J2.6

The letter K and a number code were assigned to the cells isolated from pTα⁻/⁻ mice; asterisk indicates cells from pTα⁺ mice. The two productive V(D)J joints are numbered arbitrarily 1 and 2 for each cell. N regions are determined by alignment of the sequences with the known germline sequences of Vβ (Arden et al., 1995), Dβ (Clark et al., 1984), and Jβ (Gascoigne et al., 1984; Malissen et al., 1984) genes. The Dβ germline sequences are underlined.

Cytoplasmic Staining for TCRβ and Cell Sorting

Total thymocytes were derived from C57BL/6 mice and pTα⁻/⁻ mice. Cells from C57BL/6 mice were enriched for the CD4⁻CD8⁻ subset by negative depletion of cells positive for CD4 or CD8 using Dynabeads (Dynal, Oslo, Norway). For extracellular–intracellular double staining, cells were first incubated with culture supernatant of monoclonal antibody 2.4G2 to block FCγRII/III. Cells were then stained for CD25 surface antigens with phycoerythrin-labeled anti-CD25 (3C7, Pharmigen, San Diego, CA) at optimal concentration. After washing in phosphate-buffered saline (PBS), cells were fixed in PBS plus 2% paraformaldehyde for 15 min at room temperature, followed by two washing steps in PBS. Cells were then permeabilized in 0.5% saponin for 10 min at room temperature and washed in PBS. Intracellular staining with fluorescein isothiocyanate–conjugated anti-pan-TCRβ (H57–597) diluted in PBS plus 0.5% saponin was performed for 20 min at room temperature, followed by two washing steps in PBS and two periods of 15 min each on a rocking platform in PBS plus 2% fetal calf serum plus 0.5% saponin on ice. Finally, cells were washed in PBS plus 2% fetal calf serum. Stained samples were analyzed and sorted twice on a FACS Vantage (Becton Dickinson, San Jose, CA). Cells were gated on small cells using forward scatter as an index of size. Sorted CD25⁺ TCRβ⁺ populations were reanalyzed on a FACScan (Becton Dickinson) to check for purity.

Single-Cell PCR

Approximately 10⁴ CD25⁺ TCRβ⁺ sorted cells were transferred into Petri dishes. Single cells were picked under a microscope using a microcapillary, deposited into PCR tubes containing 20 μl of PCR buffer (Perkin Elmer, Norwalk, CT), and stored at −30°C. To prepare genomic DNA, 1 μl of proteinase K (2.5 mg/ml) was added to each sample containing a single cell, and the tubes were incubated for 45 min at 55°C followed by 5 min at 94°C. Most of the TCRβ gene rearrangements were amplified by a seminested two-step PCR protocol (Löffert et al., 1996). In the first step, both V(D)J rearranged alleles were amplified simultaneously by addition to each tube of 40 μl of a mixture containing dNTP, buffer, and Taq polymerase at 0.5 U per sample (Perkin Elmer), 15 5′ primers (3 pmol of each) homologous to 13 Vβ gene families and to Dβ1 and Dβ2 genes, in combination with two 3′ primers (3 pmol of each) that primed downstream of the Jβ1 and Jβ2 cluster sequences, respectively (Table 1). The first round of amplification was done in a final volume of 60 μl for five cycles in which the annealing temperature decreased from 68°C to 60°C, followed by 25 cycles of amplification (30 s at 94°C, 1 min at 58°C, 1 min at 72°C), and finally 5 min at 72°C. For the second round of amplification, 1–1.5 μl of the first PCR product were transferred into separate tubes, each containing a single 5′ primer in combination with the nested Jβ2 or Jβ1 3′ primer (10 pmol of each), dNTP, buffer reaction, and 1 U of Taq polymerase in a final volume of 20 μl. Amplification was then carried out for 35 cycles following the procedure of the first PCR. Vβ and Jβ were identified by migration of the total PCR product on a 1.8% ethidium bromide–stained agarose gel, and positives were purified using Geneclean III (Bio 101, Vista, CA). Direct sequencing of the PCR products was performed using the Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Warrington, UK) and sequenced by automated sequencing (Applied Biosystems).

Statistics

The rearrangement data in Table 4 (top) were analyzed by Fisher's exact test using C-Stat 1.0 (Oxtech, Oxford, UK). A probability of $P < 0.05$ was considered significant.

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