

RNA fusions involving CD28 are rare in peripheral T-cell lymphomas and concentrate mainly in those derived from follicular helper T cells

Peripheral T-cell lymphomas (PTCL) comprise usually aggressive neoplasms of mature T cells with a heterogeneous molecular background. Over the past years, several genomic studies stemming from different research groups have shown evidence that mutation-induced activation of the T-cell receptor (TCR) signaling pathway is involved in the pathogenesis of several PTCL entities.^{1,7} Notably, recurrent activating point mutations in *CD28* which encodes a major TCR costimulatory receptor have been reported, mainly in angioimmunoblastic T-cell lymphoma (AITL) (10-15% of the cases).^{2,3} Recently, four different ribonucleic acid (RNA) fusions involving the carboxy-terminal part of *CD28* with the N-terminal part of *CTLA4* or *ICOS* were discovered by RNA sequencing analysis of AITL,^{2,8} cutaneous T-cell lymphoma (CTCL)^{4,9,10} and adult T-cell lymphoma/leukemia (ATLL).¹ While these data suggest that overall these fusions are rare (5-11%), a high frequency of *CTLA4(ex3)_CD28(ex4)* fusion (38% of the cases) was reported in a cohort of 120 PTCLs from Asia (including 50 AITL, 39 PTCL-not otherwise specified [-NOS] and 31 extranodal natural killer/T-cell lymphoma [ENKTCL]) via reverse transcription polymerase chain reaction (RT-PCR) analysis of formalin-fixed paraffin-embedded (FFPE) samples.⁸ However, by reanalyzing published whole exome sequencing and RNA sequencing (RNAseq) data (43 AITL, 16 PTCL-NOS and 43 ENKTCL), Gong *et al.* found only one AITL positive case for the *CTLA4(ex3)_CD28(ex4)* fusion.¹¹ In order to clarify these discrepant findings and to assess the prevalence of all depicted *CD28* RNA fusions, we designed a RT-PCR assay to detect these four *CD28* fusions which we then applied to a large series (n=273) of diagnostic frozen biopsy samples representative of various PTCL entities (Figure 1A). We found that these rearrangements are generally rare in PTCL (prevalence 4.8%), relatively more frequent in follicular helper T (TFH)-derived entities (6.5%), and most commonly represented by the *ICOS(ex1)_CD28(ex2)* fusion (73% of the cases).

Our study group comprised 110 AITL and 28 other nodal lymphomas of TFH derivation (24 TFH-like PTCL^{12,15} and four follicular [F]-PTCLs) (Online Supplementary Figure S1) plus 135 samples of different PTCL entities (Table 1) collected in the frame of the genomic network from the Lymphoma Study Association (LYSA).¹⁴ Samples were divided equally between our two laboratories for molecular screening. RT-PCR amplifications of the *CD28* fusions and *ACTB* (internal control) on total messenger (m)RNAs extracted from frozen tumor samples were analyzed by agarose gel electrophoresis (Online Supplementary Figure S2). All positive cases (n=13) and a subset of negative cases (n=58; 22%) were cross validated in both laboratories.

Results are summarized in Table 1. Overall, *CD28* fusions were detected in 13/273 (4.8%) cases. Among primarily nodal PTCLs, the highest prevalence was found in the group of AITL and other TFH-derived neoplasms with 9/138 positive samples (6/110 AITL, 3/24 TFH-like PTCL and none of the four F-PTCL; 6.8% in total), only 2/63 PTCL-NOS (3.2%) harbored a fusion, while no fusions were detected in any of the ALCLs (n=9). One of 26 CTCL and 1/6 ATLL cases were found to be positive. None of the non-cutaneous extranodal PTCL tested (18 ENKTCL, ten enteropathy associated T-cell lymphoma [EATL], three hepatosplenic T-cell lymphoma [HSTL]) were positive. A total of 15 fusions were amplified; the most common rearrangement being *ICOS(ex1)_CD28(ex2)* (11/15; 73% of the fusions), followed by *CTLA4(ex3)_CD28(ex4)* (3/15 fusions) while *CTLA4(ex2)_CD28(ex4)* was identified in a single CTCL sample. Interestingly, two cases (one TFH-derived PTCL and one CTCL) harbored two different *CD28* RNA fusions, an observation previously made in one ATLL sample.¹

