Breakpoint sites disclose the role of the V(D)J recombination machinery in the formation of T-cell receptor (TCR) and non-TCR associated aberrations in T-cell acute lymphoblastic leukemia

Nicole S.D. Larmonie,[1] Willem A. Dik,[1] Jules P.P. Meijerink,[2] Irene Homminga,[2] Jacques J.M. van Dongen,[1] Anton W. Langerak[1]*

[1]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam; [2]Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Rotterdam, The Netherlands

ABSTRACT

Aberrant recombination between T-cell receptor genes and oncogenes gives rise to chromosomal translocations that are genetic hallmarks in several subsets of human T-cell acute lymphoblastic leukemias. The V(D)J recombination machinery has been shown to play a role in the formation of these T-cell receptor translocations. Other, non-T-cell receptor chromosomal aberrations, such as *SIL-TAL1* deletions, have likewise been recognized as V(D)J recombination associated aberrations. Despite the postulated role of V(D)J recombination, the extent of the V(D)J recombination machinery involvement in the formation of T-cell receptor and non-T-cell receptor aberrations in T-cell acute lymphoblastic leukemia is still poorly understood. We performed a comprehensive *in silico* and *ex vivo* evaluation of 117 breakpoint sites from 22 different T-cell receptor translocation partners as well as 118 breakpoint sites from non-T-cell receptor chromosomal aberrations. Based on this extensive set of breakpoint data, we provide a comprehensive overview of T-cell receptor and oncogene involvement in T-ALL. Moreover, we assessed the role of the V(D)J recombination machinery in the formation of chromosomal aberrations, and propose an up-dated mechanistic classification on how the V(D)J recombination machinery contributes to the formation of T-cell receptor and non-T-cell receptor aberrations in human T-cell acute lymphoblastic leukemia.

Introduction

T-cell differentiation is characterized by the tightly regulated process of T-cell receptor (TCR) gene rearrangement, also referred to as V(D)J recombination. V(D)J recombination in TCR genes occurs in a precise order: TCRD, TCRG, TCRB, TCRA.[1] The V(D)J recombination process can be divided into two different but equally important phases. In the first phase, recombination activating gene (RAG) protein complexes consisting of heterodimeric RAG1 and RAG2 proteins, recognize, capture and bind recombination signal sequences (RSSs) that flank V, D and J genes.[2] Each RSS comprises a heptamer and a nonamer sequence which are separated by either a 12 or 23 nucleotide spacer and recombine according to the 12/23 rule.[3] Following capture of an RSS by the RAG complex, DNA double-strand breaks (DSBs) are induced, resulting in coding ends (CEs) that immediately form hairpins, and blunt signal ends (SEs) that are rapidly fused into signal joints (SJs).[2,4,5] The two hairpinned CEs are kept closely together by the Ku70-Ku80 proteins that associate with the DNA-PKcs, which binds Artemis. In the second phase, the DNA hairpins are nicked by endonuclease activity of Artemis, followed by deletion of nucleotides from the germline sequences and non-templated insertion of *de novo* nucleotides by terminal deoxynucleotidyl transferase (TdT),[5] and, finally, by joining of the CEs into coding joints (CJs). The entire repair process is orchestrated by components of the non-homologous end-joining (NHEJ) pathway within the post-cleavage synaptic complex (PCSC).[4,5]

Efficient recombination is restricted to recognition of RSSs by the RAG proteins and the subsequent repair of RAG-induced DSBs is confined to the PCSC. Despite these restrictions, molecular studies on translocation breakpoint (BP) sites have provided proof of the involvement of the V(D)J recombination machinery in the formation of aberrant recombinations.[5] Aberrant recombination between TCR genes and oncogenes gives rise to chromosomal translocations that are common in immature T-lymphoid malignancies, such as T-cell acute lymphoblastic leukemias (T-ALL). These aberrant recombinations result in juxtaposition of oncogenes in the vicinity of TCR *cis*-acting regulatory elements such as enhancers, or in removal of negative regulatory elements (NRE) from the oncogene promoter.[6] As a result, expression of the involved oncogene, which often encodes for a transcription factor, becomes deregulated. The deregulation of the oncogene is considered to be an early key event in T-ALL leukemogenesis.[6] An overview of the TCR translocations, translocation partners and occurrence in T-ALL is given in Table 1.

Two TCR translocation mechanisms, designated as 'Type 1' and 'Type 2', have been postulated.[4,5] In 'Type 1' translocations, BP site sequences that are located outside the TCR loci,

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Correspondence: a.langerak@erasmusmc.nl

and resemble RSSs (also referred to as cryptic RSSs; cRSSs), are erroneously targeted by the RAG proteins. As a result, non-TCR sequences are introduced into the V(D)J recombination process and coupled to the TCR locus.[5] 'Type 2' translocations are formed, when DSBs at sites devoid of cRSSs located within the oncogene locus, are repaired and ligated to TCR genes during the V(D)J recombination process via the NHEJ pathway.[5] Non TCR-associated chromosomal aberrations such as deletional aberrations (*SIL-TAL1*) and insertions (*HPRT1*) have also been appointed as V(D)J recombination-mediated events based on the presence of cRSSs at BP sites.[28-31]

Until now, TCR-associated translocation mechanisms have mainly been evaluated for only a few BP sites by means of *ex vivo* experiments,[4,6,31,32] basically confirming the concept of RAG mistargeting to cRSSs. These oncogenes and their respective BP sites were usually chosen for their high frequency in T-ALL and also because of the probability that they would function as a cRSS based on structural criteria.[32]

Despite the postulated role of V(D)J recombination, it is still not clear to what extent the V(D)J recombination machinery is mechanistically involved in the formation of TCR and non-TCR aberrations in T-ALL. Here we examined 117 molecularly defined BP sites and their sequences from 22 different TCR translocation partners as well as 118 BP sites from non-TCR aberrations in our T-ALL cohort and T-ALL cases described in literature. Based on this large and comprehensive *in silico* and *ex vivo* evaluation of BP sites, on analysis of TCR loci and oncogene involvement, and on analysis of pre- and post-translocation configurations of the TCR translocations, we critically re-evaluated the role of the V(D)J recombination machinery in the formation of chromosomal aberrations.

LMO2, TAL1, and TLX1 are the predominant TCR translocation partners and show clear translocation BP clusters

In the 117 TCR translocations evaluated, a total of 22 different oncogenes were identified as TCR translocation partner. *LMO2* (15%), *TAL1* (11%), and *TLX1* (25%) loci were most frequently involved in these translocations, while other TCR translocation partners were less frequently observed (approx. 32% of total, and each <5%) (Table 2 and *Online Supplementary Table S2*). The translocation frequencies reported here are based on the available molecular data, and may, therefore, not reflect true prevalence of these oncogenic TCR translocations in T-ALL. Translocation BP sites in the *LMO2*, *TAL1* and *TLX1* loci appeared to be localized in distinct regions and were either densely clustered in breakpoint cluster regions (BCRs; defined as more than two BP sites within a region of 50 bp from each other, and sharing a common translocation DSB type) or localized as single BP sites (Figure 1, Table 2, and *Online Supplementary Table S2*). The *LMO2* locus has two distinct BCRs (hereafter referred to as BCR_1 and BCR_2),

Table 1. TCR-oncogene translocation occurrence in T-ALL.

