

## BRIEF REPORT

# Reconstruction of rearranged T-cell receptor loci by whole genome and transcriptome sequencing gives insights into the initial steps of T-cell prolymphocytic leukemia

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## Abstract

T-cell prolymphocytic leukemia (T-PLL) is an aggressive tumor with leukemic presentation of mature T-lymphocytes. Here, we aimed at characterizing the initial events in the molecular pathogenesis of T-PLL and particularly, at determining the point in T-cell differentiation when the hallmark oncogenic events, that is, *inv(14)(q11q32)/t(14;14)(q11;q32)* and *t(X;14)(q28;q11)* occur. To this end, we mined whole genome and transcriptome sequencing data of 17 and 11 T-PLL cases, respectively. Mapping of the 14q32.1 locus breakpoints identified only *TCL1A*, which was moreover

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significantly overexpressed in T-PLL as compared to benign CD4+ and CD8+ T-cells, as the only common oncogenic target of aberrations. In cases with t(14;14), the breakpoints mapped telomeric and in cases with inv(14) centromeric or in the 3'-untranslated region of *TCL1A*. Regarding the T-cell receptor alpha (TRA) locus-*TCL1A* breakpoint junctions, all 17 breakpoints involved recombination signal sequences and 15 junctions contained nontemplated (N-) nucleotides. All T-PLL cases studied carried in-frame TRA rearrangements on the intact allele, which skewed significantly toward usage of distal/central TRAV/TRAJ gene segments as compared to the illegitimate TRA rearrangements. Our findings suggest that the oncogenic TRA-*TCL1A*/*MTCP1* rearrangements in T-PLL occur during opening of the TRA locus, that is, during the progression from CD4+ immature single positive to early double positive thymocyte stage, just before physiologic *TCL1A* expression is silenced. The cell carrying such an oncogenic event continues maturation and rearranges the second TRA allele to achieve a functional T-cell receptor. Thereafter, it switches off *RAG* and *DNTT* expression in line with the mature T-cell phenotype at presentation of T-PLL.

#### KEYWORDS

cell-of-origin, T-cell prolymphocytic leukemia, transcriptome sequencing, TRA-*TCL1A*, whole genome sequencing

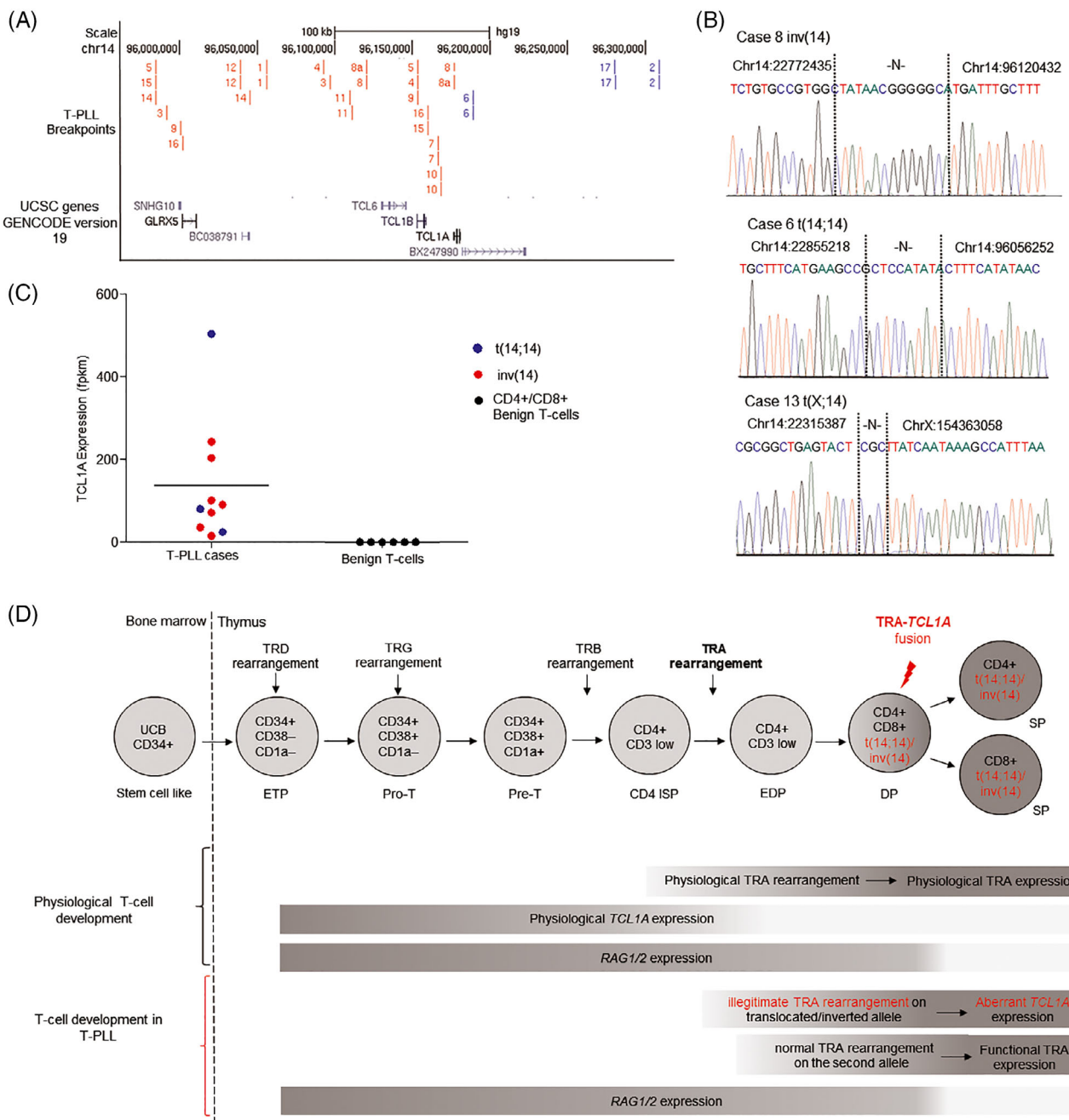
T-cell prolymphocytic leukemia (T-PLL) is a rare T-cell leukemia affecting patients with an average age at diagnosis of 65 years. According to the *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissue*, the postulated normal counterpart of the tumor cell in T-PLL is “an unknown T-cell with a mature (post-thymic) immunophenotype. Strong CD7 and co-expression of CD4 and CD8 and weak membrane CD3 may suggest that T-PLL arises from a T-cell at an intermediate stage of differentiation between cortical thymocytes and mature T lymphocytes.”<sup>1</sup> Here, we challenged this postulate by using somatic genetic analyses rather than gene or protein expression data.

