New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling

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To gain more insight into initiation and regulation of T cell receptor (TCR) gene rearrangement during human T cell development, we analyzed TCR gene rearrangements by quantitative PCR analysis in nine consecutive T cell developmental stages, including CD34⁻ lin⁻ cord blood cells as a reference. The same stages were used for gene expression profiling using DNA microarrays. We show that TCR loci rearrange in a highly ordered way (TCRD-TCRG-TCRB-TCRA) and that the initiating D62-D63 rearrangement occurs at the most immature CD34⁻CD38⁻CD1a⁻ stage. TCRB rearrangement starts at the CD34⁺CD38⁻CD1a⁻ stage and complete in-frame TCRB rearrangements were first detected in the immature single positive stage. TCRB rearrangement data together with the PTCRA (pTα) expression pattern show that human TCRβ-selection occurs at the CD34⁺CD38⁻CD1a⁻ stage. By combining the TCR rearrangement data with gene expression data, we identified candidate factors for the initiation/regulation of TCR recombination. Our data demonstrate that a number of key events occur earlier than assumed previously; therefore, human T cell development is much more similar to murine T cell development than reported before.

T cells develop from progenitors that migrate from the bone marrow into the thymus (1). Thymocytes are subdivided roughly as being double negative (DN), double positive (DP), or single positive (SP), based on the expression of the CD4 and CD8 coreceptors (1). The DN stage is heterogeneous and can be subdivided into four distinct subsets in mice based on the expression of CD44 and CD25. In humans, three distinct DN stages can be recognized: a CD34⁺CD38⁻CD1a⁻ stage that represents the most immature thymic subset and the consecutive CD34⁺CD38⁺CD1a⁻ and CD34⁺CD38⁻CD1a⁺ stages. Human DN thymocytes mature via an immature single positive (ISP CD4⁺) and a DP stage into CD4⁺ or CD8⁺ SP T cells that express functional T cell receptors (TCR) and that exit the thymus (1).

A hallmark of T cell development is the generation of T cells that express a functional TCR, TCRαβ or TCRγδ. During T cell development, the variable domains of TCRβ, TCRγ, and TCRδ (located within TCRβ) genes are assembled following rearrangement of variable (V), diversity (D), and joining (J) gene segments by a process called V(D)J recombination (2). V(D)J recombination uses the RAG1 and RAG2 enzymes that selectively target recombination signal sequences that flank V, D, and J segments (2).

Studies in T cell acute lymphoblastic leukemias suggest that recombinations of TCR genes are sequential between the different genes (TCRD > TCRG > TCRB > TCRA) as well as within a particular gene (e.g., TCRD: D62-D63, D62-J61, V6-J61) (2, 3), which is supported by limited data that were obtained from normal human T cell subsets (4). Therefore, the timing and efficiency of rearrangement of
Figure 1. RQ-PCR analysis of TCRD and TCRG gene rearrangements in human T cell development and mature T cells. (A) Schematic diagram of the human TCRD gene complex. The six "classical" Vβ gene segments (blue) are scattered between Vα gene segments (black). Because
various TCR genes must be determined by the accessibility of gene segments to RAG enzymes. Evidence suggests that promoter and enhancer activity that is controlled by transcription factors regulate V(D)J recombination by modulating chromatin structures and rendering gene segments accessible to RAG cleavage (5, 6).

For obvious reasons T cell development mainly is studied in the mouse. Real-time quantitative PCR (RQ-PCR) and DNA microarray techniques allow careful analysis of small cell numbers. In this study we assessed the precise TCR gene configuration and the gene expression profiles of thymic subsets by RQ-PCR and Affymetrix DNA microarrays. By combining these two techniques we aimed for the identification of factors that play a role in regulating human TCR gene recombination.

RESULTS AND DISCUSSION

Definition of T cell populations

CD34+ lineage negative “stem cell-like” cells were obtained from umbilical cord blood (UCB) and CD34+CD38-CD1a-CD95-, CD34+CD38-CD1a-ISP, CD34+CD38-CD1a-ISP, CD34+CD38-ISP, CD34+CD1a-ISP, CD34+CD1a+, CD34+ISP, CD34+DP CD3+, SP CD4+, and ISP CD8+ subpopulations were obtained from thymi which represented consecutive stages of T cell development (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20042524/DC1). TCRαβ- and TCRγδ-expressing cells were obtained from thymic samples and peripheral blood mononuclear cells. Cells from five donors were pooled to reduce intrasample variation and all subsets were isolated twice from different donor pools.