Protein family	Gene	Chromosome band	Chromosomal Aberrations*	Occurrence in T-ALL	References
Basic helix-loop- helix transcription factors (bHLH)	*TAL1*	1p32	t(1;14)(p32;q11)/ t(1;7)(p32;q34)	~4%	(7, 8)
	TAL2	9q34	t(7;9)(q34;q34)	~2%	(9)
	LYL1	19p13	t(7;19)(q34;p13)	~7%	(7)
	OLIG2	21q22	t(14;21)(q11;q22)	2%	(7)
	MYC	8q24	t(8;14)(q24;q11)	~1%	(7)
Lim only domain (LMO) proteins	*LMO2*	11p13	t(11;14)(p13;q11)/ t(7;11)(q34;p15)	~6%	(6, 10)
	LMO1	11p15	t(11;14)(p15;q11)/ t(7;11)(q34;p13)	~2%	(11)
	LMO3	12p12	t(7;12)(q34;p12)	<1	(12)
Homeobox proteins	*TLX1*	10q24	t(10;14)(q24;q11)/ t(7;10)(q34;q24)	5-10%C, ~30%A	(13)
	TLX3	5q35	t(5;14)(q35;q11)	20-25%C,~5%A	(14, 15)
	HOXA cluster	7p15	t(7;14)(p15;q11)/ inv(7)(p15;q34)	~3%	(14, 16)
	NKX2-1	14q13	t(7;14)(q34;q13)/ inv(14)(q13;q32)	<1%	(17), **
	NKX2-4	20p11	t(20;14)(p11;q11)	<1%	**
	NKX2-5	5q35	t(5;14)(q35;q32)	<1%	(18)
Other	*NOTCH1*	9q32	t(7;9)(q34;q34)	<1%	(19, 20)
	CCND2	12p13	t(7;12)(q34;p13)/ t(12;14)(p13;q11)	<1%	(21)
	MYB	6q23	t(6;7)(q23;q34)	~3%	(22)
	LCK	1p34	t(1;7)(p34;q34)	<1%	(23)
	BCL11B	14q32	inv(14)(q11;q32)	<1%	(24)
	TCL1A	14q32	t(7;14)(q34;q32)/ inv(14)(q11;q32)	<1%	(25, 26)
	BMI1	10p12	t(7;10)(q34;p12)	<1%	(27)

*Chr.14q11: TCRD locus. Chr.7q34: TCRB locus. **Larmonie et al., *unpublished data, 2013.* [C]Childhood. [A]Adulthood.

with approximately 40% (7 of 17) of the BP sites located in a BCR. Approximately half (6 of 13) of the *TAL1* BP sites and 67% (33 of 49) of the *TLX1* BP sites formed a BCR (Figure 1). *LMO2*, *TAL1* and *TLX1* BCRs localize in the direct vicinity of a transcription starting site (TSS), at the 5'-end of the coding region of the gene while, in contrast, the *TAL2* BCR localized at the 3'-end of the oncogene. The localization of BCRs in the vicinity of a TSS suggests a localized accessibility at these sites, presumably as a consequence of transcription activity that in turn is associated with a bias towards mutation formation.[67] The majority (approx. 90%) of the BP sites are found outside of the coding regions of the genes, with approximately 65% being localized at the 5'-end of the gene and approximately 25% at the 3'-end, while approximately 10% are localized within the coding regions (Figure 2A). This indicates that the localization of a translocation BP site, particularly the BCR, is oncogene-specific, which is most likely associated to the selective advantage gained from the translocation, dependent on the function of the transformed gene. Even though the exact position of the BP site within the coding region of the oncogene locus and the resulting selective advantage ultimately determine whether the outcome of a translocation will be the development of a T-ALL, this still does not explain why specific BP sites become recurrently involved in aberrations while others do not.

Table 2. Oncogene, TCR locus and translocation type involvement in 117 TCR translocations BP sites.

Gene	BP (region)	Distance to TSS (nt)	Evaluated cases (n)	TCR involvement (n)			Translocation type (n)		Reference
				TCRD	TCRA	TCRB	Type 1*	Type 2	
LMO2	5'of BCR_2	-6,902/-5,654	2	2	0	0	1	1	(6, 33)
	BCR_2	-1,849/-1,846	3	3	0	0	0	3	(6)[R]
	5'of BCR_1	-1,659	2	1	0	1	0	2	(6, 33)
	BCR_1	-384/-392	4	4	0	0	4	0	(6, 34, 35)
	3'of BCR_1	+169/+34,160	6	6	0	0	3	3	(6, 36, 37)[R]
			17	**16**	**0**	**1**	**8**	**9**	
TAL1	5'of BCR	-3,208/-817	3	3	0	0	0	3	(38-40)[R]
	BCR	+427/+431	6	6	0	0	6	0	(38, 40, 41)
	3'of BCR	+ 10,780/+53,085	4	3	0	1	1	3	(42-45)
			13	**12**	**0**	**1**	**7**	**6**	
TLX1	5'of BCR	- 11,129/-3,158	6	4	0	2	1	5	(46-48)
	BCR	-509/+19	35	35	0	0	0	35	(46, 49-50)
	3'of BCR	+1,313/+30,539	8	1	0	7	2	6	(30, 46, 51)
			49	**40**	**0**	**9**	**3**	**46**	
BCL11B	n.a	+48,651i	1	1	0	0	0	1	(24)
BMI1	n.a.	-277,473	1	0	0	1	0	1	(27)
CCND2	n.a.	- 61,328	1	0	0	1	1	0	(21)
MYB	n.a.	+53,891/+53,903	2	0	0	2	0	2	(22)
		+144,584	1	0	0	1	1	0	(22)
HOXA6	n.a.	-2,179	1	1	0	0	0	1	(16)
HOXA9	n.a.	-2,992/-501i	2	0	0	2	0	2	(12, 52)
LCK	n.a.	+20,253/+25,085	2	0	0	2	1	1	(23, 53)
LMO1	n.a.	-9,644	1	1	0	0	0	1	(54)
		-5,258	2	2	0	0	2	0	(10, 55)
LMO3	n.a.	+224,944	1	0	0	1	0	1	(12)
LYL1	n.a.	+8,444	1	0	0	1	0	1	(54)
		+787	1	0	0	1	0	1	(56)
MYC	n.a.	+5,96/+10,318	4	0	4	0	1	3	(57-60)
		+239,565	1	1	0	0	0	1	(58-61)
NKX2-1	n.a.	+4,286	2	1	0	1	0	2	(17)
NKX2-4	n.a.	-494	1	1	0	0	0	1	Current Study
NKX2-5	n.a.	+35,271	1	1	0	0	0	1	(18)
NOTCH1	n.a.	+39,552/+42,969	5	0	0	5	2	3	(19, 62, 63)
OLIG2	n.a.	+84,224	1	0	1	0	0	1	(64)
TAL2	n.a.	+ 31,795/+31,766	3	0	0	3	3	0	(65)
TCL1A	n.a.	-145,630/-99,998i	2	0	1	1	0	2	(25-26)
TLX3	n.a.	-50,034	1	1	0	0	0	1	(66)
			38	10	6	22	11	27	–
Total			**117**	**78**	**6**	**33**	**29**	**88**	–

