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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

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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 developmental stages of both murine and human thymocyte development, molecular analyses of the TCRβ locus, and lineages 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 developmental stages of both murine and human thymocyte development, molecular analyses of the TCRβ locus, and lineages choice, and a prolonged developmental window for β selection and γδ lineage commitment.
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 TCRβ, with TCRα rearrangement occurring later at the DP stage (4, 12, 17, 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 TCRδ and γ rearrangements and the significant onset of complete TCRβ 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 αβγδ lineage cells in TCRβγδ−/− 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 αβγδ 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 phenotypically 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, allopredoccyanin anti-CD34, PE anti-γδTCR, FITC anti-CD8, and PE anti-αβ TCR obtained from BD Pharmingen; PE and PE-Texas Red anti-CD8α, PE-Cy5 and allopredoccyanin anti-CD4, PE anti-CD34, FITC and PE anti-γδ TCR, and FITC and allophycocyanin anti-CD3 obtained from Caltag Laboratories; purified or PE-labeled anti-CDβ obtained from Serotec; purified or PE anti-TCRβ obtained from Ancell; and the relevant isotype control Abs. Purified anti-CDβ 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 Biotec), followed by sorting to >98% purity on a MoFlo Cell sorter (Cytomation). Human thymic αβ cells were prepared by sorting anti-human αβ TCR-stained thymocytes to a purity of at least 98%. Human CD34+ thymocytes were enriched with anti-CD34 magnetic beads (Dynal BioTech), 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 ISPs were 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 CD8+ cells using EasySep CD8 beads. CD8α-depleted cells were then stained for CD3, CD8a, and CD3 expression and CD4 ISPs were sorted as CD4+CD8a− cells. EDPs were isolated from the fraction of cells bound to EasySep CD8 beads by staining with Abs to CD8α, CD8β, CD3, and CD4, then sorting for CD4+CD8a−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 TCRβ and TCRγ loci were performed as previously described (43) using a strategy to detect a germ-line DNA amplicon which is deleted upon TCR recombination, similar to that described for quantitative Southern blotting (17, 18, 44, 45). The amount of germline DNA detected was normalized to an ampiclon not deleted during TCR rearrangements, and the values were used to calculate the percentage of germline DNA remaining in each population. The percentage of germline DNA at the Vβ locus in γδ cells was corrected for the presence of TCRβ excision circles (β-TREC) (43).

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 1 min 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 1 min reverse primers 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 TCRβ rearrangements

Unfractionated thymocytes were stained with PE anti-αβ TCR and single TCRαβ+ 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 I (γ and δ), and 1 U of JumpStart TaqDNA Polymerase in a 50-μl reaction under the following cycling conditions: 94°C, 3 min, 36 cycles of 94°C, 20 s; 60°C, 40 s; 72°C, 30 s with a final elongation of 5 min at 72°C. The first-round PCR products (8 μl) were dephosphorylated with shrimp alkaline phosphatase (SAP; Roche Applied Science) and treated with Exonuclease I (Exol; Epicentre) to remove leftover primers in a 10-μl reaction of 3.6X 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 Exol, incubated at 37°C for 60 s, followed by 85°C for 15 min. A second round of nested PCR for TCRδ was then performed using 2 μl of the SAP/Exol-treated products from each cell with 15 ng each of the forward and reverse δ “inside” primers (Table I), 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, 30 s; 72°C, 20 s with a final elongation of 5 min at 72°C. Products were analyzed by electrophoresis on 2.5% agarose gel. PCR products were dephosphorylated with shrimp alkaline phosphatase (SAP; Roche Applied Science) and purified using a JumpStart PCR Purification Kit (Sigma-Aldrich). Purified PCR products were sequenced.