The genetic hallmark of T-PLL is an inv(14)(q11q32) or t(14;14)(q11;q32) involving the T-cell receptor (TCR) alpha/delta gene locus (TRA/TRD) at 14q11 and the *TCL* gene cluster at 14q32.1. Few T-PLL cases harbor an alternative translocation t(X;14)(q28;q11) involving, besides TRA/TRD, the *MTCP1* gene at Xq28 which encodes a paralog of *TCL1A*.<sup>2-4</sup> Both the TRA/D-*TCL1A* and TRA/D-*MTCP1* fusions are considered as disease initiating “primary” events in T-PLL. Translocations and inversions involving TCR loci are generally believed to result from aberrant V(D)J recombination events and lead to activation of oncogenes that are juxtaposed to the TCR locus enhancer elements.<sup>5</sup> TCR rearrangements occur in a sequential and coordinated manner during both normal and leukemic T-cell development.<sup>6</sup> Therefore, genomic characterization of TCR translocations can provide an insight into the timing of such oncogenic events.

To identify the cell in which the hallmark translocation/inversion involving TRA/TRD in T-PLL occurs, we investigated the genomic composition of the functional and illegitimate TRA/TRD rearrangements in a series of bona-fide T-PLL using an unbiased approach. In particular, we generated whole genome sequencing (WGS) data of 13 T-PLL

cases and supplemented these with transcriptome sequencing (RNA-seq) of 11 of these 13 T-PLL cases. Moreover, we mined WGS data from four previously published cases of T-PLL<sup>7</sup> (Supporting Information Table 1). These four previously published cases also contained one of the 13 cases in which we herein independently generated WGS data (labeled as case #8 and #8a). Finally, we performed WGS and RNA-seq in the cell line SUP-T11 previously shown to carry a TRA/TRD-*TCL1A* juxtaposition leading to *TCL1A* overexpression.<sup>8</sup> This cell line has been reported to derive from a patient with T-ALL. Nevertheless, it lacks expression of markers for a precursor phenotype (TdT, CD34, CD1a) as well as of CD4, CD8 and TRG/D whereas other T-cell markers (sCD2, sCD3 and sCD7 and TRA/B) can be readily detected. These phenotypic data suggest that the diagnosis of T-PLL needs to be considered as differential to T-ALL (Supporting Information Figure 1). Thus, a total of 17 cases with WGS data entered the analyses (for details on patient cohort see Supporting Information Tables 1 and 2). In addition, to compare gene expression in T-PLL to normal peripheral T-cells, we performed RNA-seq on FACS-sorted CD4+ and CD8+ T-cell populations obtained from three separate blood donors each (Supporting Information Figure 2). The study was performed in agreement with the guidelines of the Institutional Review Boards of Christian-Albrecht-University Kiel (B295/11) and Ulm University (21/16).