**Based on cRSS functionality determined by* in silico *and/or* ex vivo *analysis.* [I]*Inversion; n.a.: not applicable.* [R]*Sequences found in the GenBank database.*

TCR translocations in T-ALL mostly involve the TCRD locus

The majority (approx. 67%) of all TCR translocations involved the TCRD locus. Approximately 94% of *LMO2*, 92% of *TAL1*, and 82% of *TLX1* translocations occurred during an attempted TCRD gene rearrangement (Figures 1 and 2A, and *Online Supplementary Table S2*). The BP sites associated to the *TLX1* BCR exclusively concerned TCRD translocations, while all BP sites located at the 3'-end of the *TLX1* exon 3 exclusively involved the TCRB locus (Figure 1). Approximately 58% of BP sites of the less frequent T-ALL TCR translocation partners translocated to the TCRB locus (Figure 2B). Interestingly, particular oncogenes have been observed to almost exclusively translocate with a specific TCR locus (Tables 1 and 2). Considering this exclusivity, and the fact that TCR loci recombine at specific stages of thymocyte development, one could hypothesize that synchronous accessibility of a specific oncogene locus and the TCR locus may play an important role in determining specific oncogene-TCR locus interactions and translocation probability during V(D)J recombination. In addition, since the actual physical contact between translocation partners is required for the eventual formation of a translocation, it is likely that the probability of translocation is likewise determined by nuclear proximity.

Analysis of the location of the BP site in relation to the TCR locus involved in the translocation showed that approximately 82% of all TCRD translocations involved the 5'-end of the oncogenes, while 5'-regions of oncogenes were involved in only approximately 36% of the TCRB translocations (Figure 2A). The observed prevalence of TCRD involvement at the 5'-end of oncogenes, together with the high frequency of TCRB translocations (approx. 49%) at the 3'-end of oncogenes, agrees with the theory of oncogenic regulation being dependent on the actual TCR locus involved.[46,68] The high prevalence of TCRD associated translocations, and the association of the BP sites involved with specific oncogene regions, suggests a particular proneness of the TCRD locus for aberrant recombinations and/or a selective leukemogenetic advantage upon involvement of the TCRD locus in translocations. While the majority of translocations (approx. 67%) involve the TCRD locus, TCRA was only involved in approx. 5% (n=6) of the translocations and exclusively concerned *TCL1A*, *OLIG2* and *MYC* translocations. TCRG locus involvement was not observed in any of the translocations analyzed and has so far never been reported in TCR translocations in T-ALL. The lack of TCRG translocations is remarkable, as most reported trans-rearrangements (inter-TCR gene rearrangements) do involve the TCRG locus,[69,70] suggesting that the locus can be implicated in

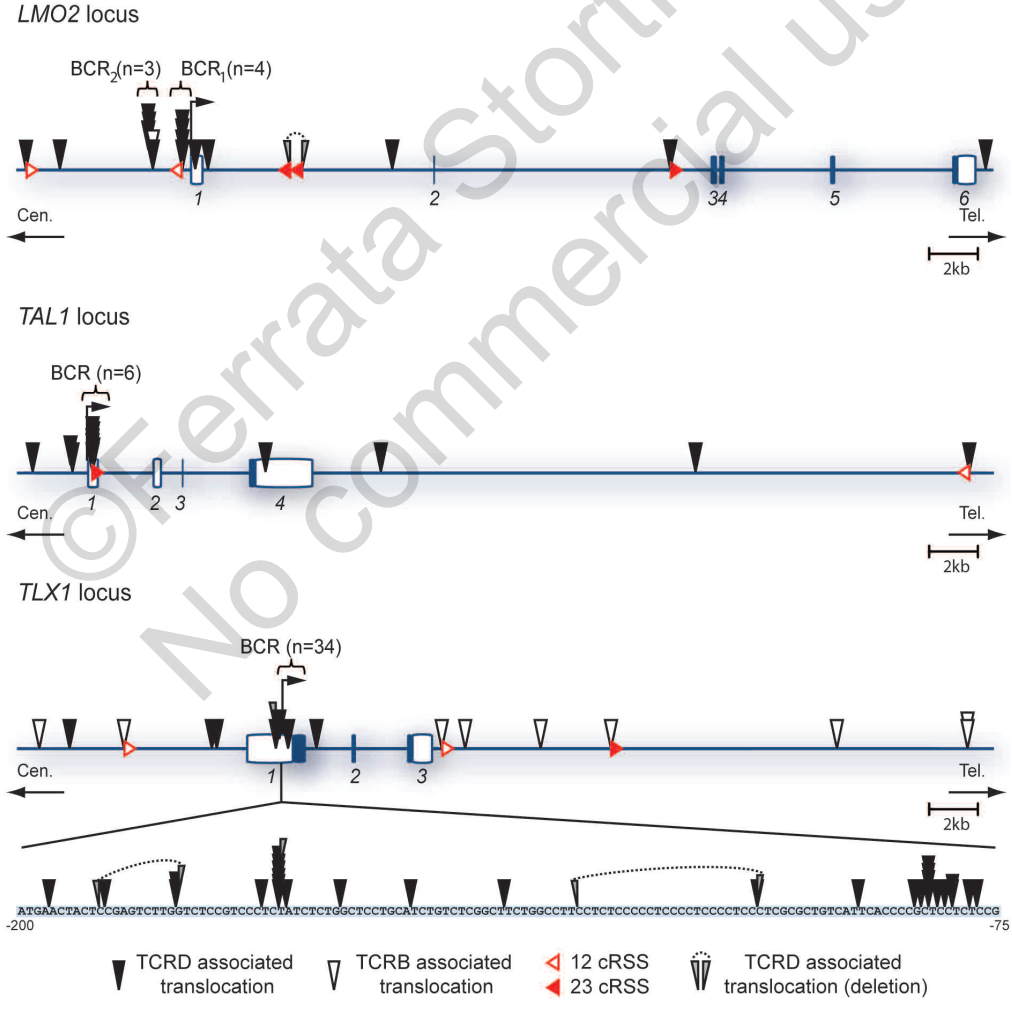

Figure 1. Overview of T-ALL associated BP position and cRSS in *LMO2*, *TAL1* and *TLX1* locus. The position of every BP analyzed in this study is given for *LMO2*, *TAL1* and *TLX1* respectively. For each BP, the associated cRSS is given. Positions of the most upstream and most downstream BP are given relative to the TSS. Breakpoint cluster region (BCR) (a region containing >2 BP sites with ~50bp between adjacent BP sites).

