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During thymocyte differentiation, TCRα genes are massively rearranged only after productively rearranged TCRβ genes are expressed in association with pTα and CD3 complex molecules within a pre-TCR. Signaling from the pre-TCR via the CD3 complex is thought to be required to promote TCRα gene accessibility and recombination. However, αβ+ thymocytes do develop in pTα-deficient mice, showing that TCRα-chain genes are rearranged, either in CD4+CD8− or CD4+CD8+ thymocytes, in the absence of pre-TCR expression. In this study, we analyzed the TCRα gene recombination status of early immature thymocytes in mutant mice with arrested thymocyte development, deficient for either CD3 or pTα and γc expression. ADV genes belonging to different families were found rearranged to multiple AJ segments in both cases. Thus, TCRα gene rearrangement is independent of CD3 and γc signaling. However, CD3 expression was found to play a role in transcription of rearranged TCRα-chain genes in CD4−CD8+ thymocytes. Taken together, these results provide new insights into the molecular control of early T cell differentiation.


**TCRA Gene Rearrangement in Immature Thymocytes in Absence of CD3, Pre-TCR, and TCR Signaling**

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3 Abbreviations used in this paper: DN, double negative; CIC, clonotype-independent CD3 complex; DP, double positive; HS, hot spot; RAG, recombination-activating gene; SP, single positive; wt, wild type.

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accessibility of the TCRD locus (31–33). To explain the differential temporal use of the proximal TCRD and TCRG genes in DN and DP cells, respectively, it was proposed (in human) that blocking element a8 1, situated between the DC and AJ segments, prevents the upstream E6 to promote accessibility to the AJ genes in DN cells (34). In this context, the block over the AJ region would be released in late DN/early DP at the time of E6 activation, after pre-TCR signaling.

In addition to these results, most of the data available to date indicate that TCR gene coding genes are not rearranged before the DN to DP transition. Because the complex structure of the TCRD locus makes TCR gene rearrangements difficult to study at the genomic level, most of these studies relied on Northern blot analysis (20, 35, 36), rather than direct analysis of rearrangement events at the DNA level. TCR genes were found expressed only after the thymocytes did exit the DN compartment. Furthermore, mimicking pre-TCR signaling by injection of anti-CD3e Abs not only induces DP thymocyte differentiation, but also increases TCR gene germline transcription, an event thought to reflect accessibility of these genes (37). In addition, TCRB, TCRC, and TCRG, but not TCRD genes were found rearranged and transcribed in CD3e-deficient mice (15, 17, 38, 39). Collectively, these data were interpreted as evidence that pre-TCR-derived signals are required to induce accessibility and rearrangement of TCRD genes. However, TCRDβ− γ− thymocytes still develop in pTε-deficient mice, in absence of pre-TCR, and we showed that the TCRα chains expressed in pTε− γ− thymocytes display features identical to that found in wild-type (w) animals (40). Pre-TCR-independent DP thymocyte differentiation can be achieved through the influence of TCRδ or TCRγδ chain expression (41, 42). Nonetheless, analysis of pTε × TCRδ double-deficient animals showed that DP thymocytes still develop in these mice, and that almost all of them have been β selected (43). This phenomenon was postulated to happen through pre-TCR-independent early rearrangement and expression of TCRα chains, able to substitute for pTε and form a TCRαβ that promotes the progression of TCRαβ-expressing DN thymocytes to the DP and SP compartments. One other study found rearranged TCRα chain genes in sorted immature DN3 wt thymocytes (44). However, it was later shown that a pre-TCR can potentially be expressed in DN2 thymocytes (45), and that the DN3 population includes thymocytes that have already undergone β selection (46). Thus, TCRα rearrangements found in wt DN3 thymocytes may result from pre-TCR signaling. Therefore, it has not yet been formally demonstrated whether TCRα chain genes can be rearranged before pre-TCR expression, and, if so, how they are induced.

In this study, we analyzed the occurrence of TCRα gene rearrangement in different models of genetically modified mice, in which thymocyte differentiation is blocked at or before the CD44−CD25− stage. Our results show that TCRα gene rearrangement does take place in immature thymocytes, independently of the main signals governing thymocyte differentiation, namely CD3, pre-TCR, TCR, and γε expression and signaling. Furthermore, we also show that CD3 is required for TCRα rearrangements to be transcribed at these early stages of development.