### Intracellular TCRβ expression and cell cycle analysis

The following populations of thymocytes were assessed for the expression of intracellular TCRβ (TCRβic): DN CD34+/CD1a- IC, DN CD34+/CD1a+, CD34+/CD4+ IC, CD34+/CD4+DP, and CD34+/CD8+ DP blasts, and CD3+/CD8+ DP blasts. Enriched populations of CD4+ and CD8+ DP cells were obtained by depletion of CD3- and CD8α-expressing cells with EasySep beads as described above. Enriched populations of DP cells were obtained from cells binding to CD8α beads during the above depletions. TCRβic expression on DP cells was analyzed on unfractionated thymocytes. Staining for TCRβic expression was performed essentially as described previously by fixing cells with 1% formaldehyde and permeabilizing with 0.5% saponin (45). Purified anti-TCRβiAb was added at the same time as other Abs to cell surface proteins to block surface staining of TCRβ. Samples were analyzed using an LSR II flow cytometer and CellQuest software (BD Biosciences). Isotype controls were used to set gates, and the percentages of TCRβic-expressing cells were obtained by subtracting the percentages of cells stained intracellularly with an isotype control Ab.

The following populations of cells were analyzed for cell cycle status by staining with propidium iodide (PI): CD34+/CD4+ TCRβic, CD34+/CD4+ TCRβic, CD34+CD8+ TCRβic, and CD34+CD4+ TCRβic. These populations were sorted to >98% purity from CD3+CD8α+ cells prepared as described above. Sorted cells were resuspended in 285 μl of a 50:50 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 (500 μg/ml, Qiagen) 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.

### 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 TCRβ 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’ β segment and Dβ1 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→Dβ rearrangements enable a cell to produce a functional TCR chain, immature D→β 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...
**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 values 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 $χ^2$ 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 TCRα rearrangement and δ-TRECs are lost with cell division, we expected that many 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 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 (TCRβ*~) expression was performed. Cells were enriched for early thymocyte populations as

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**FIGURE 1.** The TCRβ variable gene segments remain largely in germline configuration in human γδ thymocytes. Genomic DNA was isolated from purified human γδ and αβ thymocytes and the percent of the Jβ locus remaining in germline configuration was determined by real-time PCR as described in Materials and Methods. For the γδ DNA samples, these percentages were corrected for the contribution of β-TRECs. These values were subtracted from 100 to give the percent of the Jβ locus rearranged. The percent of TCRβ rearranged in each DNA sample is shown as the mean ± SD from six replicates.

**FIGURE 2.** The TCRγ locus is highly rearranged in human αβ and γδ thymocytes. Genomic DNA was isolated from purified human thymic γδ and αβ cells and the percent of the γ locus remaining in germline configuration was determined by real-time PCR as described in Materials and Methods. These percentages were subtracted from 100 to give the percent of the γ locus rearranged, which is shown as the mean ± SD for six replicates.
described in Materials and Methods, and stained for TCRβic in combination with markers to define each subpopulation. Representative histograms are shown in Fig. 4A and the percentages of TCRβic+ cells from individual experiments are shown in Fig. 4B. TCRβic expression was virtually undetectable in the earliest DN CD34+CD1a- population and only small percentages of cells were TCRβic+ 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 TCRβic were detectable in the CD4 ISP subsets, with higher percentages of TCRβic-expressing cells correlating with the down-regulation of surface CD34. On average, 85% of EDPs displayed a high level of TCRβic expression. CD34low and CD3+ DP cells (expressing CD4 and CD8αβ) showed only slightly increased percentages of TCRβic+ 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 TCRβic 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 TCRβic expression (Fig. 4B).

To determine whether the expression of TCRβic 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 TCRβic had significantly higher proportions of cells in the (S + G2-M) phases of the cell cycle compared with the corresponding TCRβ−ic populations (28% compared with 2.4–4.8%). These data indicate that many of the TCRβic+ 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 In-Frame</th>
<th>% Productive</th>
<th>Total No. Vβ-Jβ2 Sequences</th>
<th>No. Vβ-Jβ2 In-Frame</th>
<th>% Productive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88</td>
<td>18</td>
<td>20.5</td>
<td>68</td>
<td>4</td>
<td>5.8</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>16</td>
<td>25.8</td>
<td>201</td>
<td>23</td>
<td>11.4</td>
</tr>
<tr>
<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>23.6d</td>
<td>372</td>
<td>44</td>
<td>11.8d</td>
</tr>
</tbody>
</table>

*Vβ-Jβ1 sequences correspond to Vβ20.1, Vβ12.3, and Vβ3.1 joins with Jβ1.1−1.5 gene segments, though sequences were primarily Jβ1.6.