Firstly, we focused on the architecture of illegitimate TRA/TRD rearrangements leading to the hallmark aberrations. Using WGS data we identified an inv(14)(q11q32) in 13/17 cases, t(14;14)(q11;q32) in 3/17 cases (including SUP-T11) and t(X;14)(q28;q11) in 1/17 cases. We detected two breakpoints (one on the Watson and one on the Crick strand based on both derivatives/breakpoints) at the TRA/D locus and *TCL1A*/*MTCP1* gene locus for all the cases (Figure 1A,



**FIGURE 1** Oncogenic rearrangements in T-PLL and their effect on oncogene expression. A, Within the UCSC genome browser (GENCODE version 19), track bars indicate breakpoint locations at the 14q22 locus in T-PLL cases ( $n = 16$  without case 13 with  $t(X;14)$ ) and SUP-T11. Cases with  $inv(14)$  are indicated in red and cases with  $t(14;14)$  in blue (T-PLL Breakpoints track). B, Examples of Sanger sequences verifying  $inv(14)$ ,  $t(14;14)$  and  $t(X;14)$  fusion sequences in three T-PLL cases. The dotted lines mark the breakpoints. The -N- indicates the N-nucleotides inserted at the fusion junction of the sequences. C, Dot-plot showing the RNA expression values in fPKM (fragments per kilobase of transcript per million mapped reads) of *TCL1A* which is significantly higher expressed in seven T-PLL cases harboring  $inv(14)$  (red dots) and two T-PLL cases and SUP-T11 harboring  $t(14;14)$  (blue dots) as compared to benign CD4+ and CD8+ T-cells ( $n = 6$ ) (black dots) ( $P = 0.001$ , Mann-Whitney test). D, Schematic representation of differentiation stages of the physiological thymocyte development in humans and of the proposed thymocyte development in T-PLL. Stages in which the TRD, TRG, TRB and TRA rearrangements occur are indicated by arrows. The stages at which *TCL1A* and *RAG* genes are expressed physiologically in normal T-cells and aberrantly after the occurrence of hallmark alteration in T-PLL have been indicated with gradient colors, respectively (white: no expression, dark gray: high expression). Similarly, physiologic and the illegitimate TRA rearrangements and consequent TRA expression occurring in normal T-cells and T-PLL, respectively, have been indicated by the gradient colors. The TRA-*TCL1A* fusion most likely occurs in double positive cortical thymocyte stage as highlighted in red. CD4 ISP, CD4+ immature single positive; DP, double positive; EDP, early double positive; ETP, early T-cell precursor; SP, single positive

Supporting Information Figure 3). The findings in case #8 subjected to WGS independently twice were in complete agreement. Nevertheless, we verified the fusion sequences from WGS by polymerase chain reaction-based Sanger sequencing in 14/17 T-PLL samples (including SUP-T11), excluding the already published cases<sup>7</sup> (Supporting Information Tables 3 and 4). We could detect and validate both breakpoints in 11/14 cases and one out of the two breakpoints in the remaining three (cases #6, #10 and #12) (Figure 1B, Supporting Information Table 5). With regard to the fusion sequences in 14q32.1, the breakpoint junctions of 12/13 T-PLL cases carrying an *inv(14)* were located centromeric of *TCL1A* (ENST00000554012.1) at a median distance of 41719 bp (range 5745-132339 bps). In the remaining case (#8 or #8a) with *inv(14)*, we observed a breakpoint in exon 4 which disrupted the 3'-untranslated region (3'UTR) of *TCL1A*. In contrast, the breakpoints of three T-PLL cases with *t(14;14)* cases mapped 7634-128460 bp telomeric of *TCL1A* (Figure 1A, Supporting Information Figure 4). Remarkably, both breakpoint clusters of *inv(14)* and *t(14;14)* leave only *TCL1A* as an oncogenic target common to all these changes, as transcriptional units neighboring *TCL1A* were not consistently juxtaposed to the TRA/TRD enhancers in all cases. In line with the genomic findings, RNA-seq data available in 10 T-PLL cases with *inv(14)* or *t(14;14)* showed significantly higher *TCL1A* transcript levels as compared to benign CD4+ and CD8+ T-cells ( $P = 0.001$ , Mann-Whitney test) (Figure 1C, Supporting Information Figure 5). Both breakpoints on Xq28 in the T-PLL case with *t(X;14)* mapped to intron 1 of *MTCP1* (ENST00000362018.2) (Supporting Information Figure 6). *MTCP1* overlaps with *CMC4* and both were found to be higher expressed in the T-PLL case with *t(X;14)* as compared to CD4+ and CD8+ benign T-cells (Supporting Information Figure 6).