Materials and Methods

Mice

CD3ε3/4/5 (15) and RAG-2−/− mice (13) were maintained in the animal facility of the Commissariat à l‘Energie Atomique (Grenoble, France), and Eβ−/− × TCRγ−/− (47, 48) and γε−/− × pTε−/− mice (45) in the animal facilities of the Center d’Immunoéologie de Marseille Luminy (Marseille, France) and of the Centre National de la Recherche Scientifique/Centre de Développement des Techniques Avancées (Orléans, France), respectively. All mice used were between 4 and 10 wk of age. C57BL/6 mice were used as wt control.

RNA preparation and RT-PCR

Total RNA and cDNA from Eβ−/− × TCRγ−/− and CD3ε3/4/5 thymi were prepared as previously described (40, 48). PCR were performed using primers specific for the ADV2, ADV3, or ADV7 family genes (CDNAU, ADV3UP, or ADV7UP, respectively) and for the AC region (MTA). Thy-1 cDNA was amplified with the Thy-1UP and Thy-1DO primers as a positive control. PCR products were migrated on 1.5% agarose gels and probed by Southern blot hybridization using 32P-labeled primers specific for the AC region or Thy-1 (C2r and Thy-1UP2 primers, respectively). Primer sequences are shown in Fig. 1A.

A second nested PCR was performed on the CDNAU/MTA products with a CDNAU-Bam primer and an EcoRI-containing C primer (Co1) to introduce restriction sites to facilitate cloning. The conditions for all the PCR were 5 min at 94°C, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, and finally 10 min at 72°C.

Cell sorting

The following Abs were used for staining: FITC-conjugated anti-CD25 (7D4), PE-conjugated anti-CD4 (GK1.5) and anti-CD44 (IM7), biotin-conjugated anti-B220 (RA3-6B2), anti-CD8 (53-6.7), anti-CD3e (2C11), anti-CD11b (M1/70), tri-I-A^3 (25-9-17), and anti-TCRβ2 (H57-597). All the Abs and PE-conjugated streptavidin were purchased from BD PharMingen (Le Pont de Claix, France).

Single-cell suspensions of CD3ε3/4/5 thymocytes were prepared and incubated with the indicated Abs, as previously described (40). CD25+ thymocytes were electronically sorted on a FACStar® (BD Biosciences, San Diego, CA) using CellQuest software. The purity of sorted cells, assessed by reanalysis, was >99%.

Detection of TCRD locus rearrangements

Genomic DNA was extracted and amplified using primers situated downstream of different AJ gene segments (AJ56, AJ48, AJ27, or AJ23) in combination with primers specific for the ADV2, ADV7, ADV8, or ADV10 family genes (CDNAU, ADV7UP, VA3L, or ADV10UP, respectively). A control PCR was performed on the AC region using NM78 and MTCAD2O primers. The sequence of the primers and the location of AJ-specific oligonucleotides used to amplify ADV-AJ rearrangements are shown in Fig. 1. Multiplex PCR were performed with the Expen High Fidelity PCR system (Roche Diagnostics, Meylan, France), as follows: 5 min at 94°C, followed by 26 cycles consisting of 1 min at 94°C, 1 min at 58°C, 6 min at 72°C, and finally 10 min at 72°C. With 26 cycles, the PCR was in the linear phase of amplification (data not shown). PCR products were migrated on 1.6% agarose gels and probed by Southern blot hybridization. The probes used to detect the TCRα rearrangements were specific of the AJ gene segments (AJ56p, AJ48p, AJ27p, AJ23p). Each of these oligonucleotides was first used individually to probe ADV2-AJ PCR products amplified from wt thymocyte DNA. In each case, only the expected were revealed. Therefore, the PCR was performed on the AJ segments using NM78 and MTCAD2O primers. The conditions used for the PCR were 5 min at 94°C, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and finally 10 min at 72°C.