Determination of TCR gene rearrangements by RQ-PCR and GeneScan analysis

TCR gene rearrangement analysis was performed in duplicate and on the two independently purified subsets (average is shown). The primers and TaqMan probes are listed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20042524/DC1) and do not amplify germline DNA.

TCRD

The first TCRγ rearrangements (Vγ-γ1.1/2.1) were detected in the CD34+CD38-CD1a- thymic subset, earlier than we reported previously (4), and one stage after the initiation of TCRD rearrangement. These rearrangements reached maximum levels of CD34+CD38+CD1a+ thymocytes and decreased to a relatively constant level in subsequent subsets (Fig. 1 G). Vγ-γ1.3/2.3 rearrangements were observed first in CD34+CD38-CD1a- cells after which they increased rapidly and by far exceeded Vγ-γ1.1/2.1 rearrangements from the ISP stage onward (Fig. 1 H). Peripheral TCRγδ- and TCRαβ+ T cells revealed lower Jy1.3/2.3 usage than their thymic counterparts (Fig. 1 H). In thymocytes, Jy1.2 was used at very low frequency, but massive positive selection for the Jy1.2 segment occurred in peripheral TCRγδ+ T cells (Fig. 1 I; reference 8).

TCRB

Dβ1-Jβ1 rearrangements were detected first at low levels in the CD34+CD38-CD1a- population and increased thereafter from CD34+CD38-CD1a- to CD4+ ISP cells (Fig. 2 B). Dβ2-Jβ2 rearrangements were detected first at low levels in CD34+CD38-CD1a- cells, one differentiation stage after Dβ1-Jβ1 rearrangements (Fig. 2 B). The seemingly lower levels of Dβ-Jβ in DP CD3+ probably are caused by variation within the lower range of detection of our assay and likely do not represent a true decrease.

Because of the complexity of the TCRβ locus, Vβ-Jβ rearrangements were determined by using nonquantitative

Vβ4, Vβ5, and Vβ6 also are recognized as Vα gene segments, their Vα gene code is given in parentheses (adapted from reference 9). (B) Analysis of Dβ2-Dβ3 rearrangement. (C) Analysis of Dβ4-Dβ3 rearrangement. (D) Analysis of Vβ1-Jβ1 and Vβ2-Jβ1 rearrangements. (E) Analysis of Vβ3-Vβ3 rearrangement. (F) Schematic diagram of the human TCRγ gene complex. Only the rearrangeable Vγ gene segments are depicted in blue (functional Vγ) or gray (nonfunctional Vγ). For the Jγ gene segments both nomenclatures are used (adapted from reference 9). (G) Analysis of Vγ to Jγ1.1 and Jγ2.1 rearrangements. (H) Analysis of Vγ to Jγ1.3 and Jγ2.3 rearrangements. (I) Analysis of Vγ to Jγ1.2 rearrangements.
Figure 2. RQ-PCR and GeneScan analysis of TCRB rearrangements in human T cell development and in mature T cells. (A) Schematic diagram of the human TCRB gene complex. (B) Analysis of DJβ to Jβ rearrangements. (C) Schematic diagram of VB-Jβ rearrangements as determined by GeneScan analysis.
GeneScan analysis (9). The CD34⁺CD38⁻CD1a⁺ thymocytes contained low levels of the first Vβ-Jβ rearrangements in which Jβ2 gene segments were used exclusively (Fig. 2 D), much earlier than we described before (4). From the ISP subset onward, Vβ-Jβ rearrangements were in-frame as shown by the triplet peaks (Fig. 2 E) and were retained throughout all subsequent stages of development (Fig. 2, E–H). Previous reports suggested that TCRβ-selection in humans is initiated at the ISP/DP stages of T cell development (4, 10, 11), but the three-nucleotide spacing of the peaks in our GeneScan analysis suggest that selection for in-frame TCRB already occurs at the transition from CD34⁺ CD38⁺CD1a⁺ to ISP. From the DP CD3⁻ fraction, Vβ-Jβ1 rearrangements also were present, although they were less abundant than Vβ-Jβ2 rearrangements (Fig. 2 F; tube A). For Vβ-Jβ rearrangements, usage of Jβ2 gene segments was preferred over Jβ1 (Fig. 2, D–H). TCRγδ⁺ thymocytes contained no Vβ-Jβ1 rearrangements but did contain Vβ-Jβ2 rearrangements (Fig. 2 H).