aberrant V(D)J rearrangements. It has been shown that in TCRB translocations, the TCRB enhancer is involved in driving the oncogene, whilst in TCRD translocations, oncogenic expression is mostly driven by the TCRD internal promoter.[68] Interestingly, the TCRG locus carries both enhancer and silencer sequences. Signaling via the pre-TCR, which plays a role in T-ALL development,[71] can activate the TCRG silencer.[72] The apparent lack of TCRG translocations in T-ALL could thus be the result of pre-TCR mediated activation of the TCRG silencer located in *cis* of the TCRG locus that was involved in a translocation, resulting in dampening of oncogenic activation. It might well be that the positive and negative selection phases of TCRαβ thymocytes are implicated in the low occurrence of TCRA locus translocations seen in T-ALL. TCRA translocations are formed at the latest thymocyte developmental stage. It is reasonable to think that upon translocation, the window for acquiring additional mutational hits is small, thus limiting the chance for tumorigenic transformation. Since the majority (>90%) of the TCRαβ thymocytes do not survive selection but undergo apoptosis, the majority of the TCRA translocations will, therefore, be lost.

TCR gene involvement discloses window of translocation occurrence

Analysis into TCRD gene involvement showed that approximately 42%, 28% and 28% of the TCRD translocations occurred during an attempted Dδ-Dδ, Dδ-Jδ or Vδ-Dδ recombination, respectively, indicating a preference for aberrant recombinations to occur during Dδ-Dδ rearrangements. The TCRB translocations predominantly (approx. 76%) occurred during Dβ-Jβ recombinations, whilst only approximately 17% of the translocations occurred during Vβ-Dβ recombination. Five of the TCRA translocation events were formed during attempted Vα-Jα recombinations, and in one case, the translocation seemed to have occurred during simultaneous induction of DSBs at two Jα genes. It should be noted that oncogenic transformation might not manifest at the exact stage in which translocation occurs, but rather at a later developmental stage,[7] usually after acquisition of subsequent mutational hits.[73]

Since most of the TCRD and TCRB translocations involved the Dδ and Dβ genes, respectively, this would suggest an increased chance for the occurrence of an erroneous recombination during the earliest phases of TCRD and TCRB recombination. These findings show that the window of opportunity for translocation formation from the perspective of the TCRD locus is mainly during the DN stages of thymic development (Dδ-Dδ, Dδ-Jδ recombinations), whereas the TCRB locus is most likely involved in translocations during the ISP and DP3- stages of thymocyte development (Dβ-Jβ recombinations)[1] (Figure 3). However, based on the relative occurrence of TCRD and TCRB translocations and the high frequency of Dδ-Dδ miscoupling in T-ALL, we conclude that, overall, the majority of TCR translocations most likely occur during attempted Dδ-Dδ recombinations, in the DN1 and DN2 developmental stages (Figure 3).

TCR translocations in T-ALL are mostly formed via the 'Type 2' translocation pathway

In silico testing of the 117 BP sites based on recombination information content (RIC) threshold scores (see *Online Supplementary Design and Methods*) identified a cRSS at only 27 BP sites (23%) that were found within the *LMO2*, *TLX1*, *TAL1*, *TAL2*, *MYC*, *NOTCH1*, *LMO1*, *C-MYB* and *LCK* oncogene loci (Table 2, *Online Supplementary Table S2*). *Ex vivo* studies in our laboratory confirmed that BP sites associated to *TLX1*, *LMO1*, *LMO3*, and *LYL1* that were devoid of a cRSS were not induced by RAG (*Online Supplementary Table S3*), thus sup-

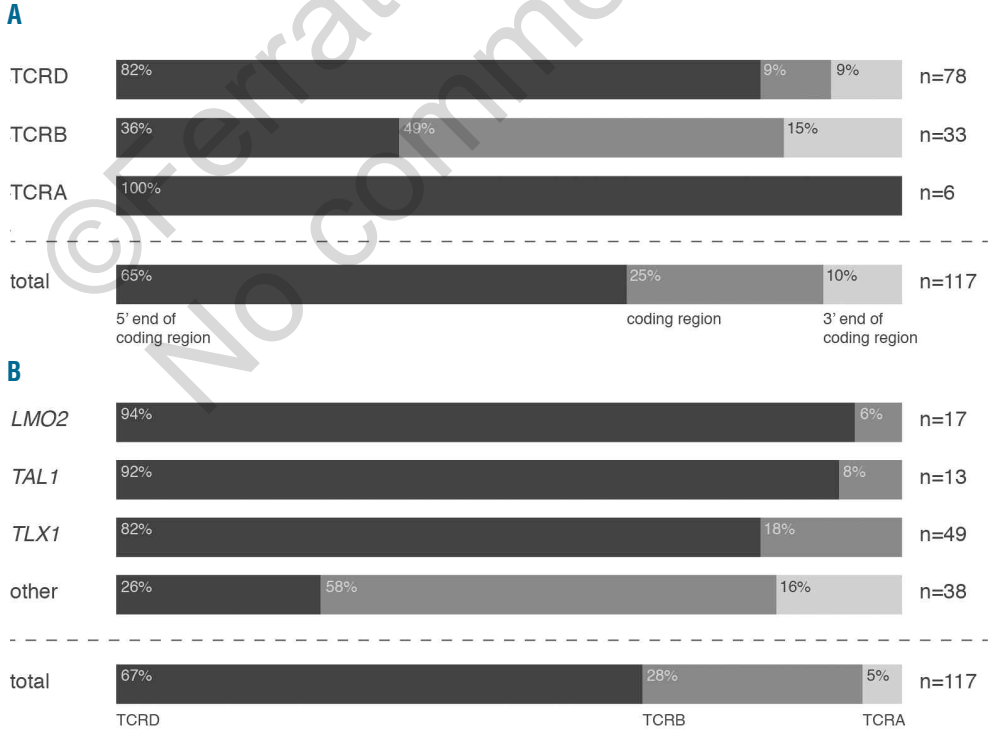

Figure 2. Pie charts illustrating percentages of TCR and oncogene translocation occurrence in T-ALL. (A) Pie charts illustrating percentages of BP site localization relative to the functional regions of the oncogenes involving all BP sites or involving a particular TCR locus. (B) Pie charts illustrating percentages of TCRD, TCRB, and TCRA related translocations involving *LMO2*, *TAL1*, *TLX1* and the rest of the analyzed translocation partners.