Cloning and sequencing

The PCR products from Eβ−/− × TCRγ−/− mice were cloned in the EcoRI and BamHI sites of pBlueScribe KS (Stratagene, La Jolla, CA). AC-positive clones were sequenced using the Thermo Sequenase premixed cycle sequencing kit (Amersham, Les Ulis, France) and analyzed on a Vistra 725 DNA Sequencer (Vistra Systems; Molecular Dynamics, Bonndoufle, France). Rearrangements amplified from CD3ε-deficient and γε × pTε-double-deficient mice were ligated in the pSTBlue-1 vector using the unique PstI site. Madson Biotechnology, ADV2- or ADV7-positive clones were sequenced by Genome Express S.A. (Grenoble, France). ADV and Al gene segments were identified by comparison with the published sequences (22, 23).
Results

Detection of numerous TCRA gene rearrangements by multiplex PCR assay

To analyze the TCRA gene rearrangement status at the DNA level, we designed a PCR assay allowing amplification of ADV genes rearranged to multiple successive AJ genes. This multiplex PCR assay is performed by using ADV-specific primers in combination with primers located downstream of different AJ gene segments (AJ56, AJ48, AJ27, and AJ23), as shown in Fig. 1B. The PCR products were then resolved on an agarose gel, transferred onto a nylon membrane, and hybridized to a mix of AJ-specific probes (AJ56p, AJ48p, AJ27p, AJ23p), as described in Materials and Methods. We chose AJ segments located in the hot spot (HS) 1 (AJ56 and AJ48), and in the HS2 (AJ27 and AJ23). These HS could represent entry points for recombination used early and late during development, respectively (40, 49–51). Wild-type thymus DNA was amplified using primers specific for the ADV2, ADV8, and ADV10 family members in combination with the four AJ primers. At least four rearrangements were easily detected in each reaction (Fig. 2). The same pattern is observed when an ADV-specific primer is used to probe amplified ADV-AJ rearrangements in place of the mix of AJ-specific probes (data not shown). The use of probes specific for AJ59, AJ51, AJ31, and AJ17, individually, and longer resolution of PCR products on agarose gels showed that the bands in the upper part of the gel are true TCRA gene rearrangements, with the exception of the top upper band in the AJ56 lane (Fig. 2 and data not shown). AJ60, AJ59, AJ51, AJ29, and AJ25 genes, described as pseudogenes, were never found, whereas rearrangements using the AJ61 pseudogene were. No rearrangements were found in RAG-2−/− mice, as expected (Fig. 3). In our assay, the DNA input is 200 ng per PCR, or 800 ng for the set of four reactions. Cells contain ~6 pg DNA. Thus, with a set of four PCR, we analyze ADV-AJ rearrangement in ~1.3 × 10^6 cells. To estimate the sensitivity of our assay, titration experiments were performed. DNA prepared from TCRαβ+ SP wt thymocytes was serially diluted in RAG-2−/− thymocytes/DNA and a constant amount of these dilutions (200 ng per PCR) were used as matrix. The PCR products were then resolved on an agarose gel, transferred onto a nylon membrane, and probed with the mix of four radiolabeled AJ probes, as described. ADV2-AJ rearrangements could be detected when as little as 0.3% TCRαβ+ SP thymocyte DNA is present in the matrix (data not shown), which corresponds roughly to 400 cell equivalents (0.3% of 1.3 × 10^6 cells).

We wanted to determine whether TCRA gene rearrangement takes place in immature thymocytes, before pre-TCR expression. It was recently shown that a pre-TCR can be expressed as early as the DN2 stage of differentiation (45), and no convenient marker exists that allows reliable separation of immature thymocytes in pre-TCR+ and pre-TCR− subsets. Furthermore, the very high sensitivity of our multiplex PCR assay hindered a direct analysis of TCRA gene status in sorted cells, given the limits of cell-sorting procedures. To circumvent these problems, we analyzed TCRA gene rearrangement in mice devoid of mature αβ and γδ thymocytes, in which pre-TCR expression is abolished because of genetic inactivation of either CD3ε or γε and pTα genes.