TCRA

Because of large numbers of rearrangeable Vα (~54) and Jα (6) gene segments (2), we could not design a multiplex RQ-PCR for reliable quantification of all Vα-Jα rearrangements. Instead, we aimed for an alternative approach in which we used different indirect measures to study TCRA recombination. TCRA recombination is initiated by the transcription of T-early α (TEA) in order to open the 5’ site of the Jα cluster, which is followed by TCRD deleting rearrangements, particularly the δREC-ψJα rearrangement. These initiating events are followed by multiple, consecutive Vα-Jα rearrangements (7, 12).

To study initiation of TCRA rearrangement we determined the level of TEA-Cα transcripts as well as the occurrence of δREC-ψJα rearrangements. TEA-Cα transcripts started to increase in CD34⁺CD38⁺CD1a⁺ cells and reached peak levels in ISP and DP cells after which they declined again (Fig. 3 B). δREC-ψJα rearrangements were detected first in ISP cells and reached peak levels in SP and TCRαβ⁺ thymocytes (Fig. 3 C). These data show that TCRA rearrangement already has started in the ISP cell population but that there are still cells within the CD3⁺ DP population that start rearrangement of the (most likely) second TCRA allele. Although TEA-Cα transcripts and δREC-ψJα rearrangements are good measures for initiation of TCRA rearrangement, they cannot be used for quantification of the actual TCRA rearrangements. TEA-Cα is an mRNA product that cannot be extrapolated simply to the actual level of TCRA rearrangements. Quantification of δREC-ψJα is complex because it is influenced strongly by ongoing Vα-Jα rearrangements and the consequently produced TREC'S (containing δREC-ψJα), whereas the amount of TREC'S (and their dilution) is heavily dependent on the fraction of proliferating cells within specific subsets. Therefore, extra accumulation of TREC'S may explain the relatively high δREC-ψJα levels in nonproliferating SP cells as compared with the preceding proliferating stages.

In an attempt to quantify TCRA recombination, we determined loss of germline TCRA DNA based on the disappearance of germline ψJα as an indirect measure for TCRA rearrangements. For this approach we used DNA from CD34⁺ lin⁻ UCB cells as 100% germline reference. Germline TCRA clearly declined from the CD34⁺CD38⁺CD1a⁺ stage onward with a major decline when ISP cells progress toward DP cells (Fig. 3 D). The low levels of germline TCRA in DP CD3⁻ cells indicate that extensive TCRA rearrangement has occurred at this stage. The apparent inconsistency with the relatively low levels of δREC-ψJα in these DP CD3⁻ cells can be explained by the fact that TREC'S that contain δREC-ψJα are diluted-out rapidly in these heavily proliferating cells and that other TCRD deleting rearrangements can occur as well, such as δREC-Jα58 and Vδ-ψJα (7). Our approach is only suitable for quantification of major decreases in germline TCRA, which occur at early stages of T cell development (ISP/CD3⁻ DP); however, it is much more difficult to measure additional TCRA rearrangements accurately at the later stages of T cell development because germline ψJα (e.g., due to δREC-Jα58 rearrangements) remains detectable on TREC'S and the difficulty of detecting further decreases within the 5–10% germline TCRA that is present in these stages.

We conclude that TCRA rearrangements are initiated when thymocytes progress from CD34⁺CD38⁺CD1a⁺ toward the ISP stage—which is much earlier than reported previously (10)—and apparently are ongoing until the CD3⁺ DP stage.

