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 human, 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+CD38−), 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 TCRα, TCRβ, TCRγ, and TCRδ (located within TCRα) 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 TCR genes during normal human T cell development have not been studied in detail.

W.A. Dik and K. Pike-Overzet contributed equally to this work.

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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-, CD34+CD38-CD1a+, CD34+CD38-CD1a+, ISP CD4+, DP CD3-, DP CD3+, SP CD4+, and SP 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 D82-D83 rearrangement was detected first in the earliest CD34+CD38-CD1a- thymic subset, much earlier during development than in our previous Southern blotting–based report (4). D82-D83 rearrangements reached maximum levels in the subsequent CD34+CD38-CD1a- stage and declined to low levels in the later subsets (Fig. 1 B). The initial D82-D83 wave was followed by D82-Jβ1 rearrangements which were detectable at low levels in CD34+CD38-CD1a- cells, increased in CD34+CD38-CD1a- cells, and peaked in CD34+CD38-CD1a cells (Fig. 1 C). Complete Vβ1-Jβ1 or Vβ2-Jβ1 rearrangements were detected first in CD34+CD38-CD1a- cells, increased to peak levels in CD4+ ISP cells, and declined thereafter when the majority of thymocytes differentiated further into the TCRαβ lineage (Fig. 1 D). During TCRαβ T cell lineage development, the TCRD gene is deleted from the TCRα/D gene complex (2, 7) which results in the formation of a T cell receptor excision circle (TREC) that may contain Vβ-Jβ1 rearrangements. TRECs do not replicate on cell division; consequently, they are diluted out in proliferating, developing T cells (2). Ki67 staining demonstrated high percentages of proliferating cells within the human ISP and DP CD3- thymic subsets (unpublished data). This proliferation likely accounts for the observed decline in Vβ-Jβ1 rearrangements after the ISP stage. The Vβ1 gene segment seemed to be used preferentially in postnatal TCRγδ+ thymocytes which is in sharp contrast to the well-described preferential Vβ2 usage by peripheral TCRγδ+ T cells (Fig. 1 D; reference 8). We detected very low V6-DB3 levels in the thymic subsets; this represents a minor pathway to initiate TCRD gene rearrangement in postnatal thymus (Fig. 1 E).

TCRG

The first TCRG rearrangements (Vγ-Jγ1.1/2.1) 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 peaked in CD34+CD38+CD1a+ thymocytes and decreased to a relatively constant level in subsequent subsets (Fig. 1 G). Vγ-Jγ1.3/2.3 rearrangements were observed first in CD34+CD38-CD1a- cells after which they increased rapidly and by far exceeded Vγ-Jγ1.1/2.1 rearrangements from the ISP stage onward (Fig. 1 H). Peripheral TCRγδ- and TCRαβ+ T cells revealed lower Jγ1.3/2.3 usage than their thymic counterparts (Fig. 1 H). In thymocytes, Jγ1.2 was used at very low frequency, but massive positive selection for the Jγ1.2 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 TCRB 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 Dβ2-Jβ1 rearrangement. (D) Analysis of Vβ1-Jβ1 and Vβ2-Jβ1 rearrangements. (E) Analysis of Vβ3-Dβ3 rearrangement. (F) Schematic diagram of the human TCRG 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 Vβ-Jβ rearrangements as deter-
GeneScan analysis (9). The CD34+CD38+CD1a+ thymocytes contained low levels of the first VB-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, VB-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, VB-Jβ1 rearrangements also were present, although they were less abundant than VB-Jβ2 rearrangements (Fig. 2 F; tube A). For VB-Jβ rearrangements, usage of Jβ2 gene segments was preferred over Jβ1 rearrangements but did contain VB-Jβ2 rearrangements (Fig. 2 H).

TCRA

Because of large numbers of rearrangable Vα (~54) and Jα (61) 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 extraplated 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 (containing δREC-ψJα), whereas the amount of TREC (and their dilution) is heavily dependent on the fraction of proliferating cells within specific subsets. Therefore, extra accumulation of TREC 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+CD1a– 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 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 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

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 (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 TCRA rearrangement is that PTCA (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 TCRA recombination is initiated after TCRβ-selection has occurred (14). Here, we show that initiation of TCRA rearrangement starts in CD34⁺CD38⁺CD1a⁺ cells. Therefore, the analogous pTα expression between mice and men, our TCRC geneScan, and our TCRA recombination data indicate that human TCRβ-selection occurs at the CD34⁺CD38⁺CD1a⁺ stage instead of the previously suggested ISP/DN stage (4, 10, 11).

To identify candidate transcription factors that are involved in regulating TCR recombination, the list of 3848 probe sets was filtered based on Gene Ontology annotation (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.

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 TCR 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. Genes that are
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 TCRD, TCRG, and TCRB rearrangements, whereas cluster 3 likely contains genes that need to be expressed at a higher level when TCRA rearranges. These clusters contain DNTT (cluster 6), RAG 1/RAG 2 (cluster 3), and genes that encode factors, such as NOTCH1 (cluster 6), RORC (cluster 3), SMARCA4 (BRG1) (clusters 6 and 3), H2AFX (cluster 3), that previously were linked to regulation of TCRG, TCRB, or TCRA rearrangements (15–18).

Genes in cluster 7, 14, and 15, such as SPIB, ICSTBP1, TCF4, CREB1, ETS1, and LEF-1, may encode factors that are involved in regulating TCRA rearrangements as well as allelic exclusion of the 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 (TCRD-TCRG-TCRB-TCRA) and defined sequential rearrangement steps of TCRD, TCRG, TCRB, and initiation of 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 TCRD rearrangement starts at DN1 in mice, followed by TCRG in DN2 and TCRB in DN2, but especially DN3 (1, 19), the human CD34+/CD38+/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 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., RAG1, RAG2, and 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
to bridge gaps in our understanding of human T cell development, and also should provide insight into the development of T cell acute lymphoblastic leukemias and T-SCID.

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 prepurified 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.

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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(23). Array groups (two biologically independent arrays per group) that corresponded to the development stages were compared based on the perfect match probe intensity levels only (22), by performing a per-probe set two-way analysis of variance (with factors “probe” and “stage”). The p-values were adjusted for multiple testing using Šidák step-down adjustment (24) and all differences with adjusted p-values <0.05 were considered to be significant. For Fig. S2, expression values were log₂-transformed and the per-probe set geometric mean was subtracted. Further analysis was performed 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, . . . , 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. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042524/DC1.

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