TCRA genes are rearranged in CD3εΔ5/Δ5 mice

In CD3εΔ5/Δ5 mice, T cell differentiation is blocked at the DN3 stage. In a previous study, we were unable to detect rearranged TCRA gene transcripts in these mice (39), in accordance with previously published results (15). However, rearranged TCRβ gene transcription is reduced in CD3εΔ5/Δ5 mice (15) and similarly, TCRA transcription could have been reduced to undetectable levels. We therefore performed multiplex PCR assay to determine whether the absence of rearranged TCRA transcripts in CD3εΔ5/Δ5 mice results from an absence of recombination at the DNA level. DNA from CD3εΔ5/Δ5, RAG-2−/−, and wt thymi was amplified to identify ADV10 family member rearrangement (Fig. 3A). In CD3εΔ5/Δ5 mice, but not RAG-2−/− mice, several bands were detected, indicating the presence of TCRA rearrangements. The patterns of utilization were not identical between two CD3εΔ5/Δ5 mice, but were quite diverse in each case ( detection of 8 and 10 bands in CD3εΔ5/Δ5 of 20 possible in wt mice). AJ segments located in the HS2 are used, indicating that the entire AJ region is accessible for recombination in DN thymocytes. Finally, TCRA rearrangements
are detected in CD3ε<sup>Δ5/Δ5</sup> indifferently under either a BALB/c or a C57BL/6 background.

The presence of TCR gene rearrangement in immature B cells was previously described (52). To ensure that the TCRα gene rearrangements observed in CD3ε-deficient thymi were produced in T and not B cells, DNA from sorted CD3ε<sup>Δ5/Δ5</sup>CD25<sup>+</sup> thymocytes (purity 99%) was analyzed. Again we could detect various TCRα gene rearrangements using ADV2 family members (Fig.

**FIGURE 2.** Amplification of TCRα gene rearrangement from thymocyte genomic DNA. Thymic DNA from wt mice was amplified using ADV10 (A)-, ADV2 (B)-, and ADV8 (C)-specific primers in combination with primers located downstream of the AJ56, AJ48, AJ27, and AJ23 genes. The AJ primers used are indicated above each lane. Each of these amplifications was probed with a mix of radioactively labeled primers specific for the AJ56, AJ48, AJ27, and AJ23 genes, respectively. A, The identity of rearranged AJ genes is indicated. It was determined by comparing the migration distance of the bands with the expected size for a rearrangement, deduced from the AJ region sequence (MUSTCRA, GenBank accession no. M64239). C, The amplification yields three bands for each AJ segment rearranged (visible only for low m.w. products), because we use an ADV8-specific primer located in the first exon of ADV8 family members, which differ from each other by the size of the intron separating the two exons. *, Nonspecific signal.

**FIGURE 3.** TCRα genes are rearranged in CD3ε<sup>Δ5/Δ5</sup> mice. A, DNA from CD3ε<sup>Δ5/Δ5</sup> thymocytes on the BALB/c (TCRAD haplotype a/a) or C57BL/6 (TCRAD haplotype b/b) background and from RAG2<sup>−/−</sup> and wt thymocytes was amplified by multiplex PCR and probed as described in Fig. 2 legend, using the ADV10-specific primer (left panel). B, DNA from sorted CD3ε<sup>Δ5/Δ5</sup>CD25<sup>+</sup> thymocytes, and from CD3ε<sup>Δ5/Δ5</sup> RAG-2<sup>−/−</sup>, and wt thymi was amplified as in A, using the ADV2-specific primer (left panel). A band migrating just above the one corresponding to AJ27 rearrangement is observed in the AJ27 lane of RAG-2<sup>−/−</sup>. This band was shown by sequencing to correspond to a nonspecific amplification of a region encompassing the unrearranged ADV2S6 gene (data not shown). A and B, the AJ primers used are indicated above each lane. To estimate the DNA loading, a control PCR was performed using AC-specific primers (right panels).
3B). We detect $\sim 10$ ADV2-AJ rearrangements in $1.3 \times 10^5$ CD25$^+$ DN CD3e$^{A5\Delta5}$ thymocytes. Our assay allows us to determine the rearrangement status of 20 AJ segments of 60. One can then extrapolate that for the complete AJ region, 3 times as many rearrangements are potentially present. Similarly, because 10 rearrangements are detected for the ADV2 family, it can be estimated that 22 times as many rearrangements could be detected if one were to test all the ADV families. From these extrapolations, it results that $10 \times 22 \times 3 = 660$ rearrangements are potentially present, in $1.3 \times 10^5$ CD25$^+$ DN CD3e$^{A5\Delta5}$ thymocytes, which indicates that $\sim 0.5\%$ of these cells do have rearranged TCRA genes.

