Human $\alpha\beta$ and $\gamma\delta$ Thymocyte Development: TCR Gene Rearrangements, Intracellular TCR$\beta$ Expression, and $\gamma\delta$ Developmental Potential—Differences between Men and Mice

Michelle L. Joachims, Jennifer L. Chain, Scott W. Hooker, Christopher J. Knott-Craig and Linda F. Thompson

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Human αβ and γδ Thymocyte Development: TCR Gene Rearrangements, Intracellular TCRβ Expression, and γδ Developmental Potential—Differences between Men and Mice

Michelle L. Joachims, Jennifer L. Chain, Scott W. Hooker, Christopher J. Knott-Craig, and Linda F. Thompson

To evaluate the role of the TCR in the αβ/γδ lineage choice during human thymocyte development, molecular analyses of the TCRβ locus in γδ cells and the TCRγ and δ loci in αβ cells were undertaken. TCRβ variable gene segments remained largely in germline configuration in γδ cells, indicating that commitment to the γδ lineage occurred before complete TCRβ rearrangements in most cases. The few TCRβ rearrangements detected were primarily out-of-frame, suggesting that productive TCRβ rearrangements diverted cells away from the γδ lineage. In contrast, in αβ cells, the TCRγ locus was almost completely rearranged with a random productivity profile; the TCRδ locus contained primarily nonproductive rearrangements. Productive γ rearrangements were, however, depleted compared with preselected cells. Productive TCRγ and δ rearrangements rarely occurred in the same cell, suggesting that αβ cells developed from cells unable to produce a functional γδ TCR. Intracellular TCRβ expression correlated with the up-regulation of CD4 and concomitant down-regulation of CD34, and plateaued at the early double positive stage. Surprisingly, however, some early double positive thymocytes retained γδ potential in culture. We present a model for human thymopoiesis which includes γδ development as a default pathway, an instructional role for the TCR in the αβ/γδ lineage choice, and a prolonged developmental window for β selection and γδ lineage commitment. Aspects that differ from the mouse are the status of TCR gene rearrangements at the nonexpressed loci, the timing of β selection and maintenance of γδ potential through the early double positive stage of development.

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Since αβ and γδ cells derive from a common precursor (15, 16), the issue of when the two lineages diverge during development is of considerable interest. In both mice and humans, TCRβ rearranges first, followed closely by TCRγ and then TCRδ, with TCRα rearrangement occurring later at the DP stage (4, 12, 18). In mice, δ, γ, and β rearrangements are completed while thymocytes are still in the DN stage, and γδ thymocytes derive mainly from DN cells (1, 19). However, in humans, a substantial fraction of complete TCRβ and γ rearrangements and the significant onset of complete TCRδ rearrangements do not occur until the CD4 ISP stage (4, 12). Thus, it is likely that γδ cells diverge from the main pathway of thymocyte development at a later stage in humans than in mice. Indeed, human CD4 ISP cells have the capacity to develop into γδ cells as shown by retroviral overexpression studies (20), but the point in development when γδ potential is lost has not been reported.

As TCR gene rearrangements undoubtedly impact the process of αβ/γδ lineage commitment, several models have been proposed to explain their role in this process (for reviews, see Refs. 21–24). The instructive model asserts that the TCR plays a primary role in determining lineage fates. In this model, αβγδ T cell precursors are bipotent before TCR gene rearrangements, but formation of a functional γδ TCR instructs the cell to develop as a γδ cell, while expression of the pre-TCR complex directs the cell to become an αβ cell. This model is supported by data showing depletion of in-frame γ and δ rearrangements in murine αβ cells (15, 17, 25, 26). The stochastic or “separate lineage” model asserts that αβγδ lineage commitment is independent of, and probably precedes, TCR gene rearrangements (27–29). Rearrangements of the TCR β, γ, and/or δ genes occur in each developing thymocyte, but only cells that make productive rearrangements of the TCR genes that match the cell fate predetermined by other factors are selected to survive. Support for this model comes from studies of mice unable to assemble a pre-TCR in which αβ lineage cells develop using the γδ TCR (30–32). Factors such as IL-7R expression (33) or Notch signaling (34–37) may also play roles in directing thymocytes into either the αβ or γδ lineage. Finally, the newer signal strength model postulates that either type of TCR can direct development into both the αβ and γδ lineages, but the strength of signal is what determines lineage choice (38, 39). Strong signals promote γδ development, while weaker signaling leads to αβ commitment. This model is supported by previously unreconciled data indicating that a given TCR can promote cross-lineage development; i.e., the γδ TCR allowing the development of DP αβ lineage cells in TCRαδ−/− mice (30–32) and the αβ TCR promoting the development of DN γδ lineage cells in αβ TCR transgenic mice (40, 41).

Considerable insight into the contribution of the TCR to the αβ/γδ lineage decision can be gleaned from an analysis of the relevant TCR loci in human thymocytes (4, 12, 42). In this study, we present analyses of the extent and productivity of rearrangement of the β locus in γδ thymocytes and of the γ and δ loci in αβ thymocytes. We are the first group to use single-cell PCR to assess the productivity of both γ and δ rearrangements in single primary αβ thymocytes. Our data reveal significant differences between humans and mice that impact the role of the TCR in the αβγδ lineage choice. Furthermore, we determine the γδ developmental potential and expression of intracellular TCRβ in successive phenotype-defined thymocyte populations. These findings are integrated into a model for human αβ and γδ thymocyte development and discussed in relationship to each of the previously proposed models for αβγδ lineage divergence. Our results illustrate unique features of human thymopoiesis including depletion of in-frame β rearrangements in γδ thymocytes, a prolonged developmental window of β selection, and preservation of γδ potential through the EDP stage of differentiation.