All *CD28* chimeric proteins are expected to comprise the cytoplasmic portion of *CD28*, accounting for signal transduction and a part or the totality of the transmembrane and extracellular domains of *CTLA4*, *ICOS* or *CD28* (Figure 1A). Depending on the fusion partner, their expression is under the control of the regulatory elements of *CTLA4* or *ICOS*.^{1,8} In normal T cells, following TCR activation, *CD28* expression is downregulated while the expression of *CTLA4* and *ICOS* is induced.¹⁵ In neoplastic

Table 1. Distribution of *CD28* RNA fusions detected by RT-PCR in different peripheral T-cell lymphoma entities.

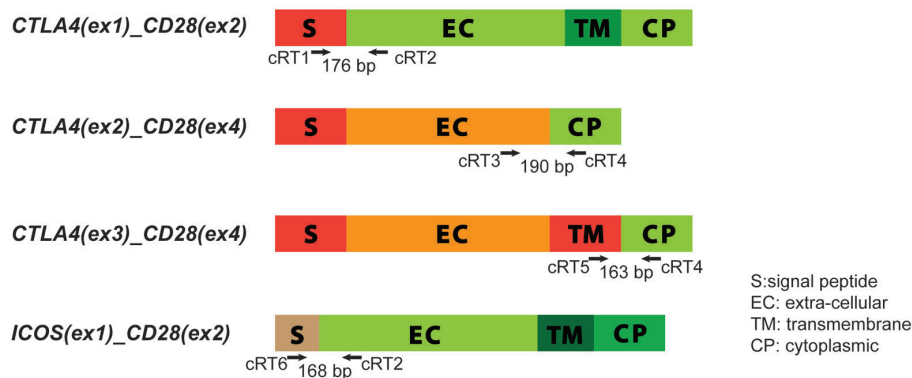
	All fusions	<i>CTLA4(ex3)_CD28(ex4)</i>	<i>CTLA4(ex1)_CD28(ex2)</i>	<i>CTLA4(ex2)_CD28(ex4)</i>	<i>ICOS(ex1)_CD28(ex2)</i>	Dual fusions
All PTCLs (273)	13/273 (4.8%)	3/273	0/273	1/273	11/273	2/273
AITL (110) TFH-like PTCL (24) F-PTCL (4)	9/138 (6.5%)	2/138	0/138	0/138	8/138	1/138*
PTCL, NOS (63)	2/63 (3.1%)	0/63	0/63	0/63	2/63	0/63
ALCL (9)	0/9 (0%)	0/9	0/9	0/9	0/9	0/9
ENKTCL (18)	0/18 (0%)	0/18	0/18	0/18	0/18	0/18
EATL (10)	0/10 (0%)	0/10	0/10	0/10	0/10	0/10
HSTL (3)	0/3 (0%)	0/3	0/3	0/3	0/3	0/3
CTCL (26)	1/26 (3.8%)	1/26	0/26	1/26	0/26	1/26
ATLL (6)	1/6 (17%)	0/6	0/6	0/6	1/6	0/6

*This case harbored *CTLA4(ex3)_CD28(ex4)* and *ICOS(ex1)_CD28(ex2)* RNA fusions. PTCL: peripheral T-cell lymphoma; AITL: angioimmunoblastic T-cell lymphoma; TFH-like PTCL: follicular helper T-cell like PTCL; F-PTCL: follicular PTCL; PTCL, NOS: PTCL, not otherwise specified; ALCL: anaplastic large cell lymphoma; ENKTCL: extranodal natural killer/T-cell lymphoma, nasal-type; EATL: enteropathy associated T-cell lymphoma; HSTL: hepatosplenic T-cell lymphoma; CTCL: cutaneous T-cell lymphoma; ATLL: adult T-cell lymphoma/leukemia.

