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

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Supplementary information

Patients and tumor samples

Frozen samples of diagnostic tissues biopsies from 273 PTCLs with associated clinical annotations were collected in the framework of the Tenomic consortium of the LYSA. Diagnoses were confirmed by at least 2 expert hematopathologists according to the criteria of the 2016-7 WHO classification. Criteria used to define TFH-like PTCL-NOS were previously reported [15]. The study was approved by the local ethics committee (CPP Ile de France IX 08-009). In total, our study group comprised 110 AITL and 28 other nodal lymphomas of TFH derivation (24 TFH-like PTCL[12] and 4 follicular (F)-PTCLs)(Supplemental Figure S2), 63 PTCL-NOS, 26 CTCL, 18 ENKTCL, 10 enteropathy-associated T-cell lymphomas (EATL), 7 ALK-negative and 2 ALK-positive anaplastic large cell lymphomas (ALCL), 6 ATLL and 3 hepatosplenic T-cell lymphomas (HSTL) Among 138 TFH-derived PTCL cases, 85 were previously characterized by targeted deep sequencing for mutations in TCR signalling related genes (*CD28, PLCG1, CTNNB1, GTF2I, PIK3R1, PDPK1, VAV1, FYN, CARD11, KRAS; STAT3, LCK, TRAF6, AKT1, PIK3R5, VAV2, MAPK3, PIK3CA, RHOA*)[3] and 53 were sequenced for a restricted panel of genes (*CD28, PLCG1, CTNNB1, PIK3R1, PDPK1, CARD11 and RHOA*) following the same protocol.

RT-PCR design and methods

Total mRNA were extracted from frozen tumoral samples using Trizol (Thermofisher, Waltham, Massachusetts, USA) and quantified using Qubit (Thermofisher). 500 ng were used to perform Retro-Transcription with the First strand cDNA synthesis kit from Thermofisher using random hexamers. PCR amplification (33 cycles) of fusions and beta-actin were then performed using 1 uL of RT and 300 nM primers (Eurogentec, Liège, Belgium) listed in the supplementary table S1 in a final volume of 20 µL. The resulting PCR products were analyzed by 2% agarose gel electrophoresis. *CTLA4*(ex3)_*CD28*(ex4); *CTLA4*(ex1)_*CD28*(ex2) and *ICOS*(ex1)_*CD28*(ex2) fusions were cloned from positive samples (Dr. Kataoka and Dr. Ko) into a eukaryotic expression vector. They were transduced into HEK293T cells to generate positive controls. To test sensitivity, RNA from pCMV6_empty transduced HEK293T cells were diluted or not with 10% to 0.0001% of RNA isolated from $5x10^6$ cells expressing *CD28* fusions. RT-PCR was then performed as described

above. Fusions were detected using as little as to 0.0001% of RNA from CD28 fusion-expressing cells (data not shown). Beta-actin was amplified in all samples as an internal control. Samples were shared equally between two different laboratories for the fusions screening. Then, all the positive cases and 58 randomly selected negative cases (21%) were crossvalidated in the two laboratories. All results were concordant between our two laboratories, with exception of one sample which was positive in one lab and negative in the other. We performed new RT-PCR on this sample starting from RNA in the two labs and found it negative, suggesting that this false positive was due to contamination.

Luciferase-based activity assay.

$10x10^6$ Jurkat cells (ATCC, clone E6-1) were electroporated with indicated constructs (10 ug) together with constructs for an *NF-kB*-Firefly luciferase reporter (5 ug), SV40 Large T Antigen (5 ug) and a Renilla luciferase construct for normalization (200 ng). 24h later, 250,000 cells/mL were stimulated in triplicates overnight with IgG control (10 ug/mL), PMA (20 ng/mL, SIGMA, Saint-Louis, Missouri, USA) and ionomycin (1 uM, SIGMA), or a combination of anti-CD3 (10ug/mL, B-B11 clone from Diaclone, Besançon, France), anti-CD28 (10ug/mL, B-T3 clone, Diaclone) and cross-linking antibody (9ug/mL, goat anti-mouse IgG (H+L) from Jackson Immunoresearch, West Grove, PA, USA). Firefly and Renilla luciferase activities were measured using the dual-glo kit from Promega (Madison, WI, USA), following the manufacturer's instructions.