Materials and Methods

Abs, cells, and cell isolations

Abs used were as follows: FITC anti-CD1a, allophycocyanin anti-CD34, PE anti-γδTCR, FITC anti-CD8, and PE anti-αβTCR obtained from BD Pharmingen; PE and PE-Texas Red anti-CD8α, PE-Cy5 and allophycocyanin anti-CD4, PE anti-CD34, FITC and PE anti-αβTCR, and FITC and allophycocyanin anti-CD3 obtained from Caltag Laboratories; purified or PE-labeled anti-CD8β obtained from Serotec; purified or PE anti-TCRβ1 obtained from Ancell; and the relevant isotype control Abs. Purified anti-CD8β was labeled with Alexa Fluor 488 (Molecular Probes). Human thymus was obtained from cardiac surgeries on infants and children at Children’s Hospital in Oklahoma City, OK under protocols approved by the Institutional Review Boards of both the University of Oklahoma and the Oklahoma Medical Research Foundation. Single-cell suspensions were made by forcing thymic tissue through a 70-μm nylon filter. Human γδ thymocytes were prepared using the Miltenyi γδ isolation kit (Miltenyi Biotech), followed by sorting to >98% purity on a MoFlo Cell sorter (Cy-tomation). Human thymic αβ cells were prepared by sorting anti-human αβTCR-stained thymocytes to a purity of at least 98%. Human CD34+ thymocytes were enriched with anti-CD34 magnetic beads (Dynal Bio-tech), and subsequently stained with Abs to CD34, CD1a, CD4, and CD8, CD34+CD4+CD8+ CD1a+ and CD34+CD4+CD8+ CD1a+ cells were then sorted to >98% purity. CD4 ISP cells isolated using a two-step procedure. Thymocyte suspensions were first depleted of CD3+ cells using EasySep CD3 magnetic beads (Stem Cell Technologies), followed by depletion of CD8α+ cells using EasySep CD8 bead. CD8α-depleted cells were then stained for CD4, CD8α, and CD3 expression and CD4 ISP were sorted as CD4+ CD3+CD8α+ cells. EDPs were isolated from the fraction of cells to bound EasySep CD8 beads by staining with Abs to CD8α, CDββ, CD3, and CD4, then sorting for CD4+ CD8α−β− CD3+ cells. HeLa cells, used as a source of germline control DNA in PCR experiments, were grown in DMEM containing 10% FCS (HyClone), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies).

Quantitation of the extent of TCR γ and β locus rearrangements by real-time PCR

Genomic DNA was prepared using the Puregene kit (Genta Systems). Primers and probes for real-time PCRs have been described previously (43) and were purchased from Applied Biosystems and Sigma Genosys. Real-time PCRs for the quantitation of germline TCR gene rearrangements were amplified by multiplex PCR from 200 ng of αβ thymocyte genomic DNA using 0.2 μg of forward and reverse primer (Table I), and 1 U of JumpStart TaqDNA Polymerase (Sigma-Aldrich) in a 30-μl reaction. Cycling conditions were: 94°C for 5 min, 35 cycles of 94°C, 30 s; 60°C, 30 s; 72°C, 30 s, with a final elongation of 5 min at 72°C. TCRβ gene rearrangements were amplified from 200 ng of γδ thymocyte genomic DNA using 1 μM forward and reverse primers (Table II) and 1 U of JumpStart TaqDNA Polymerase in a 25-μl reaction. Cycling conditions were as above, using 55°C annealing for Jβ2.7 reactions and 62°C annealing for all other reactions, followed by an extension at 72°C for 2.5 min. PCR products were gel-purified, cloned, and sequenced. Unique sequences without a premature stop codon and with a preserved amino acid joining sequence were counted as in-frame.

Amplification and sequencing of TCR gene rearrangements

TCR gene rearrangements from functional V regions were amplified using the primers listed in Tables I and II; the TCR gene nomenclature is that of the International ImMunoGenetics database (IMGT; http://imgt.cines.fr/). TCRγ rearrangements were amplified by multiplex PCR from 200 ng of αβ thymocyte genomic DNA using 0.2 μg of forward and reverse primer (Table I), and 1 U of JumpStart TaqDNA Polymerase (Sigma-Aldrich) in a 30-μl reaction. Cycling conditions were: 94°C for 5 min, 35 cycles of 94°C, 30 s; 60°C, 30 s; 72°C, 30 s, with a final elongation of 5 min at 72°C. TCRβ gene rearrangements were amplified from 200 ng of γδ thymocyte genomic DNA using 1 μM forward and reverse primers (Table II) and 1 U of JumpStart TaqDNA Polymerase in a 25-μl reaction. Cycling conditions were as above, using 55°C annealing for Jβ2.7 reactions and 62°C annealing for all other reactions, followed by an extension at 72°C for 2.5 min. PCR products were gel-purified, cloned, and sequenced. Unique sequences without a premature stop codon and with a preserved amino acid joining sequence were counted as in-frame.