porting the idea that translocations involving these sites were formed via the 'Type 2' translocation pathway. This further confirmed our previous findings of a high correlation between the RIC score obtained from *in silico* analysis and the translocation efficiency determined by means of *ex vivo* recombination substrate assay.[6] Only approximately 25% of the whole spectrum of TCR translocations in human T-ALL is driven by RAG mistargeting of cRSSs ('Type 1' translocations). Interestingly, a large fraction (approx. 36%, 16 of 78) of the TCRB translocations occurred via the 'Type 1' translocation pathway, while this was the case for only approximately 21% (12 of 33) of TCRD translocations and approximately 17% (1 of 6) of TCRA translocations (Figure 4A). In approximately 75% of cases, the involvement of the V(D)J recombination machinery is mainly apparent due to the involvement of a TCR locus in these formations. This reinforces previous notions based on smaller series that TCR translocations predominantly occur via the 'Type 2' translocation pathway.[5,32] Furthermore, this suggests that the V(D)J recombination machinery *per se* is not the driving force in the induction of DSBs at the majority of the BP sites that lead

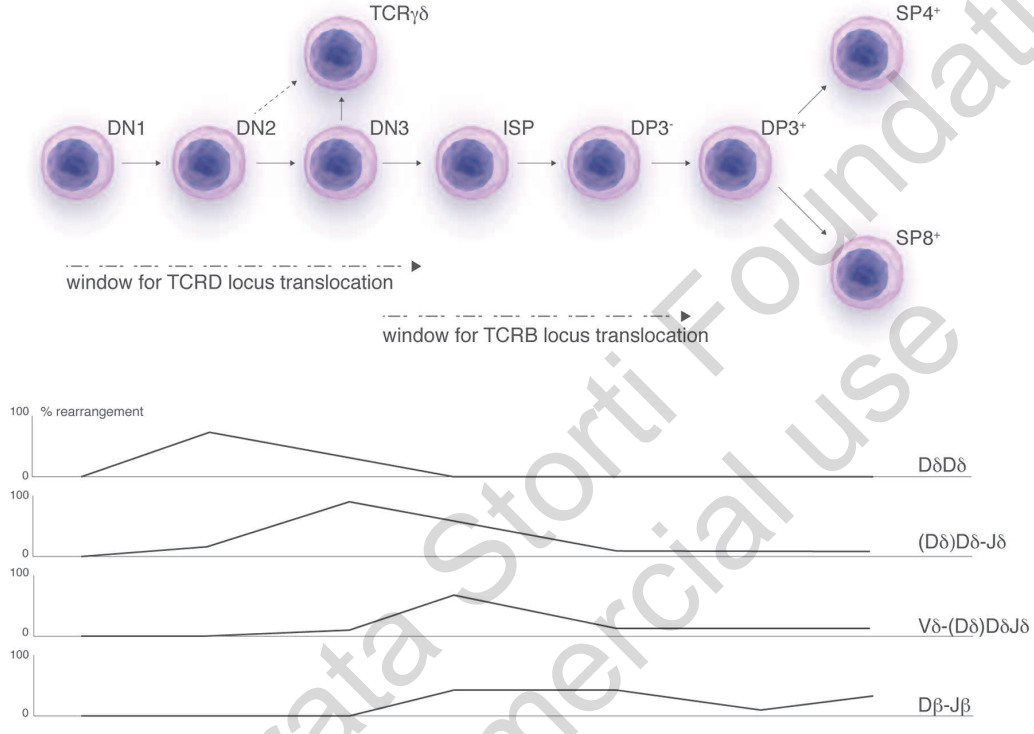

Figure 3. Window of opportunity for formation of TCRD and TCRB locus translocations. Schematic overview of consecutive stages of normal human thymocyte development. Graphs show rearrangement activity (%) during each developmental stage. The arrows indicate the window in which each recombination/ translocation combination can occur. V(D)J recombination graphs are adapted from Dik *et al.*[1] DN: double negative; ISP: immature single positive; DP: double positive; SP: single positive.

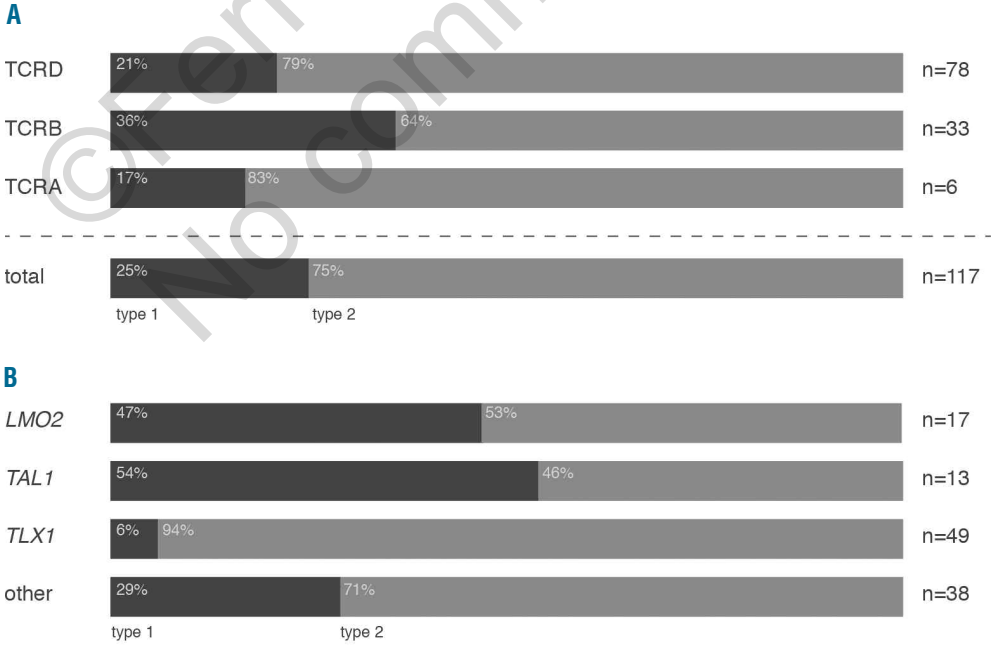

Figure 4. Pie charts illustrating percentages of translocation type. (A) Percentages of the occurrence of 'Type 1' and 'Type 2' translocations involving the TCRD, TCRB, and TCRA genes. (B) Percentages of 'Type 1' and 'Type 2' translocations involving either *LMO2*, *TAL1*, *TLX1* and the remaining translocation partners.

to TCR translocation formation.