These genomic and transcriptomic findings corroborate the consistent deregulation of the *TCL1A* and *MTCP1* oncogenes in T-PLL, in line with previous studies.<sup>9,10</sup> As juxtaposition of oncogenes to TCR or immunoglobulin (IG) loci by an illegitimate TCR/IG rearrangement is usually supposed to result in deregulated transcription of respective oncogene, it is notable that the physiological expression of *TCL1A* during T-cell development is observed up to CD4+ immature single positive (CD4 ISP) stage, whereas it is silenced in later stages of T-cell development<sup>4,7</sup> (Figure 1D).

To gain further insights into the T-cell maturation stage at which the *TCL1A/MTCP1* inversions/translocations occur we investigated the junctional sequences of the illegitimate rearrangements leading to the oncogenic fusions at the TRA/TRD locus in 14q11. We observed that all 17 T-PLL cases (including SUP-T11) have a breakpoint at a recombination signal sequence (RSS) of the J region of TRA (JA) and juxtapose the TRA enhancer (Ea) in cis to the *TCL1A/MTCP1* gene(s). In addition, we detected nontemplated ("N") nucleotides at the fusion junction of 15/17 cases (Supporting Information Table 5). Moreover, we observed that the breakpoints were more variable on the other side of the inversion/derivative chromosomes: one case breaks at TRAJ58, one case breaks at TRAJ61, seven cases break at a TRAV, one breaks at a hybrid TRAV23/DV6, three break at a TRDV2 segment, one breaks at a TRDD3 and three break between TRAV41 and TRDV2, which are located adjacent to each other. Interestingly, case

#9 with a breakpoint in TRDD3 (delta diversity 3) had a second breakpoint in TRAJ61 on the other allele. This JA gene segment is the most proximal to the TRD locus. With the exception of breaks in three cases (#1, #2, #4), also these junctions mapped close to a classical RSS. The breakpoints in these three cases were located between TRAV41 and TRDV2 (Supporting Information Table 5). Two clustered within 10 bp and one was at a distance of approximately 2 kb from the 5' end of the TRA/D gene cluster, respectively. We searched for the presence of a cryptic RSS at the proximity of the breakpoints between TRAV41 and TRDV2. Using the RSS database CNR-ITB,<sup>11,12</sup> we found that case #2 breaks at the junction of cryptic RSS 23 (cRSS-23) and that the case #1 breakpoint is 12 bp upstream of the same cRSS-23, whereas the breakpoint in case #4 is 85 bp upstream of another cRSS-23 (Supporting Information Table 6). These findings strongly suggest involvement of the RAG enzyme, especially in cases #1 and #2, targeting noncanonical RSS found in the region between TRAV41 and TRDV2. Interestingly, both were RSS-23 (rather than RSS-12), which are used by TRAV and TRDV gene segments. Finally, we investigated the expression of the TRD locus on the basis of RNA-seq data. We did not identify significant expression from any of the V(D)J gene segments of the TRD locus in the T-PLL cases. This finding is in agreement with the disruption of the TRD locus as a result of TRA locus rearrangement. Physiological TRD locus rearrangement in T-cell development starts at the early T precursor (ETP) thymic stage. After TRD rearrangement the cell proceeds to the pro and pre-T cell stage and later to the CD4 ISP stage<sup>13</sup> (Figure 1D). Our data indicate that the translocation/inversion events involving *TCL1A/MTCP1* in T-PLL occur at the point when the TRA locus opens or becomes accessible for VJ rearrangement, which leads to the excision of the TRD locus. This is the stage when the thymocyte proceeds from the double negative pre-T cell stage and develops via CD4 ISP and early double positive (EDP) stage into the double positive (DP) cortical stage of T-cell maturation.<sup>13,14</sup> Remarkably, at this stage some physiologic *TCL1A* expression is likely also maintained before the thymocyte silences the expression of this gene at later stages of T-cell development. As outlined above, oncogene expression is supposed to be a prerequisite for such enhancer hijacking events like *inv(14)/t(14;14)* in lymphatic neoplasms. Thus, the occurrence of the hallmark event coincides with and prevents physiological down-regulation of *TCL1A* (Figure 1D).<sup>7</sup>