Single-cell PCR analysis of TCRγ and TCRδ rearrangements

Unfractionated thymocytes were stained with PE anti-αβ TCR and single TCRαδ+ cells were sorted into 96-well plates containing 20 μl of 0.2 μg/μl Proteinase K (Amresco) in 1× JumpStart TaqPCR buffer. Plates
were incubated at 50°C for 50 min for Proteinase K digestion, followed by 95°C for 10 min. The first-round PCR used 15 ng each of the forward and reverse "outside" primers in Table 1 (γ and δ), and 1 U of JumpStart Taq DNA Polymerase in a 50-μl reaction under the following cycling conditions: 94°C, 3 min, 36 cycles of 94°C, 20 s; 60°C, 20 s; 72°C, 20 s with a final elongation of 5 min at 72°C. The first-round PCR product (8 μl) were dephosphorylated with shrimp alkaline phosphatase (SAP; Roche Applied Science) and treated with Exonuclease I (EXO; Epicentre) to remove leftover primers in a 10-μl reaction of 3.6× SAP buffer (25 mM Tris-HCL, 1 mM MgCl2, 0.1 mM ZnCl2, 50% glycerol, pH 7.6), 0.5U SAP, and 1 U of EXO, incubated at 37°C for 60 s, followed by 85°C for 15 min. A second round of nested PCR for TCRγ was then performed using 2.5 μl of the SAP/EXO-treated products from each cell with 15 ng each of the forward and reverse "inside" primers (Table 1), and 1 U of JumpStart TaqDNA Polymerase in a 30-μl reaction under cycling conditions of 94°C for 3 min, 36 cycles of 94°C, 20 s; 60°C, 20 s; 72°C, 20 s with a final elongation of 5 min at 72°C. Products were analyzed by electrophoresis on 2.5% agarose gels, and detectable TCRγ rearrangements were gel-purified, cloned, and sequenced. For cells with an in-frame TCRγ rearrangement, TCRγ y rearrangements were amplified using γ "inside" primers (Table 1), cloned, and sequenced as described for TCRγ.

**Intracellular TCRβ expression and cell cycle analysis**

The following populations of thymocytes were assessed for the expression of intracellular TCRβ (TCRβintracellular) in the following populations of thymocytes: DN CD34, CD4, CD8, CD34+CD4+TCRβ+, CD34+CD8+TCRβ+, CD34-CD4+TCRβ+, and CD34-CD8-TCRβ+. These populations were sorted to >98% purity from CD34+CD8α+ cells prepared as described above. Sorted cells were resuspended in 285 μl of a 1:1 mixture of FCS and PBS and then fixed with 715 μl of cold 70% ethanol added dropwise with gentle vortexing. Fixed cells were resuspended in 1 ml of PBS containing PI (50 μg/ml) and RNase A (50 μg/ml) and incubated for 20 min at 37°C. Cells were then cooled on ice and analyzed for DNA content with a FACScan flow cytometer (BD Biosciences) and CellQuest software.

**Chimeric human/mouse fetal thymic organ cultures (Hu/mo FTOC)**

Hu/mo FTOC was performed essentially as described (47). Reconstituted deoxyguanosine-treated murine fetal thymic lobes were incubated for up to 3 wk in Yssel’s medium (48) supplemented with 2% human AB serum and 5% FCS. Upon harvest, cells were counted, stained with Abs to human αβTCR and γδTCR, and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

**Results**

**The extent of TCR rearrangements in human thymocytes**

To investigate the role of the TCR in human αβ vs γδ lineage commitment, the extent of complete TCRβγ gene rearrangements was analyzed in γδ thymocytes using quantitative real-time PCR. This assay (43) quantitates the amount of germline DNA in between the most 3’ Vβ segment and DJβ1 remaining in a given sample, using a strategy analogous to that of quantitative Southern blotting previously used in analyses of murine thymocytes (17, 18, 44, 45) and includes a correction for the contribution of β-TRECs to the germline signal. In the case of γδ thymocyte DNA, only 4.6 ± 2.4% (mean ± SD) of the germline TCRβ signal was attributed to β-TRECs (data not shown). Because only complete V→DJβ rearrangements enable a cell to produce a functional TCR chain, immature D→Jβ rearrangements were not evaluated. Therefore, the percentage of germline DNA refers to the proportion of the locus whose variable genes have not rearranged. Fig. 1 shows the percentages of the TCRβ locus rearranged in six γδ