*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>
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<tr>
<td>Total</td>
<td>186</td>
<td>56</td>
<td>30.1</td>
</tr>
</tbody>
</table>

*Sequences correspond to gene rearrangements from all functional Vγ gene segments to all possible J gene segments.

### 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 TCRβic.

### 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 TCRβν 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 TCRγ and δ loci, attempting to produce a γδ TCR. Most that are successful early in development never attempt TCR V→DJβ 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
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, as 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 TCRβα (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 vs 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 shown that cloned peripheral blood γδ T cells contained a low percentage of complete V→DJβ rearrangements. Second, and more importantly, the 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 spectrototyping 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 could 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

<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>

αβ Sequences correspond to Vδ1, Vδ2, and Vδ3 joins with Jβ1–4 gene segments, though most sequences contained Jβ1.

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 CD3+CD8-CD4+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 expressing 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.
TCRβς-expressing cells. Preliminary results revealed that these cells contained ~40% productive TCRγδ rearrangements (61 of 151 sequences), a finding that by χ2 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 χ2 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.

The finding that one-third of the TCRγ rearrangements in αβ thymocytes were in-frame raised the possibility that significant proportions of αβ cells might have the potential to express a functional γδ TCR. To address this issue and to assess the impact of TCRγ rearrangements on the entry of progenitors into the potential αβ pool, we analyzed the productivity of over 100 TCRδ rearrangements in sorted single αβ thymocytes. TCRδ rearrangements were significantly less productive (14%) than would be predicted by random chance (33%) (Table V), indicating a selective mechanism for the depletion of cells with in-frame δ rearrangements during human αβ thymocyte development. A similar situation occurs during murine αβ development (15, 26). To address the issue of whether any αβ thymocytes had the potential to express a functional γδ TCR, we analyzed the productivity of TCRγ gene rearrangements in those cells with productive δ rearrangements. Since at least 93% of human αβ thymocytes had nonproductive rearrangements at the γ and/or δ locus, we conclude that the vast majority of cells that develop into αβ thymocytes are those that could not express a functional γδ TCR. As in the mouse, these data indicate an important role for the expression of a functional γδ TCR 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 DNIII 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−CD4−CD8α−β− (EDP) stage (Fig. 5). Even though ~85% of EDP cells expressed TCRβς (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 TCRβς in human thymocyte populations. DN CD34−CD1a− cells were the first population to have a small percentage of cells expressing TCRβς (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-Tα protein expression is not appreciably detectable before the CD4 ISP stage (62), so it is possible that TCRβς expression is not synchronously linked to the expression of pre-TCR and β selection. We did find significant percentages of TCRβς+ 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 TCRβς+ cells in the DN CD34−CD1a− 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<sup>−</sup> had larger percentages of cells in the (S + G<sub>2</sub>-M) phases of the cell cycle compared with TCRβ<sup>+</sup> cells, consistent with their having passed β selection. The percentage of TCRβ<sup>−</sup> 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<sup>+</sup>CD8α<sup>−</sup>β<sup>+</sup>) to DP (CD4<sup>+</sup>CD8α<sup>−</sup>β<sup>−</sup>) 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<sup>+</sup>CD1a<sup>−</sup> 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 recombination 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 assays and retroviral transduction 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


FIGURE 6. Model for human αβ and γδ thymocyte development. Human αβ and γδ thymocytes develop from a common precursor. γδ developmental potential decreases with increasing maturation, but is retained until at least the EDP stage. Decreasing γδ potential is paralleled by increasing proportions of cells expressing TCRβ<sup>−</sup>. Thus, cells undergo β selection and commitment to the αβ lineage as they progress through several phenotypic stages (see Discussion for detailed explanation).