TCRA rearrangements found in CD3e-deficient mice are diverse

To characterize the molecular nature of TCRA rearrangements found in CD3e-deficient mice, TCRA rearrangements using either the ADV2 or ADV7 family members, rearranged to AJ56, AJ48, AJ27, or AJ23, were cloned and sequenced. Sequencing showed that the ADV2 and ADV7 members used in the CD3e$^{A5\Delta5}$ mouse analyzed are diverse (Fig. 4). Multiple junctions were identified, which present nucleotide addition and/or deletion. Insertions of $>13$ nucleotides at the ADV-AJ junction are observed for three sequences. One of these is clearly DD2 gene segment. In three other junctions, a stretch of four or five nucleotides matching DD1 or DD2 sequence can also be identified. Rearrangement of a DD segment to AD and AJ segments was found in other studies conducted in wt animals (unpublished results). Finally, analysis of the sequences indicates that the thymocytes were not selected for in-frame TCRA rearrangements, as expected because of absence of surface expression.

Thus, all the results obtained with the CD3e$^{A5\Delta5}$ mice show that multiple TCRA rearrangements can be completed as early as the CD44 $^{+}$CD25$^+$ stage of differentiation in absence of signaling through pre-TCR, TCR, or clonotype-independent CD3 complex (CIC).

TCRA genes are rearranged in absence of the y chain and pTa

Overlapping signals derived from growth factors, cytokines, and Ag receptors are essential for early T cell differentiation. Mice deficient for the common cytokine receptor $\gamma$-chain (y) and pTa exhibit thymic hypoplasia ($4 \times 10^4$ thymocytes) and a complete block of development at the CD44$^{+}$CD25$^+$ stage. Despite this low cellularity and early arrest, limited TCRB gene rearrangements could nonetheless be found in total thymic DNA from these mice (45). Thus, we next analyzed whether ADV2-AJ recombination is also present.

Thymic DNA from $\gamma$-$\times$ pTa double-deficient mice was amplified using the AJ primers in combination with the ADV2 family-specific primer (Fig. 5). A clear signal was obtained with the two of three $\gamma$-$\times$ pTa$^{-}$ mice shown. Although certain mice did not present TCRA rearrangements involving ADV2 family members, a signal was obtained using either ADV7- or ADV10-specific oligonucleotides (Fig. 5 and data not shown). Only one sample of five tested negative for both ADV2 and ADV10 (Fig. 5 and data not shown), a result that does not preclude the presence of rearrangements involving other ADV families.

We determined the nature of the TCRA rearrangements amplified in these mice. Because of the faint signal intensity obtained with the $\gamma$-$\times$ pTa$^{-}$ mice, a nested PCR protocol was used to obtain sufficient material for cloning. Two samples showed rearrangements with the different AJ primers were used. Various TCRA gene rearrangements were sequenced (Fig. 6). Five different ADV2 family members were identified, with nucleotide deletion and/or addition at the ADV-AJ junction.

Our results show that TCRA gene rearrangements are detected as early as the CD44 $^{+}$CD25$^+$ stage of differentiation, in absence of signaling from cytokine receptors using $\gamma$- and the pre-TCR.

**FIGURE 4.** Sequence of TCRA gene rearrangements from CD3e$^{A5\Delta5}$ mice. TCRA gene rearrangements using AJ56, AJ48, AJ27, or AJ23 segments in combination with ADV2 or ADV7 family members were amplified, cloned, and sequenced from CD3e$^{A5\Delta5}$ thymocytes. The extremities of ADV and AJ genes, the DD1 or DD2 genes, and the N and P nucleotides are shown. ADV and AJ segments identified are indicated. Nucleotides are written in small letters. Sequences were considered as DD1 or DD2 genes in the case of homologies of at least four nucleotides. In-frame (+) and out-of-frame (−) sequences are indicated.
studied in $E B^+\times T C R^+\times$ and wt mice. As expected, no rearranged transcripts were detected in CD3e$^{\Delta S}\Delta S$ and RAG2$^{-/-}$ mice. Thus, rearranged TCRA genes can be transcribed in DN thymocytes in absence of pre-TCR and TCR expression. Cloning and sequencing of ADV2-AC amplification products revealed a high proportion of ADV-DD-DJ rearrangements spliced onto the AC segment (data not shown), as described in mice bearing the same mutation in the DC segment and wt mice (53). Nonetheless, bona fide ADV-AJ-AC transcripts (15–35%) were also identified, and sequencing showed utilization of different AJ gene segments and ADV2 family members in the two $E B^+\times T C R^+\times$ mice analyzed (data not shown). Thus, rearranged TCRA genes can be transcribed in immature DN thymocytes, in absence of pre-TCR and TCR expression. Together with the absence of TCRA chain transcripts in CD3e$^{\Delta S}\Delta S$, this result strongly argues in favor of a role for CIC complexes in the control of this transcription.