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**Table I. Primers for single cell PCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Outside Primer Sequence</th>
<th>Inside Primer Sequence</th>
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</thead>
<tbody>
<tr>
<td>Vγ1 Fwd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TGACGCACTACAGGAGGGGA</td>
<td>ACTTGGACATTACACAGAGGA</td>
</tr>
<tr>
<td>Vγ1p Fwd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CCACTTCAGGAGGAGGGGA</td>
<td>CCTTCTTTACACAGGAGGGGA</td>
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<tr>
<td>Vγ9 Fwd</td>
<td>GTATAGGACGGATCACCATTT</td>
<td>ATATGATTGACGAGGAGAG</td>
</tr>
<tr>
<td>Vγ10 Fwd</td>
<td>CCTGTTGACGACCTTATGCA</td>
<td>GACCGACAGGTTAAAGAG</td>
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<tr>
<td>Vγ1 Fwd</td>
<td>CTCAGGCTGCAATTGTGACAG</td>
<td>CCTGCTGGAAGATGTCGACAG</td>
</tr>
<tr>
<td>Jy1.1 + 2.1 Rev</td>
<td>ATTCAGGCTGCAATGTTGACAG</td>
<td>CCAGGAGATGATGAGAGAG</td>
</tr>
<tr>
<td>Jy1.2 Rev</td>
<td>CGAGGGCTGCAATGTTGACAG</td>
<td>ATAGAGCTGGAAGGTGACAG</td>
</tr>
<tr>
<td>Jy1.3 + 2.3 Rev</td>
<td>TGCATATACGGCAAAAGCAAG</td>
<td>CTCCTATTATACGGTGAAG</td>
</tr>
<tr>
<td>Vδ1 Fwd</td>
<td>ACCCTGGAGCTCCCTGATGA</td>
<td>TGTCAGAACAGCACTTCAAC</td>
</tr>
<tr>
<td>Vδ2 Fwd</td>
<td>AATCTGAGCAAGGACAGACA</td>
<td>GCAAGAACCTGTCAGATCAT</td>
</tr>
<tr>
<td>Vδ3 Fwd</td>
<td>CAAACTGACCGAGGAGGAG</td>
<td>CAAAGAACCATGGAGGAG</td>
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<tr>
<td>Jδ1 Rev</td>
<td>CCTCTAGACGGATGGTTCTTT</td>
<td>AGTCAGAACAGCACTTCAAC</td>
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<tr>
<td>Jδ2 Rev</td>
<td>GACCTACGCTCAAACCAAACCT</td>
<td>CACAGGAGATGATGAGAGAG</td>
</tr>
<tr>
<td>Jδ3 Rev</td>
<td>CGAGGGCTGCAATGTTGACAG</td>
<td>ATAGAGCTGGAAGGTGACAG</td>
</tr>
<tr>
<td>Jδ4 Rev</td>
<td>TGCCTCTCTAATCTGGTGAACT</td>
<td>CTCCTGAGATGATGAGAGAG</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Fwd, Forward; Rev, reverse.

---

**Table II. Primers for population sequencing of TCRγ and TCRβ**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Vγ1 Fwd</td>
<td>ACTGTTACCTACAGGAGGGGA</td>
</tr>
<tr>
<td>Vγ9 Fwd</td>
<td>ATATTGATGAGTGAGGAGGGGA</td>
</tr>
<tr>
<td>Jy1.1 Rev</td>
<td>ATAGATCAGTAGGAGGACAG</td>
</tr>
<tr>
<td>Jy1.2 Rev</td>
<td>ATATGAGCTGCAATGTTGACAG</td>
</tr>
<tr>
<td>Jy1.3 Rev</td>
<td>GATGAGGAGCTGCAATGTTGACAG</td>
</tr>
<tr>
<td>Jy1.2 Rev</td>
<td>GGTAGAACCTTGGATCCAGT</td>
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<tr>
<td>Jy1.3 Rev</td>
<td>CTGGATACCTGCAATGTTGACAG</td>
</tr>
<tr>
<td>Vβ12.3 Fwd</td>
<td>ATTTACGTTTAACAAACAGTTGAGC</td>
</tr>
<tr>
<td>Vβ3.1 Fwd</td>
<td>ATATTTGAATGAGCATCAGTACAG</td>
</tr>
<tr>
<td>Vβ20.1 Fwd</td>
<td>CGTGCAGTGGCTCCTCTCAGAG</td>
</tr>
<tr>
<td>Jβ2.7 Rev</td>
<td>CCGCGCAGAATGATTGTC</td>
</tr>
<tr>
<td>D5-Jβ16 Rev</td>
<td>ACCCTGAGCTGGCTCTCTCTCAG</td>
</tr>
<tr>
<td>TCRJ2.3 Rev</td>
<td>GATGCCGCACTGGCTCTCTCAG</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fwd, Forward; Rev, reverse.
(8.7 ± 6%) and two αβ (58 ± 4%) thymocyte isolates. The data indicate that most γδ cells had not undergone any complete TCRβ rearrangements. The observed extent of TCRβ rearrangement in αβ thymocytes (55–60%) was slightly less than the value predicted for an allelically excluded thymocyte population (70%; Ref. 45), perhaps because some rearrangements occurred by inversion rather than by deletion.

Next, the extent of TCRγ rearrangements was analyzed in populations of human αβ thymocytes using a similar real-time PCR strategy (43). Fig. 2 shows the percentages of the TCRγ locus rearranged in six αβ (97 ± 2%) and two γδ (95 ± 1%) thymocyte isolates. This indicates that the TCRγ locus rearranges on both alleles in nearly all developing thymocytes, regardless of αβ/γδ lineage development.

Analysis of TCR gene rearrangement productivity

To assess the potential of the rearranged TCR genes to generate expressed TCR proteins, the productivity of TCRβ and TCRγ rearrangements was analyzed in γδ and αβ thymocytes, respectively. TCR gene rearrangements were amplified by PCR of genomic DNA using primers specific for V- and J-gene segments (Table II), then cloned and sequenced. As shown in Table III, productive TCRβ rearrangements using either the Jβ1 or the Jβ2 cluster were underrepresented in all three isolates of γδ cells relative to the value expected if such rearrangements were to occur randomly (33%), suggesting that the small percentage of γδ cells with TCRβ rearrangements may have undergone some type of selection, either through lineage commitment or survival. Interestingly, gene rearrangements using the Jβ2 cluster, the more commonly used cluster in human peripheral blood T cells (49), were statistically more likely to be nonproductive, compared with those using the Jβ1 cluster (p = 0.0002, by χ² analysis). In contrast, in two independent αβ thymocyte isolates, approximately one-third of the TCRγ gene rearrangements were in-frame (30%, Table IV), suggesting that the presence of an in-frame γ rearrangement did not preclude αβ development.