The illegitimate TRA/TRD rearrangements in T-PLL prevent expression of a functional TCR alpha or delta in tumor cells from the affected allele. Taking into consideration that T-PLL cells nevertheless not only express a functional T-cell receptor but also seem to rely on that,<sup>15-17</sup> we investigated the second allele of chromosome 14 in which the TRA/TRD locus remains unaffected by the cancer-related aberration. As outlined above, we failed to detect significant expression of TRD V and J segments in the analyzed T-PLL cases. Thus, we studied the configuration of the normally rearranged TRAV and TRAJ by investigating the expression at the TRA locus using the RNA-seq data and combining this information with genomic data for the TRA segments expressed in T-PLL cases (Supporting Information methods). Having thus reconstructed the message for the expressed TRA, we compared the usage of TRAV/TRAJ gene segments in the

physiological rearrangement and those detected in the translocations/inversions. In the functional TRA rearrangements, the TRAV/TRAJ gene segments are generally located distal or central, with just a few being located proximal in the locus (Table 1). However, as detailed above, in the illegitimate rearrangements the affected segments are generally proximal with even one case that uses TRDD3 gene segment and the most proximal TRAJ61 gene segment during recombination (Table 1). Remarkably, this difference between the usages of distal/central gene segments in normal TRA rearrangements as compared to proximal gene segments in inversions/translocations is significant ( $P < 0.05$ , Fisher's exact test) (Supporting Information). These findings suggest that illegitimate TRA/TRD translocations/inversions occur at first opening of the TRA locus and that the T-PLL (primed) cells use the most proximal TRAV/J gene segments. As these do not result in a productive TRA due to the oncogenic aberrations, the TRAV-J rearrangements take place on the other allele involving progressively more distal TRAV/J gene segments, until a productive rearrangement occurs. To assess the functionality of the TRA derived from the physiologic rearrangement of the second allele, we finally

investigated this for any mutations in the rearranged TRA gene segments. We found that none of the nucleotides was mutated in TRAV and TRAJ and that the rearranged sequences were in frame; thus, there was no hint for deleterious changes in the expressed TRA (Supporting Information Table 7), in keeping with expression of a surface TRA protein. Hence, we conclude that T-PLL cells express an intact T-cell receptor alpha chain derived from TRA rearrangements taking place after the illegitimate TRA rearrangement resulting in oncogenic activation.

As the T-PLL (primed) cell is obviously able to further recombine its TCR, we wondered whether traces of this process can be observed in T-PLL. To this end, we investigated the available RNA-seq data for expression of the members of the RAG complex (RAG1 and RAG2).<sup>18,19</sup> In line with previous data,<sup>13,15</sup> we found that the T-PLL cases investigated and carrying the hallmark aberrations are devoid of RAG1 (mean expression of 0.1 fpkm, range 0-0.1), RAG2 (mean expression of 0 fpkm, range 0-0) and DNMT (mean expression of 0 fpkm, range 0-0) gene expression. The latter codes for terminal deoxynucleotide transferase (TdT), which is widely expressed in ALL,

**TABLE 1** Analysis of the TRA locus on the intact and inverted/translocated allele of the T-PLL cases with available RNA-seq and WGS data (the rearranged TRAV and TRAJ segments are listed)