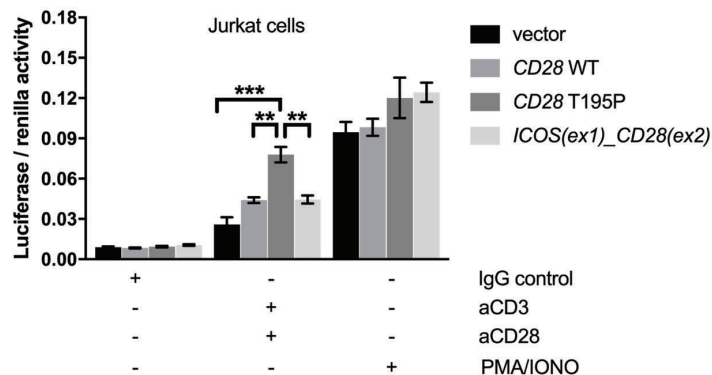
cells harboring *CD28* fusions, it can be anticipated that signals which normally induce the expression of *CTLA4* or *ICOS*, would induce the expression of the fusion proteins whose triggering mimics *CD28* signalling. Thus, the overall consequence of these fusions would be continuous or prolonged *CD28* co-stimulatory signaling. It has been demonstrated that Jurkat cells transduced with the *CTLA4(ex3)_CD28(ex4)* fusion showed enhanced proliferation and interleukin (IL)-2 production upon *CTLA4* binding.^{8,9} To further investigate the signaling induced by the *ICOS(ex1)_CD28(ex2)* fusion, we used a nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B)

luciferase-based reporter assay to assess its activity in comparison to mutant (T195P) and wild-type *CD28* (Figure 1B). The chimeric protein is composed of the *ICOS* signal peptide fused to the extracellular, transmembrane and cytoplasmic domains of *CD28*. In the mature form, the signal peptide is cleaved, resulting in *CD28* expression at the cell surface. Upon *CD3* and *CD28* co-stimulation, *CD28_T195P* induced higher levels of luciferase expression than *CD28_WT* ($P=0.01$) (as previously described),² while the *ICOS(ex1)_CD28(ex2)* fusion delivered an activatory signal similar in amplitude to that of the *CD28_WT* form (Figure 1B).

A



B



C

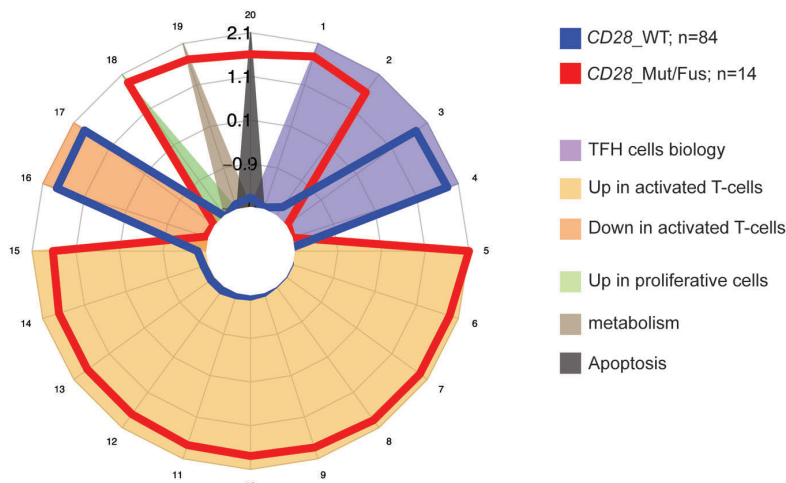


Figure 1. Biological impact of *CD28* alterations. (A) Schematic representation of the different *CD28* RNA fusions and PCR design for their screening. Arrows indicate the approximate positions of oligonucleotide primers on the indicated RNA fusions. (B) NF- κ B luciferase reporter assay in Jurkat cells monitoring the activity of a *CD28* T195P activating mutant and the *ICOS(ex1)_CD28(ex2)* fusion, compared with *CD28* WT. Data are represented as mean \pm SEM from four independent experiments. (C) Spider plot representation of gene sets differentially enriched in patients with (red) ($n=14$) or without (blue) ($n=84$) alterations (mutations or fusions) in *CD28*. A total of 304 signatures, including 23 of 50 hallmark signatures and nine signatures from the curated and immunogenic signature collection of the Molecular Signatures Database (MSigDB), all signatures of interest in lymphoid biology (Signatures database, Staudt's lab), and two manually annotated sequences linked to TCR signalling were tested. The list of signatures that reached statistical significance is provided in *Online Supplementary Table S2*. WT: wild-type; Mut/Fus: mutations or fusions.