**Discussion**

In this study, we analyzed the occurrence of TCRA chain gene recombination in DN thymocytes, in three different mouse models in which early T cell differentiation is blocked at or before the CD44+CD25+ stage of maturation. The data presented in this study clearly show the presence of a diverse set of TCRA rearrangements in all the mice analyzed, demonstrating that TCRA, β, γ, and δ chains can be produced during the early phases of T cell differentiation in the thymus, in absence of any signaling via the CD3 complex either as part of the pre-TCR, the TCR, or as CIC, or via γc-containing cytokine receptors and the pre-TCR.

Until now, most of the available data indicated that TCRA gene rearrangement only begins after pre-TCR signaling, at the DN/DP transition. Our results now show that ADV-AJ recombination is not so bluntly regulated, but is rather modulated during thymocyte differentiation. This event already takes place in a fraction of thymocytes during early DN stages, before being induced in most of the cells while they mature to the DP stage. In CD3-deficient animals, we estimated that ~0.5% of immature thymocytes do have rearranged TCRA genes. This low abundance and the randomness of ADV-AJ recombination probably explain why different patterns of ADV-AJ rearrangements are observed in different CD3e$^{\Delta S}\Delta S$ mice. However, this figure may be higher in wt mice, as we cannot exclude that CD3, although not necessary, participates in the regulation of gene accessibility when expressed. In addition, it must be stressed out that this number is only an estimate, since 1) all ADV gene families and all AJ segments may not rearrange to the same extent (it is known, for example, that ADV2 genes are used in ~12% of peripheral T cells), and 2) in our calculation, each PCR product corresponds to only one rearrangement, whereas ADV-AJ rearrangements are diverse (Fig. 4).

In pTa$^{-/-}$ mice, 40% of the few DP cells that can develop express an intracellular TCRβ chain. The proportion of β-selected cells is nearly null in pTa$^{-/-}\times$ TCRα$^{-/-}$ mice, but raises to 95% in pTa$^{-/-}\times$ TCRδ$^{-/-}$ animals, indicating that pTa$^{-/-}$ thymocytes that are not β selected differentiate under the influence of γδ.

**Figure 5.** TCRA gene rearrangements in $\gamma c^{-/-}\times p T a^{-/-}$ thymi. DNA from $\gamma c^{-/-}\times p T a^{-/-}$, RAG2$^{-/-}$, and wt thymi was amplified by multiplex PCR as performed as in Fig. 4 using the ADV2-specific primer (left panel). The AJ primers used are indicated above each lane. The lanes corresponding to the $\gamma c^{-/-}\times p T a^{-/-}$ and RAG2$^{-/-}$ mice were exposed longer for better visualization. The control PCR was performed as in Fig. 2 (right panel).

**Figure 6.** Sequence of TCRA gene rearrangements from $\gamma c^{-/-}\times p T a^{-/-}$ mice. TCRA gene rearrangements using AJ48, AJ27, or AJ23 segments in combination with ADV2 family members were amplified, cloned, and sequenced from $\gamma c^{-/-}\times p T a^{-/-}$ thymi. The extremities of the ADV and AJ genes, and the N and P nucleotides are shown. ADV and AJ segments identified are indicated. P nucleotides are written in small letters. In-frame (+) and out-of-frame (−) sequences are indicated.

**Figure 7.** Rearranged TCRA genes are transcribed in $E B^+\times T C R^+\times$ mice. Rearranged TCRA transcripts from 4-wk-old CD3e$^{\Delta S}\Delta S$, EB$^+\times$ TCRδ$^{-/-}$, RAG-2$^{-/-}$, and C57BL/6 mice were amplified by RT-PCR using primers specific for three different ADV families in combination with an AC-specific primer. A control PCR was performed using Thy-1-specific oligonucleotides. The lane “no cDNA” corresponds to the same PCR amplifications performed without cDNA as a negative control. The PCR products were probed with radioactively labeled primers specific for the AC region or Thy-1.