TCRδ and γ rearrangements in single αβ thymocytes

To determine whether cells expressing an αβ TCR ever had the chance to make a functional γδ TCR and to become a γδ cell earlier in development, TCRδ and γ rearrangements were cloned and sequenced in single αβ thymocytes. Because the δ locus is deleted during TCRδ rearrangement and δ-TRECs are lost with cell division, we expected that most of the cells would not contain detectable δ sequences. Therefore, to avoid sequencing γ rearrangements from many cells that would not have detectable δ rearrangements, we sequenced δ first. For those cells with detectable δ sequences, we then cloned and sequenced γ rearrangements. Out of a total of ~1100 single cells analyzed, 134 cells had detectable δ rearrangements (Fig. 3, lower panel). Only 19 (14%) of these sequences were in-frame (Table V). Assuming that the productivity of the δ rearrangements that were deleted and lost is similar to that of the sequences we obtained, these data suggest that between 14% (if one allele per cell is rearranged) and 26% (if both δ alleles are rearranged) of αβ thymocytes had at least one δ allele rearranged in-frame. From the 19 cells containing in-frame TCRδ rearrangements, we were able to amplify and sequence 21 of 38 γ alleles (Fig. 3, upper panel), only 3 of which (14%) were productively rearranged. If both γ alleles are rearranged in almost all cells as suggested by our real-time PCR data, this frequency of in-frame alleles should lead to ~26% of cells with at least one γ allele in-frame. Therefore, the percentage of αβ thymocytes with rearrangements of both δ and γ in-frame would be predicted to be between 3.6% (14% of 26%) and 6.8% (26% of 26%), depending upon the average number of δ alleles rearranged per cell. Thus, our analyses indicate that the vast majority of αβ thymocytes could not have expressed a γδ TCR at some point earlier in development. It is impossible to know whether those few αβ cells with in-frame rearrangements of both δ and γ could have produced a functional γδ TCR, as not all combinations of γ and δ chains can make a functional receptor (50).

Intracellular TCRβ expression in developing human thymocytes and evidence for β selection

To correlate the status of TCRβ gene rearrangements in developing thymocytes with expression of TCRβ protein, a comprehensive analysis of intracellular TCRβ (TCRβIC) expression was performed. Cells were enriched for early thymocyte populations as
Cells through the EDP stage can give rise to γδ thymocytes in hu/mo FTOC

The γδ and αβ developmental potential of CD34+CD1a+, CD4 ISP, and EDP thymocytes was assessed in hu/mo FTOC. After 1–3 wk, the cells were harvested and stained with Abs to αβ and γδ TCR. All three populations generated large numbers of αβ TCR-expressing cells (Fig. 5B). Fig. 5A shows representative histograms of surface γδ TCR expression on gated populations of (DN + CD8 SP) cells, fractions enriched for γδ T cells. The percentage of γδ TCR+ cells in this subpopulation is highest in cultures initiated with CD34+CD1a+ thymocytes, but significant levels of γδ TCR- cells were also observed in cultures derived from both CD4 ISP and EDP cells. Fig. 5B shows the absolute numbers of γδ TCR+ and αβ TCR+ cells generated from each subpopulation in four separate experiments. Although the absolute numbers of γδ TCR- cells declined as the input cells were more mature, the EDP population had not lost its ability to yield γδ cells even though an average of 85% of EDP cells expressed TCRβγ.

**Discussion**

We present here a detailed characterization of events in human thymocyte development impacting the αβ/γδ lineage decision. Our findings and the work of others are summarized in a model for human αβ and γδ thymocyte development (Fig. 6). Our data are most compatible with an instructive role for the TCR in αβ vs γδ lineage commitment, with γδ development as the default pathway for human thymocyte differentiation. This conclusion comes from several observations: first, the majority of γδ thymocytes have their TCRβγ genes in germline configuration (Fig. 1); second, the majority of αβ thymocytes have both their γ alleles rearranged (Fig. 2); third, αβ thymocytes are depleted of in-frame δ rearrangements (Table V); and finally, productive TCRγ and δ rearrangements are rarely found in single αβ cells. These data suggest that virtually all human thymocytes first rearrange their TCR γ and δ loci, attempting to produce a γδ TCR. Most that are successful early in development never attempt TCR V→DJδ gene rearrangements. However, those cells unable to produce a functional γδ TCR early in development progress into the CD4 ISP and EDP stages when the majority of complete TCRβ gene rearrangements take place and most β selection occurs. If γ and δ continue to rearrange, this scenario would be reminiscent of the competitive instructional model of thymocyte development (21, 22), in which the first productive TCR complex to be expressed and signal (i.e., the γδ TCR or the pre-TCR) determines the lineage fate. This could explain the relatively long window of time in which cells are still able to commit to the γδ lineage (Fig. 5). The presence of some in-frame TCRβ rearrangements in γδ thymocytes would be described in *Materials and Methods*, and stained for TCRβγ in combination with markers to define each subpopulation. Representative histograms are shown in Fig. 4A and the percentages of TCRβγ- cells from individual experiments are shown in Fig. 4B. TCRβγ expression was virtually undetectable in the earliest DN CD34+CD1a- population and only small percentages of cells were TCRβγ+ at the next CD34+CD1a+ stage. These findings agree with previously published data indicating that complete V→DJβ rearrangements are not readily detectable until the CD4 ISP stage (4, 12). However, modest to significant levels of TCRβγ were detectable in the CD4 ISP subsets, with higher percentages of TCRβγ-expressing cells correlating with the down-regulation of surface CD34. On average, 85% of EDPs displayed a high level of TCRβγ expression. CD3-/- and CD3+ DP cells (expressing CD4 and CD8αβ) showed only slightly increased percentages of TCRβγ- cells when compared with EDPs. These data indicate that by the time the cells reach the EDP stage, the majority are either in the process of, or have undergone, β selection. Fig. 4B shows that the major increase in TCRβγ expression begins in the CD4 ISP stage, and reaches a maximum by the EDP stage. It is also interesting to note the degree to which individuals vary in the timing and extent of TCRβγ expression (Fig. 4B).