TLX1 associated translocations are mostly governed by the 'Type 2' translocation pathway. Contrary to current assumptions, 3 (6%) of the *TLX1* translocations seem to have resulted from the 'Type 1' translocation, as these BP sites are associated with functional cRSSs as defined by *in silico* analysis based on RIC threshold levels. Interestingly, these three cRSS-related *TLX1* translocations are all TCRB-associated translocations (Figure 1). Importantly, the *TLX1* BCR and *LMO2* BCR_2 did not associate to a cRSS, while both the *LMO2* BCR_1 and the *TAL1* BCR translocations did. Other translocation partners mostly (>71% of cases) translocated via the 'Type 2' translocation pathway (Figure 4B). This clearly shows that the majority of TCR translocations are not caused by RAG-cRSS interactions. The presence of a cRSS at a BP site is the most obvious genetic feature known to render DNA susceptible to DSB induction involved in TCR translocations. The fact that the *TLX1* BCR and the *LMO2* BCR_2 are not associated to a cRSS suggests that these sites must be associated with another type of sequence-specific feature rendering these particular sites recurrently susceptible to DSB inductions.

cRSSs in oncogene loci are not randomly involved in T-ALL translocations

cRSSs are distributed throughout the entire human genome at an average density of one cRSS per 500 bp.[74] The *LMO2* locus comprised 109 cRSS, of which only 5 (<4%) were associated with a BP site. The 12 bp spacer cRSS with the highest RIC score (-29.21) was identified at the *LMO2* BCR_1. Interestingly, the 23 bp spacer cRSS with the highest RIC score (-48.74) was not associated to any of the *LMO2* BP sites in our study, while the position and orientation of this cRSS could have caused the oncogenic activation of *LMO2* upon involvement in a translocation (*Online Supplementary Figure S1*). Of the 56 cRSSs identified in *TAL1*, the *TAL1* BCR did associate to its most efficient 23 bp spacer cRSS (RIC score: -46.34), while no BP sites were associated to the 12 bp spacer cRSS with the highest RIC score (-31.10). Of the 139 *TLX1* cRSSs identified, the UPN474 BP site was associated to the most efficient 23 bp spacer cRSS (RIC score: -47.754), while no BP sites were associated to the 12 bp spacer cRSS with the highest RIC score (-29.66). Interestingly, the *TLX1* and *LMO2* 12 bp spacer cRSSs as well as the *TLX1* and *TAL1* 23 bp spacer cRSS have comparable RIC scores, implying

Table 3. Determination of RAG involvement at BP sites involved in non-TCR chromosomal rearrangements.

Mutation	Oncogene gene	Distance to TSS	*In silico* determined cRSS present at BP	RIC score	Fusion partner	Distance to TSS	*In silico* determined cRSS present at BP	RIC score	Reference
Deletion									
SIL-TAL1	*TAL1*	-2.109/-2.129*	CACAGCC-23-AAGGAAAAG	-46.62	*SIL*	+396/+420*	no	–	(8, 29, 39, 97)
		-3.792/-3.802*	CACAGAG-12-GCCAAAACT	-28.12		+405/+420*	no	–	(8, 97)
		+5.292*	CACACAC-12-GATAGAAAC	-38.63		+407*	no	–	(8)
			CACACAC-23-ATAGAAACA	-50.05					
		-2.190*	CACAGCC-23-AAGGAAAAG	-46.62		+1.067*	no	–	(97)
		-6.187*	no	–		+407*	no	–	(97)
LMO2-RAG2	*LMO2*	+2,687*	no	–	*RAG2*	+3,110*	no	–	(98)
		-31,943*	no	–		-71,278*	no	–	(98)
Insertion/ Deletion									
HPRT[¶]		+687*	CAGTGTG-23-GGCAAAACC	-36.92	$RSS^{V\alpha24}$/ $RSS^{J\alpha29(ins.)}$	nd	CACAGTG-23-ACGCAAACC /CACAGTG-23-ACTCAAACC	-27.57 /-27.83	(28, 30)
	HPRT1 Deletions/ Insertions	nd	nd	nd	$RSS^{V\alpha22}$/ $RSS^{J\alpha45(ins.)}$	nd/nd	CACAGTG-23-ACACAAACC / CACTCTG-12-ACATAAACT	-25.91 /-23.23	(30, 96)
		nd/ nd	nd/nd	nd/ nd	$RSS^{V\alpha34}$/ $RSS^{J\alpha44(ins.)}$	nd/nd	CACAGCG-23-CTCCAAACC /CACTGTG-12-ACAGAAACC	-39.11 /-21.54	(30, 96)
		+684 /+20.693$^{(del)*B}$	CAGTGTG-12-GGCAAAACC/ CACACAC-12-ACAAATACA	-36.92 -28.28	BICD1 $^{(ins.)(del)B}$	+7,693/ +8,282*	-/ CAAAGTG-23-TGCGCCCGG	-56.46	(28, 30)
		33,400/33,422	no	–	–	–	–	–	(95)
		33,359/23,551	no	–	–	–	–	–	(95)
		11,405/17,892	no	–	–	–	–	–	(95)
		22,664/29,064	no	–	–	–	–	–	(95)
		13,588/26,981	no	–	–	–	–	–	(95)
	HPRT1 Deletions	14,949/27,861	no	–	–	–	–	–	(95)
		1,980/28,526	no	–	–	–	–	–	(95)
		23,322/27,499	no	–	–	–	–	–	(95)
		13,092/13,447	no	–	–	–	–	–	(95)
		+667/+679 (n=15)	CAGTGTG-12-GGCAAAACC	-36.92	–	+20,658 /+20,679	CACACAC-12-ACAAATACA/ CACACAC-23-TTTGTGTGT	-28.28 -57.793	(99)
		+672/+677 (n=2)	CAGTGTG-12-GGCAAAACC	-36.92	–	+18,581 /+18,590	CACACAC-12-TCTTAATCC CAGAGAG-23-TAAAAAGTG	-36.19 -63.91	(99)
		+678 (n=1)	CAGTGTG-12-GGCAAAACC	-36.92		+20,998	–	–	(99)

[¶]*HPRT1 associated insertions shown are mutations found in normal human T cells, *The precise BP position is not known due to type of break, No: no functional cRSS found at that BP position according to the RIC algorithm analysis., -: no RIC score, nd: not determined, $X/Y^{(del)}$: Deletion between 5'BP and 3'BP position, $X^{(ins.)}$:Insertion of that particular sequence in to another locus.* [B]*The presence of a cRSS tested at both BP sites.*

similar recombination efficiency. However, none of these *TLX1* cRSSs are associated to a *TLX1* BCR, even though they are located within a region (at the 3'end of the *TLX1* locus) known to be involved in translocations (*Online Supplementary Figure S1*). These observations imply that particular cRSSs involved in T-ALL translocations are preferentially targeted over other highly efficient cRSSs found within the same region and that particular cRSSs do not become engaged in T-ALL translocations, whilst other BP sites in the vicinity of these allegedly highly recombination efficient cRSSs are associated to non-RAG-cRSS mediated breaks. Moreover, this shows that the targeting of cRSSs for involvement in a T-ALL translocation is not random and is independent of the recombination efficiency of a cRSS, implying that other (genetic or epigenetic) factors are eventually decisive for break susceptibility at these particular sites.