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T cells, and that early expression of a TCRαβ compensates for the absence of pre-TCR and promotes DN/DP progression and β selection (43). We previously found that rearranged TCRα genes expressed in pTα−/− mice are diverse, as in wt mice (40). However, in this study, we could not formally rule out that the TCRα chain transcripts sequenced were produced in those DP thymocytes that did differentiate under the influence of γδ+ thymocytes. The results presented in this work demonstrate for the first time, at the DNA level, the occurrence of diverse TCRα gene rearrangements in a small subpopulation of immature pre-TCR-deficient thymocytes. In addition, we show that TCRα gene rearrangement is independent not only of pre-TCR expression, but also of expression of either the CD3 complex, as CIC or associated with a γδ TCR, or γε-containing cytokine receptors. Altogether, these findings provide strong support to the hypothesis that TCRα rearrangements produced in DN thymocytes are responsible for DP thymocyte development in absence of pTα, at least partially in pTα−/− animals, and probably totally in pTα × TCRδ double-deficient mice. Furthermore, the low abundance of thymocytes having rearranged TCRα genes probably explains why DP thymocyte number in pTα−/− mice is only ~2% of the wt level (14). Because all of the T lymphocytes produced in pTα−/− × TCRδ−/− animals use a TCRα chain generated in early thymocytes, these mice constitute an ideal model to determine whether T lymphocytes using these chains fully participate in all immune functions, or whether they constitute a T lymphocyte subset with special homing or functional properties. In a normal situation, the contribution of this differentiation pathway to αβ T lymphocyte development is probably low compared with the pre-TCR pathway. Indeed, we must keep in mind that, in wt mice, the majority of TCRα rearrangements take place in DP cells and, even if our results show that the pre-TCR is not required for TCRα gene rearrangements in DN thymocytes, pre-TCR-induced proliferation vastly increases the number of cells that will rearrange their TCRα genes.

TCRα gene rearrangement in early thymocytes does, however, impact γδ T lymphocyte development. In adult mice, αβ+ and γδ+ thymocytes are generated from the same precursor population (2). Numerous studies have tried to decipher the mechanisms responsible for the dual fate of these precursors, and two major models have emerged (see Ref. 3 for review). In the separate lineage (or stochastic) model, lineage decision is imposed first, and TCR genes rearrange later. If the TCR isotype then expressed matches the lineage (e.g., γδ TCR in γδ thymocytes and pre-TCR in αβ thymocytes), the cell survives and differentiates. Otherwise, it dies. In the competitive rearrangement (or instructive) model, the choice is dictated by the nature of the TCR isotype expressed, either γδ or pre-TCR, that transduces different signals to the developing thymocytes. In both models, only the γδ TCR and the pre-TCR receptors are thought to be instrumental for cell fate, differentiation, and/or survival. The finding that TCRα genes can be rearranged and expressed in the same DN2/DN3 population(s) as TCRδ, G, and B genes now offers the distinct possibility that an αβ TCR can also play a role in lineage commitment, at least in two ways. First, it gives developing thymocytes a new pathway to become an αβ T lymphocyte, either by rescuing committed precursors from death (stochastic), or by delivering differentiation signals (instructive). Second, it reduces the probability for a precursor to become a γδ T lymphocyte. Indeed, the unique genomic organization of the TCRδ locus is such that any ADV-AJ rearrangement event will excise TCRδ genes from the chromosome. The progenitors will therefore be allowed only one attempt to rearrange TCRδ genes on the remaining allele, and have only one chance of three to succeed, compared with five of nine if both alleles are available. Unlike αβ thymocyte development, which includes a phase of intense proliferation, γδ T lymphocyte development is linear. Thus, in both cases, and irrespective of the lineage commitment model considered, the net result of TCRα gene rearrangement in early thymocytes will be a depletion of γδ T lymphocytes. However, it should be noted that occurrence of ADV-AJ recombination in a T cell progenitor does not per se preclude its development toward the γδ lineage (44). Finally, in addition to their biological significance, our findings do have another important consequence regarding the study of αβγδ lineage commitment: since TCRα rearrangements can be detected as early as the DN2 stage of differentiation, they can no longer be considered a marker for αβγδ divergence operating after the pre-TCR.