Bioinformatical analysis

Gene expression profiles were produced using the Affymetrix U133 Plus 2.0 microarray chip[3,12]. These were normalized using the Robust Multiarray Average (RMA) implementation in the Affy package from R-Bioconductor. Differential expression of genes was calculated using the R package limma. Biological variation due to the presence or absence of *RHOA* mutations (G17V or K18N) was controlled for by adding a binary column into the design matrix of the linear model. This took the value 1 if *RHOA* was mutated and 0 otherwise.

Pathway analysis was performed using the Broad Institute's Gene Set Enrichment Analysis (GSEA) software on the pre-ranked setting. For each, this was done using a gene list ordered by the negative log of the raw p-value output from limma, multiplied by the sign of the fold

change, indicating the degree of difference between the two conditions in question. A total of 344 signatures were tested, including 271 signatures of interest in lymphoid biology (Staudt, lymphochip.nih.gov/ signaturedb/) and 73 from the curated and immunogenic signatures collections in the Molecular Signatures Database (MSigDB, software.broadinstitute.org/gsea/msigdb/). GSEA pre-ranked was run using these gene sets with command-line parameters "-norm meandiv -nperm 1000 -scoring_scheme weighted -metric Signal2Noise -make_sets true -rnd_seed timestamp -set_max 500 -set_min 5". Relevant pathways among those with a significant FDR (under 0.05) were selected.

Radar plots were produced using the radarchart function from the R package, fmsb. The Normalised Enrichment Score (NES) produced by GSEA was plotted for each selected gene set.

Supplementary Figure S1. RT-PCR analysis of *CD28* RNA fusions. Agarose gel electrophoresis of RT-PCR products. PCR products were analyzed by 2% agarose gel electrophoresis. *CTLA4*(ex3)_*CD28*(ex4); *CTLA4*(ex1)_*CD28*(ex2) and *ICOS*(ex1)_*CD28*(ex2) fusions were cloned from positive samples into an eukaryotic expression vector. They were transduced into HEK293T cells to generate positive controls. HEK293T cells transduced with empty vector were used as a negative control (CTRL-). Here, positive control (CTRL+) is a mix of RNA from HEK293T cells transduced with empty vector (99,99%) or cloned fusion (0.01%). Beta-actin was amplified in all samples as an internal control and produced the higher molecular weight fragment. Arrows on the right indicate the expected length of amplicons. As *CTLA4*(ex2)_*CD28*(ex4) was not cloned, *CTLA4*(ex3)_*CD28*(ex4) was used as control since primers amplifying *CTLA4*(ex3)_*CD28*(ex4), amplified also *CTLA4*(ex2)_*CD28*(ex4), with a higher molecular weight PCR product. No positive sample was observed for *CTLA4*(ex1)_*CD28*(ex2) (not shown).

Supplementary Figure S2. Summary of the cohort of TFH-derived PTCLs examined for CD28 fusions. The cohort of 138 TFH-derived PTCL samples screened by RT-PCR for *CD28* RNA fusions comprised 85 cases previously analysed by targeted deep sequenced for a panel of TCR signalling-related genes (*CD28, PLCG1, CTNNB1, GTF2I, PIK3R1, PDPK1, VAV1, FYN, CARD11, KRAS; STAT3, LCK, TRAF6, AKT1, PIK3R5, VAV2, MAPK3, PIK3CA, RHOA*)[3] and 53 additional cases sequenced for a restricted panel of genes (*CD28,*