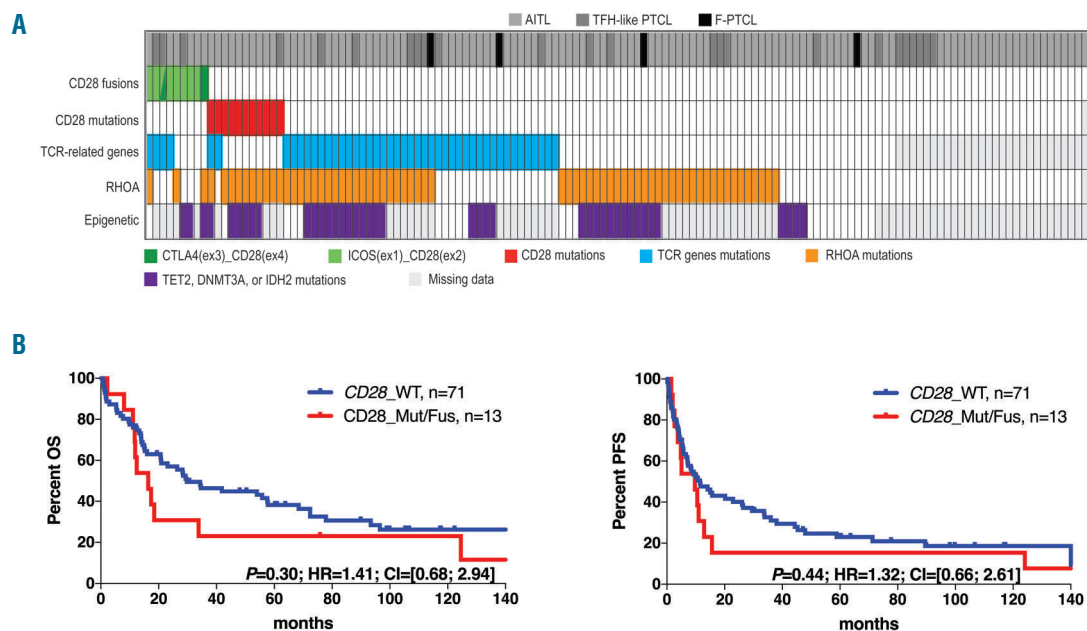


Figure 2. CD28 mutations and fusions in AITL and other TFH-derived PTCLs. (A) Landscape of genetic alterations of CD28, other TCR signaling-related (*PLCG1*, *CTNNB1*, *GTF2I*, *PIK3R1*, *PDPK1*, *VAV1*, *FYN*, *CARD11*, *KRAS*; *STAT3*, *LCK*, *TRAF6*, *AKT1*, *PIK3R5*, *VAV2*, *MAPK3*, *PIK3CA*), *RHOA*, and epigenetic modifiers (*TET2*, *DNMT3A* or *IDH2*) in 138 AITL and other PTCLs of TFH derivation. (B) Overall survival (OS; left panel) and progression-free survival (PFS; right panel) of patients with (red) or without (blue) CD28 mutations or fusions. Analyses are restricted to the 84 patients with AITL or other PTCLs of TFH derivation treated with anthracyclin-based chemotherapy. TFH-like PTCL: follicular helper T-cell like peripheral T-cell lymphoma; F-PTCL: follicular PTCL; AITL: angioimmunoblastic T-cell lymphoma.