To determine whether the expression of TCRβγ correlated with the onset of β selection, the cell cycle status of subpopulations of CD4 ISP cells was determined. Fig. 4C shows that both CD34- and CD34+ ISP populations expressing TCRβγ had significantly higher proportions of cells in the (S + G2-M) phases of the cell cycle compared with the corresponding TCRβ- populations (28% compared with 2.4–4.8%). These data indicate that many of the TCRβγ- cells within the CD34+ and CD34- CD4 ISP subsets have undergone β selection. Therefore, our data suggest that β selection is an asynchronous, ongoing process that occurs throughout several phenotypic stages of thymocyte development and whose onset is not strictly correlated with the expression of CD4 and especially CD8.

**Table III. Productivity of TCRβ gene rearrangements in human γδ thymocytes**

<table>
<thead>
<tr>
<th>γδ Isolate</th>
<th>Total No. Vβ-Jβ1 Sequences</th>
<th>No. Vβ-Jβ1 Sequences In-Frame</th>
<th>% Productive Vβ-Jβ1</th>
<th>Total No. Vβ-Jβ2 Sequences</th>
<th>No. Vβ-Jβ2 Sequences In-Frame</th>
<th>% Productive Vβ-Jβ2</th>
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<td>3</td>
<td>58</td>
<td>15</td>
<td>25.8</td>
<td>103</td>
<td>17</td>
<td>16.5</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>49</td>
<td>23.6</td>
<td>372</td>
<td>44</td>
<td>11.8</td>
</tr>
</tbody>
</table>

*a Vβ-Jβ1 sequences correspond to Vβ20.1, Vβ12.3, and Vβ3.1 joins with Jβ1.1–1.6 gene segments, though sequences were primarily Jβ1.6.

*b Vβ-Jβ2 sequences correspond to Vβ20.1, Vβ12.3, and Vβ3.1 joins with Jβ2.1–2.3 and Jβ2.7 gene segments.

*By χ² analysis, *p* = 0.03 for the comparison 23.6 vs 33.3 and *p* = 0.0002 for the comparison 23.6 vs 11.8.

*By χ² analysis, *p* = < 0.0001 for the comparison 11.8 vs 33.3.

**Table IV. Productivity of TCRγ gene rearrangements in human αβ thymocytes**

<table>
<thead>
<tr>
<th>αβ Isolate</th>
<th>Total No. Unique Sequences</th>
<th>No. γ Sequences In-Frame</th>
<th>% Productive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>97</td>
<td>29</td>
<td>29.9</td>
</tr>
<tr>
<td>2</td>
<td>89</td>
<td>27</td>
<td>30.3</td>
</tr>
<tr>
<td>Total</td>
<td>186</td>
<td>56</td>
<td>30.1</td>
</tr>
</tbody>
</table>

*a Sequences correspond to gene rearrangements from all functional Vγ gene segments to all possible J gene segments.
expected if the pre-TCR could not be expressed and/or signal before the expression and signaling of the γδ TCR in some cells. Depletion of in-frame TCRβ rearrangements in γδ cells could be explained if those cells expressing both a γδ TCR and the pre-TCR were either deleted or diverted into the αβ lineage. Thus, thymocytes develop, the highest level of γδ potential is seen earliest in development, and as γδ potential decreases, the percentage of cells that are αβ-committed increases, as shown by the increasing expression of TCRββ (Fig. 4). These studies demonstrate that there are important features of human thymocyte development that are distinct from those described for murine cells, as discussed below.

First, the extent and productivity of complete TCRβ gene rearrangements in human versus murine γδ cells differ greatly. Previous studies with adult murine thymic γδ cells showed that the TCRβ locus was substantially rearranged, with ~15–20% of alleles displaying complete V→DJβ rearrangements (44). Analysis of the productivity of these rearrangements produced an array of results, ranging from 30 to 70% (9, 15, 44, 51, 52), depending on the origin of the γδ cell. In most cases, these data were interpreted to mean that an in-frame TCRβ chain might confer a selective survival or proliferative advantage to γδ thymocytes, and that productively rearranged TCRβ genes did not preclude γδ development.

Our findings for human γδ thymocytes are quite different. First, a much smaller percentage of the β locus showed complete rearrangements than in the mouse (8.7% on average, Fig. 1). Since our results were corrected for the presence of β-TRECs (43), this cannot be the reason for the higher percentage of germline DNA in our experiments. We did find extensive D→Jβ rearrangements in human γδ cells (data not shown), indicating that early on, at least part of the TCRβ locus was accessible to the recombination machinery. Our data are consistent with those of Couedel et al. (53) who showed that cloned peripheral blood γδ T cells contained a low percentage of complete V→DJβ rearrangements. Second, and more importantly, few complete β rearrangements detected were predominately out-of-frame (Table III), suggesting an instructional role for the pre-TCR in diversion away from the γδ lineage.