The role of the V(D)J recombination machinery in DSB induction and aberrant repair leading to TCR translocations

Ex vivo experiments have clearly proven the ability of RAG to bind and cleave cRSSs. However, our observations clearly indicate that cRSSs are not randomly targeted by RAG *in vivo*. It is well known that chromatin organization influences the ability of RAG to gain access to an authentic RSS.[75] Therefore, access to a cRSS is also decisive for the occurrence of RAG-cRSS mistargeted DSB induction at oncogene loci sites. Reactivity of the RAG protein complex is not only dependent on the recognition of a cRSS by RAG1, but it is likewise dependent on the ability of RAG2 to bind to a H3K4me3-modified nucleosome adjacent to that cRSS in order to enable cleavage at that cRSS site.[76] This suggests that epigenetic marks at a cRSS in the oncogene locus must be optimal to enable RAG-cRSS recognition and subsequent cleavage. Since epigenetic marks are site-specific, they can mediate heterogeneous accessibility throughout the locus. This differential accessibility could (at least in part) be the cause for a preferential involvement of particular cRSSs in TCR translocations.

Besides being a sequence-specific nuclease targeting (c)RSSs, RAG protein complexes are also able to function as a structure-specific nuclease, recognizing non-B DNA structures as substrate.[77,78] The ability of RAG to induce DSBs at non-B DNA structures suggests that RAG could be involved in a broader range of DSB induction mechanisms and thus be the driver of DSB inductions involved in 'Type 2' translocations.[78,79] This means that non-cRSS associated DSB inductions could still be RAG mediated, thus providing a new perspective on 'Type 2' translocations. This would also imply that RAG associated translocations do not only occur in the 25% 'Type 1' aberrations but do, in fact, occur more often. Other sequence-specific features (transposable elements, association with origin of replication, GC-content or free energy levels[80-82]) could also be involved in making these 'Type 2' sites more susceptible to DSB induction, on top of the vulnerability level provided by a cRSSs or by the DNA conformational susceptibility.

Particular RAG mutations have been shown to cause the formation of unstable RAG post-cleavage synaptic complexes (PCSC) that allow DSBs to participate in the error-prone alternative NHEJ repair pathway.[83] Although we do not have any indications about this, we cannot formally exclude the possibility that such RAG mutations could be the cause of the T-ALL derived translocations that we evaluated. However, identification of pre- and post-translocation TCR recombination configurations of derivate chromosomes, as seen in T-ALL, provides evidence that RAG proteins are able to initially target an authentic RSS prior to mistargeting a cRSS in 'Type 1' translocations. Furthermore, such analyses also show that correct joining of CEs within the same cell is possible prior to or subsequent to 'Type 2' translocation formation. Ongoing recombination following the translocation event is a phenomenon that has been previously described for *TAL2* translocations and that is thought to contribute to the post-translocation oncogenic effect.[84,85] The fact that normal recombination can precede or follow a translocation event in the very same cell implies that nothing is intrinsically wrong with the V(D)J recombination machinery in these aberrant cells.

Irrespective of the ability of RAG to induce DSB at either cRSS sites, or DNA structures similar to non-canonical (non-B) DNA structures, or other sequence-specific features,[77] the question remains whether RAG-induced DSBs actually stimulate translocation formation. RAG-cRSS complexes have been shown to cause PCSC instabil-

Table 4. Conditions to determine the involvement of the V(D)J recombination mechanism in the formation of chromosomal aberrations and to define the aberration type.

V(D)J recombination associated aberration Type	TCR involvement	cRSS involvement	Condition			Frequency*	Typical aberrations
Type 1A	+	+	TCR locus with RSS	AND	locus with cRSS	~14%	TCR-*LMO2*
Type 1B**	–	+	locus with cRSS	AND	locus with cRSS	–	*HPRT1-BICD***
Type 2A	+	–	TCR locus with RSS	AND	locus without cRSS	~40%	TCR-*TLX1*
Type 2B	–	+	locus with cRSS	AND	locus without cRSS	~40%	*SIL-TAL1* (frequent)
Type 3***	–	–	locus without cRSS	AND	locus without cRSS	~2%	*SIL-TAL1* (rare types)

**Percentages based on both TCR translocation and non-TCR molecularly defined aberrations in T-ALL analyzed in this study, **so far not (yet) identified in T-ALL, ***with untemplated nucleotides at junctional regions, ◀ :RSS, ◁ :cRSS, ‖ : no cRSS.*

ity, which in turn inhibits repair at these sites via the classical homologous recombination pathway[86] and promotes repair via the alternative NHEJ pathway. Since RAG protein-complexes are known to 'shepherd' broken ends into the NHEJ repair pathway,[87] off-target RAG-induced DSBs could be more prone to involvement in the DSB repair process at the V(D)J recombination site. Such susceptibility to repair-mistakes and translocations has also been observed in patients with the repair deficiency syndromes Nijmegen breakage syndrome (NBS) (NBN gene) and ataxia telangiectasia (AT) (ATM gene).[88,89] Even though ATM-deficient mice have been shown to carry an increase in TCRD/A translocations,[90,91] it should be stressed that this proneness to translocations only causes a generalized defect in the damage response upon DSB acquisition, and does require RAG activity for actual induction of translocations involving the TCR loci.[92]

The involvement of the V(D)J recombination mechanism can be extended to non-TCR and non-cRSS associated aberrations

Involvement of the V(D)J recombination machinery in the formation of TCR translocations in T-ALL is clearly evident, particularly when considering the involvement of the TCR genes in these aberrations. However, V(D)J recombination activity in T-ALL is not limited to rearrangements involving TCR loci given that cross-lineage Ig gene rearrangements (mainly involving IGH) have been observed in low frequencies as well.[93,94] The attribution of the V(D)J recombination machinery in the formation of other, non-TCR related chromosomal aberrations in T-ALL is, however, less evident. We analyzed 118 BP sites of non-TCR associated aberrations observed in T-ALL by determining the presence of cRSS at the different BP sites and evaluated the potential role of the V(D)J recombination mechanism in the formation of these aberrations. *HPRT1* related insertions, though not directly implicated in T-ALL leukemogenesis, do occur in normal peripheral T-lymphocytes.[28,30,95] Even though these insertions do not seem to have direct oncogenic effect, we did analyze them to delineate the mechanism involved. *HPRT1* has frequently been shown to carry a complex deletion/insertion rearrangement, where gross regions of the TCRA locus are inserted into the *HPRT1* locus.[28,30,96] One of the three TCRA insertions identified in this study (the BP site of 2 other cases could not be identified) showed involvement of a cRSS at the *HPRT1* locus (Table 3). A *BICD* insertion (9q22) into *HPRT1* locus has also been described. In this case, both ends of the *HPRT1* BP sites carried a cRSS, while only one end of the inserted *BICD* sequence carried a cRSS. In addition to insertions, deletions are also common in the *HPRT1* locus. Analysis of the different BP sites showed that the BCRs related to the *HPRT1* deletions are associated to a cRSS; however, in approximately 50% of the deletions, no cRSSs could be identified at the deletion BP sites (Table 3).