Taking advantage of available transcript profiles for a large subset of AITL and other TFH lymphomas, we sought to assess the impact of CD28 genetic alterations (mutations and fusions) on gene expression (Figure 1C and *Online Supplementary Figure S2*). No significant difference in CD28, CTLA4 or ICOS mRNA levels was observed between six fusion-positive and 96 fusion-negative cases which had associated gene expression profiling data. Next, considering those samples harboring CD28 mutations or fusions (referred to as CD28_Mut/Fus) together versus those wild-type (WT) for CD28, the analysis of the top 100 genes differentially expressed showed the up-regulation of genes involved in PI3K, MAPK or NF- κ B signalling pathways, in actin cytoskeleton remodeling, in metabolism or apoptosis in CD28_Mut/Fus cases (*Online Supplementary Figure S3*). Accordingly, gene set enrichment analyses (GSEA) showed that the molecular signatures of CD28_Mut/Fus were differentially enriched in 20 gene sets as compared to CD28_WT, reflecting higher T-cell activation, proliferation or metabolic activity (Figure 1C and *Online Supplementary Table S2*).

We then examined how CD28 fusions integrate into the mutational landscape and impact outcome in AITL and other PTCLs of TFH derivation. Figure 2A depicts the distribution of CD28 alterations and mutations in other TCR signaling-related genes (subsequently called TCR-related genes), RHOA and epigenetic modifiers (*TET2*, *DNMT3A* or *IDH2*) in the entire cohort of AITL, TFH-like PTCLs and F-PTCLs tested for CD28 fusions (138 cases in total). CD28 fusions (9/138) and point mutations (11/138) were mutually exclusive and altogether present in 15% of the samples. While the majority of CD28 point mutations (10/11 cases, 91%) were found in AITL, six of

nine CD28 RNA fusions (67%) were present in other TFH PTCLs. CD28 mutations were mainly found in RHOA-mutated cases (10/11; 91%), but were virtually exclusive to mutations in other TCR-related genes (9/11; 82%). Conversely, CD28 fusions co-occurred with RHOA mutations in only three of nine cases (33%) whereas four of nine fusion-positive cases (44%) had mutations in other TCR-related genes.

The 84 patients who received intent-to-treat anthracyclin-based induction chemotherapy were considered for clinical outcome analysis. Of these, seven patients had CD28 mutations and six harbored a CD28 fusion. Of six CD28 fusion-positive patients, five relapsed and four died within the first year after diagnosis (two from disease, one from treatment toxicity and one from unknown causes). Considering 13 CD28_Mut/Fus versus 71 CD28 WT patients, no significant difference was observed in overall survival (OS) or progression free survival (PFS) (Figure 2B). However, focusing on the subset of 59 TFH-derived PTCL previously characterized for mutations in a large panel of TCR-related genes who received an anthracyclin based regimen, three patients carrying at least one alteration (mutation or fusion) in one or more gene(s) related to TCR signalling other than RHOA (TCR_Mut/Fus), (36 patients, 61%) showed a trend towards shorter PFS ($P=0.07$) compared to other patients who carried RHOA mutations only (RHOA_Mut) (14 patients, 24%) or were WT for all tested genes (TCR_WT) (nine patients, 15%) (*Online Supplementary Figure S4*). These data expand our previous findings of the negative connotation of these alterations on patient outcome.

In conclusion, our findings definitively confirm that the CD28 RNA fusions with CTLA4 or ICOS are overall rare

events in PTCL (4.8%). This is unambiguously demonstrated by the screening of a large cohort of frozen cases in two different laboratories. Specifically, the prevalence of the *CTLA4(ex3)_CD28(ex4)* fusion (found in two TFH-PTCLs and one CTCL) was only 1% in our cohort. The most common fusion was *ICOS(ex1)_CD28(ex2)* (73% of fusions), which was previously reported in one out of 20 AITL cases subjected to RNAseq.² In AITL and other TFH-derived PTCL, where these fusions are most prevalent (6.5% of cases), they are mutually exclusive to *CD28* mutations. Thus, taking into account both point mutations and fusions, *CD28* ranks second after *RHOA* among the altered TCR-related gene in the group of PTCLs of TFH derivation, found in 15% of the patients. Considering the patients with mutations in TCR-related genes other than *RHOA*, 29% of them harbor *CD28* alterations. Finally, within the limit of this retrospective series, the cases harboring *CD28* Mut/Fus did not differ from others in terms of OS or PFS, and had gene expression programming (GEP) enriched in signatures reflecting higher T-cell activation and higher proliferation.

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