TCRα rearrangements found in pTα−/− mice are normal (40). Recombination of TCRα genes also takes place in TCRB−/− animals. In this study, in contrast to previously published studies conducted in CD3εκθ−− mice (15, 54), we show that TCRα genes are also rearranged in absence of CD3 complex expression. Malissen and coworkers (15, 39, 54) might have failed to detect TCRα gene rearrangement in these animals because they did use an Adv8-specific oligonucleotide and, in our hands, this family seems to be rearranged at a very low level, if at all, in CD3-deficient mice (N. Pasqual, S. J. C. Mancini, S. M. Candéias, and E. Jouvin-Marche, unpublished results). Despite the presence of TCRα rearrangements, TCRα chain transcripts were not detected in CD3εκθ−−−−−− mice (Fig. 7), indicating that either the pre-TCR, the TCR, or CIC complexes are required for the transcription of rearranged TCRα genes. In contrast, TCRα transcripts were found in Eβ−−−− × TCRδ−−−− mice, which lack conventional pre-TCR and TCR expression. An alternative pre-TCR, composed of a TCRγ chain associated with pTα and the CD3 complex, was recently described (55). However, its expression would be expected to induce at least a low level of DP thymocyte differentiation. As no DP population was observed among Eβ−−−− × TCRδ−−−− thymocytes, TCRγpTα complex expression was ruled out (48). It seems therefore that no clonotypic TCR or pre-TCR can be assembled in Eβ−−−− × TCRδ−−−− mice. One can argue that ADV-AJ rearrangements in these mice result from perturbations in the local control of accessibility, because of the Neo cassette insertion near Eδ and blocking element αβ1. Even though, any ADV-AJ rearrangement event will excise the Neo cassette from the TCRD locus, and identical pattern of Eα occupancy was found in Eβ−−−− × TCRδ−−−− and RAG−−−−− thymocytes (56). Therefore, transcriptional control of rearranged TCRα genes in Eβ−−−− × TCRδ−−−− thymocytes is as in nonmanipulated animals. The presence of rearranged TCRα chain transcripts in Eβ−−−− × TCRδ−−−− mice then suggests that the absence of TCRα transcripts in CD3εκθ−−−−−− mice results from CD3 deficiency. Thus, the CD3 complex is not necessary for induction of TCRα gene rearrangement, but rather seems to be involved in the transcriptional control of rearranged TCRα genes, as previously suggested in the case of TCRB genes (57).

It is clear in our study that TCRα rearrangements are both pre-TCR and TCR independent. The CD3 complex is also dispensable, as CIC. Furthermore, although it was shown that the pre-TCR provides γε-independent signal allowing the αβ T cell differentiation in γε− mice (45), the induction of TCRα gene rearrangement is independent of overlapping γε and pre-TCR signals. Thus, none of the main molecules known to regulate thymocyte development are required. What then are the signals responsible for ADV-AJ recombination in early thymocytes? One possibility is that they appear as a byproduct of TCRD gene rearrangement, because of a faulty control of accessibility. The other possibility is of course
that TCR genes are made accessible to the recombination machinery only after a specific signal is delivered to developing immature thymocytes. In the context of the current model of enhancers as accessibility regulators, ADV/AJ gene rearrangement in early thymocytes would suggest that Eor is already active in these cells and its activation is independent of CD3- and γδ-derived signals. However, Eor is dispensable for ADV/AJ recombination. Its inactivation only impairs, but does not abolish TCRα gene rearrangement and expression (31, 32), showing that at least one other yet unidentified element is able to promote ADV/AJ recombination. Whether this element is responsible for ADV/AJ rearrangement in immature thymocytes must await its identification and elucidation of its regulation. In any case, TCRα gene rearrangements are found as early as the CD44+25+ stage of development, at the time of TCRD gene rearrangement, in mice in which T lymphocyte differentiation is blocked at an immature stage because of genetic inactivation of different signaling pathways. It remains to be formally demonstrated whether the same phenomenon also takes place during normal thymocyte development in wt mice.

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References