Analysis of BP sites of *SIL-TAL1* deletions (del(1p)) showed that approximately 99% (84 of 85) of all *TAL1* BP sites are associated with a cRSS whilst, in contrast to earlier suggestions,[31,32] none of the *SIL* BP sites tested had a cRSS in their vicinity (Table 3). Although *TAL1* also frequently translocates to TCR loci, interestingly none of the *TAL1* BP sites involved in *SIL-TAL1* deletions were in the direct vicinity of the *TAL1* BP sites involved in TCR translocations. The single *SIL-TAL1* case that lacked involvement of a cRSS at either of the BP sites, did have eight untemplated nucleotides at the junctional region,[97] suggesting the presence of TdT activity during ligation of these BP sites.

The cryptic deletion del(11)(p12p13) fuses the *LMO2* gene to the *RAG2* gene.[98] No cRSS was identified at the *RAG2* or *LMO2* BP sites. However, a potential untemplated nucleotide is present at the junctional region (Table 3). Despite the high occurrence of *LMO2* in TCR translocations, *LMO2* deletional BP sites do not co-localize with BP sites associated in *LMO2*-TCR translocations. We previously showed that *LMO2* activation was associated with the loss of the *LMO2* negative regulatory element (NRE) rather than due to juxtaposition of *LMO2* to the TCRD enhancer.[6] Furthermore, we identified the presence of one 12 bp spacer cRSS 3' of the *LMO2* NRE and three different 23 bp spacer cRSSs at the 5' side (*Online Supplementary Figure S2*).[6] Since many T-ALL cases have unresolved *LMO2* activation, and these cRSS lay in the orientation for RAG-mediated NRE deletion (*Online Supplementary Figure S1A*), we determined the mutual recombination potential of these cRSSs. To this end, the ability of these cRSS to mediate a deletion *ex vivo* was tested by means of a recombination substrate assay (*Online Supplementary Appendix*). The *ex vivo* analyses showed that none of the 12 bp / 23 bp cRSS combinations tested (*Online Supplementary Table S4* and Figure S2A) invoked such recombination. Nevertheless, the *HPRT1(-BICD1)* deletions/insertions show that interactions between cRSSs can occur.[28,30] Even though such cRSS-cRSS interactions have not yet been identified in T-ALL, involvement of the V(D)J recombination machinery in the formation of these aberrations is evident based on the presence of cRSSs at the BP sites and the insertion of untemplated nucleotides at the junctions.[28,30]

Comprehensive classification of TCR and non-TCR aberrations with respect to the role of the V(D)J recombination mechanism

Collectively, our findings indicate that the V(D)J recombination mechanism can also be involved in non-TCR aberrations, and in particular cases, potentially even in non-cRSS associated aberrations. Despite the fact that cRSS-cRSS interactions have not been observed in T-ALL so far, they are comparable to 'Type 1' (RSS-cRSS) recombinations. Hence, we propose that these cRSS-cRSS recombinations be denoted 'Type 1B' recombinations in which a second cRSS replaces the authentic TCR RSS (Table 4). Analogous to *TAL1* translocations, the V(D)J recombination machinery is also involved in *SIL-TAL1* deletions,[31] as deduced from the presence of cRSSs at the *TAL1* deletion BP sites only. The addition of random nucleotides at these junctions, a TdT-driven hallmark of the V(D)J recombination process, further confirms this involvement. Notably, it cannot be excluded that DNA polymerase mu (Polμ) can also be involved next to TdT.[100] The lack of cRSSs at the *SIL* BP sites however, suggests that, similar to 'Type 2' translocations, non-RAG induced DSBs within *SIL* are repaired via the NHEJ pathway within a RAG synaptic complex. This could be indicative of a variant of the 'Type 2' recombination pathway. In this proposed 'Type 2B' aberration (Table 4 and *Online Supplementary Figure S3*) *TAL1* would act as the carrier of the cRSS instead of the authentic TCR RSS. Although not as obvious, a role for the V(D)J recombination machinery

could also be argued in cases such as *SIL-TAL1* and *LMO2-RAG2* deletions[97,98] that, despite the lack of a cRSS at the reciprocal BP sites, do show potential non-templated nucleotides at the junctions. Such recombinations could be tentatively referred to as 'Type 3' aberrations (Table 4).

Conclusions

The activity of the V(D)J recombination machinery in thymocytes is primarily limited to the TCR locus. This restriction is determined by an interplay of sequence-specific features and TCR locus accessibility. As this process is confined to PCSCs, this, in principle, ensures the maintenance of genome stability. Nevertheless, it is clear that the V(D)J recombination machinery is involved in the formation of genomic aberrations by mediating DSB induction and erroneous repair of breaks in non-TCR loci during the recombination process. Here we demonstrate that only approximately 25% of the whole spectrum of TCR translocations in human T-ALL is driven by RAG mistargeting of cRSSs ('Type 1' translocations). In approximately 75% of cases, the involvement of the V(D)J recombination machinery is mainly apparent due to the involvement of a TCR locus in these formations ('Type 2' translocations). Non-TCR chromosomal aberrations in T-ALL do not involve RAG mistargeting either, with the exception of *SIL-TAL1* deletions, which can be considered as a 'Type 2B' aberration (*Online Supplementary Figure S3*). Thus, V(D)J recombination associated aberrations mostly result from repair mistakes rather than RAG mistargeting of cRSS. Furthermore, our findings argue that nothing is intrinsically wrong with the V(D)J recombination mechanism in these T-ALL, since pre- and post-translocation chromosome configurations indicate normal RAG targeting, repair and recombination of the involved TCR locus in the same cell.

Collectively, our results suggest that the V(D)J recombination machinery is generally not the driving force in the onset of the formation of TCR translocations and other, non-TCR genetic aberrations in human T-ALL. Rather it appears to play a role in facilitating the formation of these aberrations through repair, following any type of DSB breaks in particular oncogene regions. Based on these findings, we propose an extended and comprehensive mechanistic classification scheme on how the V(D)J recombination machinery contributes to TCR-associated and non-TCR associated aberrations in T-ALL (Table 4). When considering the frequency of cRSS associated *versus* non-cRSS associated aberrations using this new classification, we see that approximately 50% of the chromosomal aberrations in T-ALL as evaluated here are cRSS-mediated (Table 4). This is a considerably higher frequency of cRSS-mediated DSB inductions leading to chromosomal aberrations than previously thought.

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