Microarray analysis

A total of 3848 probe sets underwent a significant change between any two successive stages of differentiation. Raw microarray data can be found at http://www.ebi.ac.uk/miamexpress, MIAME accession no. E-MEXP-337, and http://franklin.et.tudelft.nl, including a gene search browser. The expression levels of these probe sets were used to calculate a correlation coefficient between all possible pairs of microarrays and revealed high correlation between biological repeats (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20042524/DC1). This allowed us to use the average expression values of the two arrays that were performed per subset (obtained from five pooled thymi) for further analysis. Hierarchical clustering of the 3848 probe sets was performed and is described in Fig. S3 and Table S2 (available at http://www.jem.org/cgi/content/full/jem.20042524/DC1).
Of special interest for TCRβ-selection and initiation of TCRα rearrangement is that PTCRA (pTα) expression increased in the CD34+CD38+CD1a+ stage, peaked in the CD34+CD38+CD1a+ and ISP stages, after which it declined (Table S2). Mouse microarray data (13) have shown a similar expression pattern of pTα, with a minor peak at DN3 and a larger peak at the DP CD3- stage. Experiments with pTα mutant mice indicate that TCRβ-selection in the mouse occurs at DN3 (14), and that TCRα recombination is initiated after TCRβ-selection has occurred (14). Here, we show that initiation of TCRα recombination starts in CD34+CD38+CD1a+ cells. Therefore, the analogous pTα expression between mice and men, our TCRβ GeneScan, and our TCRα recombination data indicate that human TCRβ-selection occurs at the CD34+CD38+CD1a+ stage instead of the previously suggested ISP/DP stage (4, 10, 11).

To determine which genes may have a role in regulating TCR rearrangement, hierarchical cluster analysis was performed (Fig. 4 A). The 446 probe sets were divided into 15 clusters; the prototypic expression patterns are depicted in Fig. 4 B. Based on the TCRα rearrangement patterns (as described above) and the prototypic gene expression patterns, we propose that clusters 3, 5, 6, 7, 14, and 15 (Fig. 4 B, asterisk) contain genes that may encode candidate factors for initiation/regulation of TCR rearrangements because the genes that are present in these clusters show higher expression at the moments at which active TCR gene rearrangement occurs.

Figure 3. RQ-PCR analysis of initiating events around TCRα rearrangement in human T cell development and mature T cells. (A) Schematic diagram of the human TCRα gene complex. The TEA element (red) forms a sterile mRNA with Cα sequences. The TCRD deleting elements δRec and ψα are also indicated. (B) Analysis of TEA-Cα mRNA expression. Due to shortage of material, TEA expression was only determined once. (C) Analysis of the TCRD deleting rearrangement δRec-ψα. (D) Analysis of “remaining” TCRα germline DNA.

(transcriptional regulation and DNA binding) which yielded a final list of 446 probe sets that encoded a total of 361 genes (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20042524/DC1). Expression of genes that are associated with T cell commitment/differentiation and/or V(D)J recombination, such as NOTCH1, HES1, GATA3, BCL11B, RAG1, RAG2, and DNTT (TdT), increased strongly in early T cell differentiation.
We propose that clusters 3, 5, and 6 contain genes that are common for the rearrangement of all four TCR genes. Cluster 5 likely contains genes that are important for the regulation of $\text{TCRD}$, $\text{TCRG}$, and $\text{TCRB}$ rearrangements, whereas cluster 3 likely contains genes that need to be expressed at a higher level when $\text{TCRA}$ rearranges. These clusters contain $\text{DNTT}$ (cluster 6), $\text{RAG 1/RAG 2}$ (cluster 3), $\text{SMARCA4 (BRG1)}$ (clusters 6 and 3), and genes that encode factors, such as NOTCH1 (cluster 6), RORC (cluster 3), $\text{H2AFX}$ (cluster 3), that previously were linked to regulation of $\text{TCRG}$, $\text{TCRB}$, or $\text{TCRA}$ rearrangements (15–18).

Genes in cluster 7, 14, and 15, such as $\text{SPIB}$, $\text{ICSBP1}$, $\text{TCF4}$, $\text{CREB1}$, $\text{ETS1}$, and $\text{LEF-1}$, may encode factors that are involved in regulating $\text{TCRA}$ rearrangements as well as allelic exclusion of the $\text{TCRB}$ locus. These are discussed in the supplemental Results and Discussion (available at http://www.jem.org/cgi/content/full/jem.20042524/DC1).