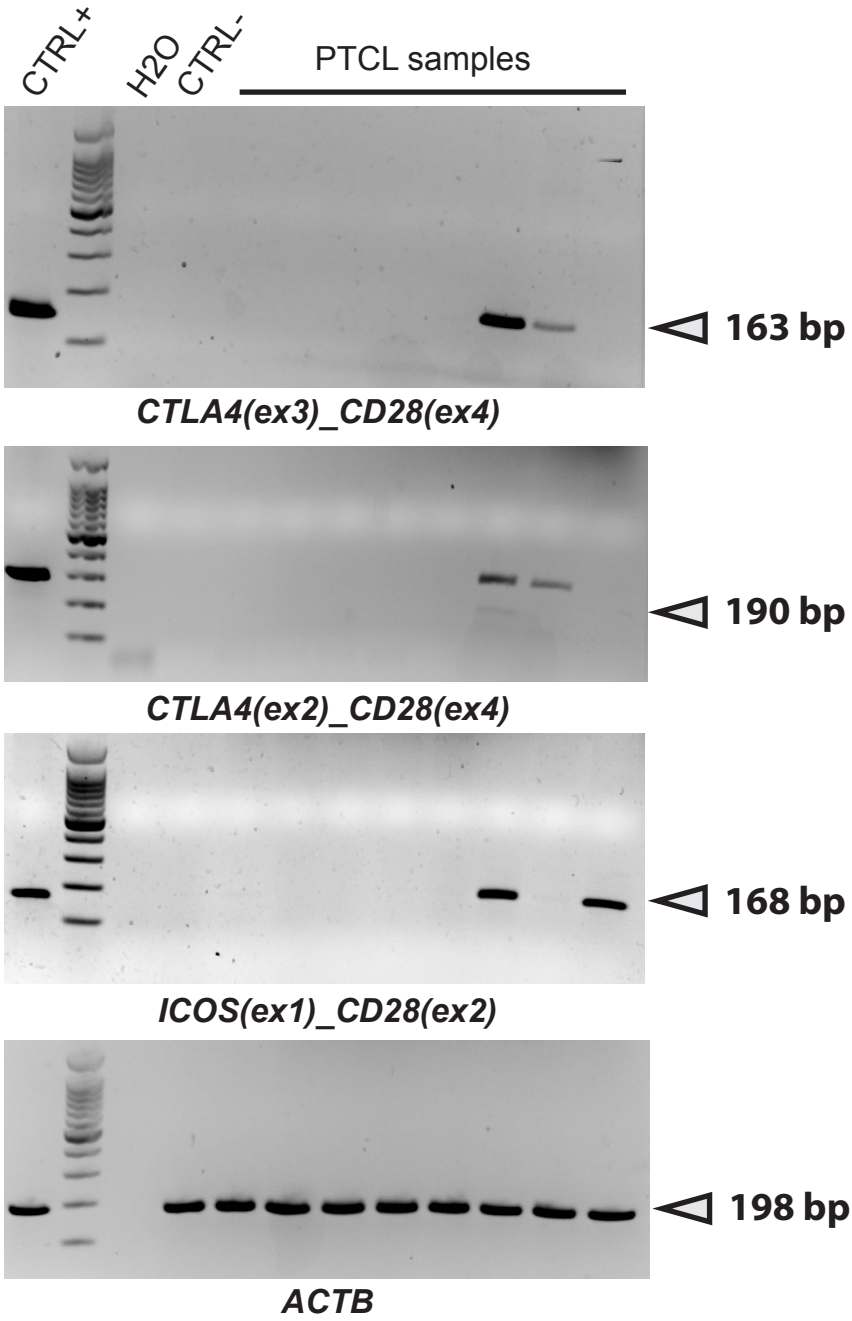
PLCG1, CTNNB1, PIK3R1, PDPK1, CARD11 and RHOA) following the same protocol. In total, 24 of the 53 additional cases could be classified as "TCR_signaling_mutated" while incomplete information on the remaining 29 cases precluded categorizing wild-type or mutated. Of the 109 cases with known TCR signalling related genes mutation status, 69 patients had received intent-to-treat anthracyclin-based chemotherapy and 92 had available GEP data.

Supplementary Figure S3. Global gene expression profiles comparison between TFH-derived PTCL samples harbouring or not CD28 genetic alterations. Heatmap of mRNA level of the top 100 differentially expressed genes in samples with or without alterations (mutations or fusions) in *CD28*. mRNA levels of *CD28, CTLA4* and *ICOS* are showed at the bottom.Genes relative to PI3K, MAPK, NFkB or Calcuim signalling pathways (*), those relative to cytoskeleton remodelling ($), those relative to metabolism (£) and those relative to protein folding (#) are highlighted.