In one of the few studies with primary human cells available to compare with ours, Margolis et al. (42) concluded that γδ cells from individual thymi followed different pathways of development, depending upon the timing of δ and β gene rearrangements. In some thymi, the β locus was rearranged in γδ cells to the same extent as in αβ cells. Furthermore, in one of nine thymi, γδ cells had predominately in-frame β rearrangements. The discrepancies between their results and ours are difficult to reconcile, but may be related to methodology, as they used PCR spectrotyping to evaluate both the extent and productivity of rearrangements, rather than quantitative real-time PCR and DNA sequencing. We may have failed to see γδ thymocytes with predominately in-frame β rearrangements, as this was an infrequent finding in their studies (one of nine samples). In any case, as there were very few TCR V→DJβ rearrangements in any of our six preparations of γδ thymocytes, we conclude that most human γδ thymocytes become lineage-committed before complete V→DJβ gene rearrangements begin. Whether the few γδ cells with complete in-frame β rearrangements die by apoptosis or are diverted to the αβ lineage is currently unknown, making the impact of productively rearranged TCRβ genes on γδ development of interest for future studies.

The second important difference between humans and mice is the status of the TCRγ locus in αβ thymocytes. We found almost complete rearrangement (97%) of the TCRγ locus in these cells, and the rearrangements showed a random productivity profile (Fig. 2, Table IV). Although the TCRγ locus has been reported to be highly rearranged in αβ lineage leukemic cells (54) and alloreactive T cell clones (55), ours is the first assessment of the extent of TCRγ gene rearrangement in normal primary human αβ thymocytes. The TCRγ locus is also highly rearranged in murine αβ cells, though the exact extent is difficult to discern from the literature, as the organization of the murine TCRγ locus precludes a straightforward analysis analogous to ours (17, 56, 57). Several reports indicated that murine TCRγ rearrangements are likely subject to allelic exclusion (58, 59), while our data and those of others suggest a lack of allelic exclusion at the human TCRγ locus (54, 55, 60). Furthermore, αβ thymocytes from mice were selectively depleted of in-frame TCRγ gene rearrangements (15, 17, 25), suggesting that αβ cells derived from thymocytes unable to productively rearrange their γ locus. In contrast, our data showed a random productivity profile of TCRγ rearrangements in αβ cells, suggesting at first glance, that TCRγ rearrangements do not influence lineage commitment. However, this would be surprising given the strong selection against cells that can express a γδ TCR during αβ development (see below). One possibility is that this selection is mediated at the level of γ and δ-chain pairing due to constraints placed on which Vγ chains can pair with Vδ proteins to produce a functional TCR. Precedence for this mechanism is found in studies of mouse γδ cells (50). A second explanation for this apparent contradiction is that V-Jγ recombination would be biased toward productive rearrangements due to microhomology-based joining (61) and that the experimental value of 30% in-frame TCRγ rearrangements could actually reflect a selection against in-frame rearrangements. To test this possibility, we analyzed TCRγ rearrangements in CD34+CD4 ISP cells, a population unlikely to be committed to the αβ lineage due to its low percentage of

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**Table V. Productivity of TCRδ gene rearrangements in single αβ thymocytes**

<table>
<thead>
<tr>
<th>αβ Isolate</th>
<th>Total No. δ Sequences</th>
<th>No. In-frame δ Sequences</th>
<th>% Productive TCRδ Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>5</td>
<td>21.7</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>7</td>
<td>13.7</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>7</td>
<td>11.7</td>
</tr>
<tr>
<td>Total</td>
<td>134</td>
<td>19</td>
<td>14.2</td>
</tr>
</tbody>
</table>

* Sequences correspond to Vβ1, Vβ2, and Vβ3 joins with Jβ1–4 gene segments, though most sequences contained Jβ1.

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**FIGURE 3.** Single-cell PCR analysis of TCRγ and δ rearrangements in human αβ thymocytes. Genomic DNA was isolated from single human αβ thymocytes and nested PCRs performed to detect either TCRγ or TCRδ gene rearrangements as described in Materials and Methods. A representative agarose gel of PCR products from 10 individual αβ thymocytes is shown. M, m.w. markers.
FIGURE 4. Intracellular TCRβ expression and cell cycle status in human thymocyte subpopulations. Thymocyte populations were enriched from neonatal thymus and stained for intracellular TCRβ expression as described in Materials and Methods. A, Cells were electronically gated on each subpopulation. DNs were defined as CD4−CD8−CD3−CD1a+ or CD1a−. ISPs were gated as CD3−CD8a−CD4−CD34− or CD34+. EDPs were defined as CD3+CD8β+CD4−CD8a+. DPs were gated as CD4+CD8β− and CD3− was used to define the blast population. The filled histograms indicate the PE-TCRβ staining in each cell population, while the isotype control (PE-IgG2a) is depicted by the black line tracing. The percentage of cells expressing TCRβ after subtracting the isotype control percentage is indicated in each histogram. B, Individual values for the percentages of TCRβ−expressing cells in various thymocyte subpopulations from up to 10 different thymi are shown. The mean %TCRβ−± SD for each population is plotted in the line graph. C, CD34−/−CD4 ISP TCRβ−/− cells were sorted and analyzed for cell cycle status by staining with PI. The percentages of cells in the (S + G2-M) phases of the cell cycle are shown.
TCRβ<sup>+</sup>-expressing cells. Preliminary results revealed that these cells contained ~40% productive TCRγ rearrangements (61 of 151 sequences), a finding that by χ<sup>2</sup> analysis is significantly different (p = 0.048) from that for αβ thymocytes (30%, Table IV). However, this value is not significantly different than a random distribution (33%, p = 0.18 by χ<sup>2</sup> analysis), raising the question of whether in-frame γ rearrangements are truly depleted in αβ thymocytes. Further experimentation will be required to determine whether human TCRγ rearrangements are subject to microhomology domain biases and to fully understand the role of these gene rearrangements in the αβ vs γδ lineage decision.