Novel insights into human T cell development

We confirm that TCR loci rearrange in a highly ordered way ($\text{TCRD}$-$\text{TCRG}$-$\text{TCRB}$-$\text{TCRA}$) and defined sequential rearrangement steps of $\text{TCRD}$, $\text{TCRG}$, $\text{TCRB}$, and initiation of $\text{TCRA}$ recombination to specific human thymic subsets. Importantly, our data show that recombination of the TCR genes occurs earlier during human T cell development than previously reported (4, 10). Given that $\text{TCRD}$ rearrangement starts at DN1 in mice, followed by $\text{TCRG}$ in DN2 and $\text{TCRB}$ in DN2, but especially DN3 (1, 19), the human $\text{CD34}^-\text{CD38}^-\text{CD1a}^-$, $\text{CD34}^-\text{CD38}^+\text{CD1a}^-$, and $\text{CD34}^+\text{CD38}^-\text{CD1a}^+$ subsets resemble murine early DN1 (CD44$^+$CD25$^-$CD117$^+$), late DN1/DN2 (CD117$^+$DN1; CD44$^+$CD25$^+$), and DN3 (CD44$^-$CD25$^+$) stages, respectively. However, the relative frequency of DN1 cells in mice is higher than that of the corresponding human subset (CD34$^+$CD38$^-$CD1a$^-$). We also demonstrate that TCRβ-selection and initiation of $\text{TCRA}$ rearrangement already occur at the CD34$^+$CD38$^+$CD1a$^+$ stage of human T cell development, instead of the ISP/DP stage (4, 10, 11), similar to the mouse (i.e., TCRβ-selection occurs at DN3 in the mouse; reference 14). Based on the TCR rearrangement data and the expression profile of key recombination and differentiation genes (e.g., $\text{RAG1}$, $\text{RAG2}$, and $\text{PTCRA}$), we show that human and mouse T cell development are much more similar than assumed previously. In addition, candidate factors for regulation of TCR recombination are identified.

We propose an updated human T cell differentiation model as shown in Fig. S5 (available at http://www.jem.org/cgi/content/full/jem.20042524/DC1). These novel data help

Figure 4. Analysis of probe sets related to transcriptional regulation. (A) Hierarchical clustering of 446 probe sets differentially expressed over the various stages and related to transcriptional regulation and DNA binding. (B) z-score trend representation for each cluster deduced from the 446 probe sets. Cluster codes (1–15) are shown in the top right corner, z-score on the y axis, subsets on the x axis. The gray area represents 1 SD. The asterisk in the top right corner indicates clusters that contain genes that are potentially important in regulating TCR rearrangements. N, indicates the number of probe sets in a cluster.
Table I. Transcription and DNA-binding–related genes with expression profiles that correlate with TCR gene rearrangements in human T cell development

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Materials and Methods

Purification of thymocyte subsets, CD34+ lineage- cells, and mature peripheral T cells. For the isolation of CD34+ Lin- UCB cells and thymocyte subsets, total mononuclear cells or thymocytes from five donors were pooled to reduce intrasample variation. CD34+ subsets were purified by magnetic-activated cell-sorted beads and further purified by cell sorting to >96% purity.

RQ-PCR analyses of TCR gene rearrangements. This essentially was performed as described previously (20). For the various TCR recombinations, cell lines/diagnostic samples were selected as clonal control DNA.

GeneScan analysis for complete and in-frame Vβ-Jβ gene rearrangements. This was done by two multiplex PCR reactions as described by the BIOMED-2 Concerted Action (9).

TEA expression by RQ-PCR analyses. Expression of TEA was defined by calculating the ratio of TEA to the average value of the control genes ABL and GUSB.

Microarray analysis. Affymetrix microarray analysis essentially was done as described previously (21) and according to Minimum Information About a Microarray Experiment guidelines (www.mged.org/Workgroups/MIAME/miame.html).

Statistical analysis. Probe intensity background was removed using robust multichip analysis (22). The intensity levels were quantile normalized.
using Genlab software, running under Matlab (http://www.genlab.tudelft.nl). After per-probe set normalization to zero mean and unit standard deviation (z-score), a hierarchical clustering (complete linkage) was calculated based on Pearson correlation. The number of clusters k was determined by looking for a local minimum of the Davies-Bouldin index calculated for $k = 1, \ldots, 30$ (25).

### Online supplemental material
All methods are described in more detail as supplemental Materials and methods online. Additional Results and Discussion, as well as Figs. S1–S5 and Tables S1–S3, are provided online, as well as supplemental Materials and methods online. Additional Results and Discussion, as well as Figs. S1–S5 and Tables S1–S3, are provided online, as well as supplemental Materials and methods online.

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