Allele	Case	TRAV gene segment			TRAJ gene segment			
		Gene segment	Deleted nucleotides	Localization of gene segment	Gene segment	Deleted nucleotides	Localization of gene segment	
Normal allele	Case #1	TRAV1-2	0	Distal ++	TRAJ18	4	Distal	
	Case #2	TRAV22	5	Central	TRAJ48	0	Central	
	Case #6	TRAV14DV4 <sup>#</sup>	9	Central/distal	TRAJ29	4	Central	
	Case #7	TRAV12-2	3	Distal	TRAJ42	3	Central	
	Case #8	TRAV6	1	Distal	TRAJ43	1	Proximal/central	
	Case #9	TRAV12-1	1	Distal	TRAJ13	0	Distal	
	Case #10	TRAV29DV5 <sup>#</sup>	3	Proximal/central	TRAJ10	4	Distal	
	Case #11	TRAV8-6	0	Central/distal	TRAJ11	7	Distal	
	Case #12	TRAV12-1	0	Distal	TRAJ31	5	Central	
	Case #13	TRAV9-2	4	Central/distal	TRAJ9	4	Distal	
	SUP-T11	TRAV25	5	Central	TRAJ40	2	Central	
	Inverted/translocated allele	Case #1	TRAV41DV2 <sup>#</sup>	0	Proximal ++	TRAJ58	2	Proximal ++
		Case #2	TRAV41DV2 <sup>#</sup>	0	Proximal ++	TRAJ61	0	Proximal ++
Case #6		TRAV38-1	2	Proximal	TRAJ52	8	Proximal	
Case #7		TRAV23DV6 <sup>#</sup>	1	Central	TRAJ46	2	Proximal	
Case #8		TRAV39	3	Proximal	TRAJ49	0	Proximal	
Case #9		TRDD3	1	TRDD3	TRAJ61	0	Proximal ++	
Case #10		TRAV35	15	Proximal	TRAJ28	1	Central	
Case #11		TRAV21	3	Central	TRAJ23	10	Central	
Case #12		TRAV10	8	Distal	TRAJ31	11	Central	
Case #13		TRAV8-4	15	Distal	TRAJ17	8	Distal	
SUP-T11		TRAV22	3	Central	TRAJ42	18	Proximal/central	

Note: #: V gene segment shared between TRA and TRD; Localization of gene segment: Localization of the recombined gene segment within the TRAV or TRAJ locus, proximal TRAV: proximal gene segment to TRAJ, distal TRAV: distal gene segment to TRAJ; proximal TRAJ: proximal gene segment to TRAV, distal TRAJ: distal gene segment to TRAV, distal ++: very distal TRAV/TRAJ gene segments.



but not T-PLL. We thus conclude that in T-PLL, the genes involved in TRA recombination are activated while the illegitimate TRA VJ rearrangement occurs, remain active during several rounds of recombination until the T-cell achieves its functional rearranged TRA and are switched off thereafter (Figure 1D).

In the present study, using WGS-based and breakpoint analysis of physiologic and illegitimate TRA rearrangements combined with whole transcriptomic expression profiling we have identified the potential time-point when the hallmark oncogenic event in T-PLL occurs. We have shown that T-cells in T-PLL go through normal early stages of thymocyte development where physiological rearrangements of TCR gamma, delta and beta gene rearrangements take place in the early T precursor (ETP), pro and pre-T cell stage, respectively. When the cell reaches the double positive (DP) stage at which the TRA locus becomes accessible, an illegitimate TRA rearrangement takes place as a result of an inversion/translocation activating the oncogenes *TCL1A/MTCP1*. At this stage, the thymocyte subsequently goes through multiple rounds of TRA rearrangements in the presence of RAG enzymes until it undergoes a rearrangement which produces a functional TCR alpha chain that is required by the T-PLL cells to survive and proliferate. Thereafter, the poised T-PLL cell seems to be able to progress through T-cell maturation steps including switching off its RAG machinery. These observations also reflect a requirement for TCR expression in T-PLL, since these leukemias could formally have a cortical thymic, sCD3 negative phenotype, with no surface TCR expression, if *TCL1* driven oncogenesis were sufficient for leukemic development in the absence of TCR expression. This is similar to the requirement for TCR expression in NPM-ALK driven anaplastic large cell lymphoma.<sup>20</sup> In contrast, *TCL1*-TCR rearrangements in (mainly sTCR negative) T-ALL are extremely rare and virtually never involve TRA.<sup>21</sup>

Thus, the present study provides detailed insights into the sequential genomic alterations that lead to and follow from hallmark oncogenic event in T-PLL. Nevertheless, it needs to be emphasized that our study does not claim that these are the "initial" steps of T-PLL pathogenesis, as this would require chronologic alignments of illegitimate TRA rearrangements with *ATM* mutations, since the latter occur in the vast majority of T-PLL.<sup>7,22</sup> The increased incidence of T-PLL in patients with germline mutations in *ATM*<sup>23</sup> and the function of the ATM protein in repair of RAG-mediated DNA breaks might suggest that ATM dysfunction, whether encoded by germline or somatic genetic changes or due to stochastic alterations in expression, might contribute to the occurrence of illegitimate TRA rearrangements in T-PLL.<sup>17,24</sup> This would suggest a model in which *ATM* alterations take place before TRA rearrangements in T-PLL, which needs to be tested in future studies.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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