A third significant difference between human and murine thymocyte development is a prolonged window of development through which β selection and γδ lineage commitment occur simultaneously. Murine thymocytes can develop into both αβ and γδ thymocytes through the DN<sub>III</sub> stage, but show greatly reduced γδ potential in the DNIV compartment as assessed by culture of sorted thymocytes in FTOC (1). Thus, in the mouse, it is well-accepted that coexpression of CD4 and CD8 marks commitment to the αβ lineage. In contrast, we show here for the first time, that γδ developmental potential persists into the later phases of human thymocyte development until at least the CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>αβ<sup>-</sup> (EDP) stage (Fig. 5). Even though ~85% of EDP cells expressed TCRβ<sup>+</sup> (Fig. 4), a small percentage with this cell surface phenotype remained uncommitted in terms of lineage decision, and still produced γδ cells in hu/mo FTOC (Fig. 5). The γδ potential in the next developmental stage (DP blasts) was greatly diminished, as γδ cells cannot be identified with certainty in hu/mo FTOC initiated with this population (data not shown). γδ potential correlated inversely with the observed expression of TCRβ<sup>+</sup> in human thymocyte populations. DN CD3<sup>+</sup>CD1a<sup>+</sup> cells were the first population to have a small percentage of cells expressing TCRβ<sup>+</sup> (Fig. 4). These data are consistent with recent work by Dik et al. (4) who also placed the onset of complete TCRβ rearrangements at this stage. However, pre-T<sub>ε</sub> protein expression is not appreciably detectable before the CD4 ISP stage (62), so it is possible that TCRβ<sup>+</sup> expression is not synchronously linked to the expression of pre-TCR and β selection. We did find significant percentages of TCRβ<sup>-</sup> cells at the CD4 ISP stage, consistent with the findings of Blom et al. (12), especially in the subpopulation that had downregulated CD34 expression (Fig. 4). In fact, the TCRβ<sup>-</sup> cells in the DN CD3<sup>+</sup>CD1a<sup>+</sup> fraction had significantly lower CD34 expression than the remainder of the population (data not shown),
suggesting that down-regulation of CD34 correlates with the onset of complete TCRβ gene rearrangements and/or β selection. Populations of CD4 ISP cells expressing TCRβ- had larger percentages of cells in the (S + G2-M) phases of the cell cycle compared with TCRβ+ cells, consistent with their having passed β selection. The percentage of TCRβ+ cells continued to increase as the cells matured and reached a plateau at the EDP and DP blast stages (Fig. 4). Our results contrast with those of Toribio’s group (11) who contend that the transition of cells from the EDP (CD4+CD8α-β-γδ-) to DP (CD4+CD8α-β-) stage marks the point of β selection. The source of this discrepancy is unknown, but could relate to different methods of cell isolation or different staining protocols. We conclude that in humans, progression of cells from a CD34+CD1a+ phenotype through stages during which CD4 and CD8 are successively up-regulated is a flexible and dynamic process during which β selection and γδ lineage commitment occur simultaneously, with the frequency of β-selected cells increasing and γδ potential diminishing.

Even though our data are most consistent with an instructional mechanism for the αβ vs γδ lineage decision, other factors besides signals from the TCR are relevant. Recent data on human thymocyte development (36, 37) showed that the retroviral expression of active intracellular Notch protein in the earliest thymocyte precursors dramatically skewed their development toward a γδ cell fate. Furthermore, molecules such as Egr1 (38) and lymphoxygen (63) have been shown to impact αβ/γδ lineage development in mice. Clearly, other molecules besides the TCR can play key roles in lineage decisions. The potential relevance of our data to the recent signal strength model of αβ/γδ lineage choice (38, 39) is currently unknown and will require clarification by further investigation.

In summary, we document a number of significant differences in the development of αβ and γδ thymocytes in humans vs mice and identify important areas for future investigation. Although our data suggest that human αβ vs γδ development is primarily influenced by the timing of TCR gene rearrangements and expression of functional TCRs, the cellular factors and signaling pathways that regulate the accessibility of TCR loci and gene rearrangement remain to be defined. For example, what factors allow D→Jβ rearrangements in developing γδ cells, but preclude V→DJβ rearrangements? What initiates V→DJβ rearrangements in those cells that fail to make a functional γδ TCR? Why are rearrangements to the Jβ1 cluster nearly twice as likely to be in-frame in developing γδ cells as those to the Jβ2 cluster? Using chromatin immunoprecipitation assay and retrotransduction of candidate molecules into human precursor cells, these questions can now be addressed. The prolonged window of β selection and γδ potential is a finding unique to human thymocytes and should prompt more studies on the development of γδ thymocytes at the phenotypic and molecular levels. The identification of factors that control the lineage commitment of cells with simultaneous productive rearrangements at the γ, δ, and β loci, and the molecular mechanism by which Notch signaling can influence the αβ vs γδ fate are particularly interesting questions. Overall, our data underscore the need to continue studies of primary human thymocytes to understand the mechanisms controlling both normal and pathological human T cell development.

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Disclosures

The authors have no financial conflict of interest.

References