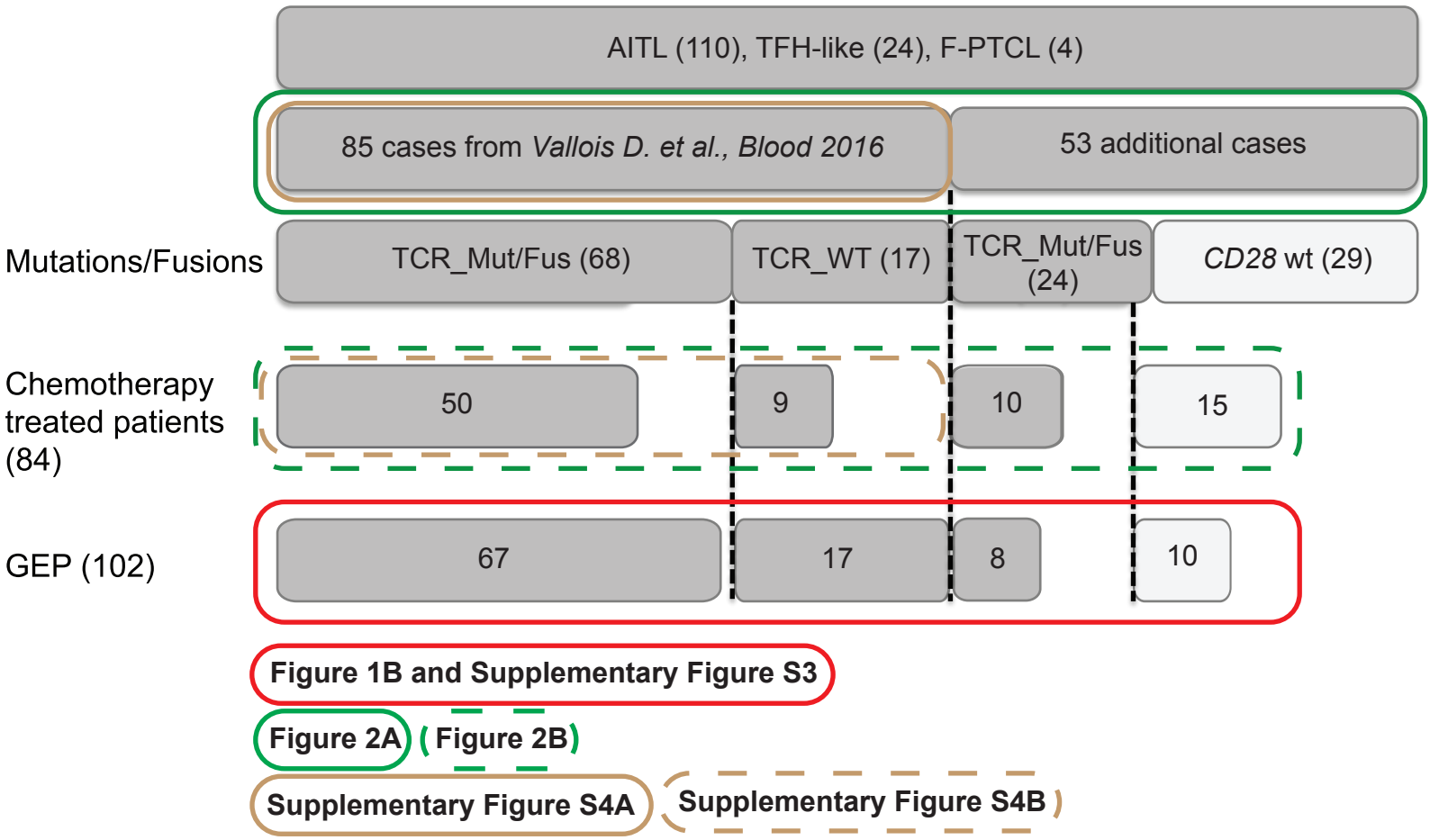
Supplementary Figure S4. Updated mutational status of TCR signalling-related genes in 85 TFH-derived PTCLs. (A) Mutational landscape Genes other than *RHOA* are ranked by decreasing alteration (mutation or fusion) frequency and show an essentially mutually exclusive pattern. In total, 53% of cases were altered in one or several TCR-related gene(s) other than *RHOA*; 27% were mutated in *RHOA* only, and 20% harboured no mutation in any of the genes tested. Five samples harboured *CD28* fusions, which are mutually exclusive to *CD28* mutations. Overall, *CD28* was therefore the most frequently altered TCR-related gene (13/85; 15%). When the 45 *TCR*_Mut/Fus; *RHOA*_Mut or WT cases were specifically considered as a denominator population, the frequency of *CD28* alterations was 29% (13/45). (B) *CD28* fusions contribute to clinical impact of genetic alterations in TCR signalling-related genes other than *RHOA*. Overall survival (left panel) and progression-free survival (right panel) of patients with (red) or without (blue) mutations and fusions in TCR signalling-related genes other than *RHOA*. Analyses are restricted to the 59 patients treated with anthracyclin-based chemotherapy. Patients harbouring genetic alterations in *CD28* or TCR signalling-related genes other than *RHOA* show a trend towards shorter PFS (10.12 vs 33.68 months; p=.07) than patients with *RHOA* mutations only or no mutation in tested genes.

Supplementary Table S1. List of RNA-specific PCR primer.

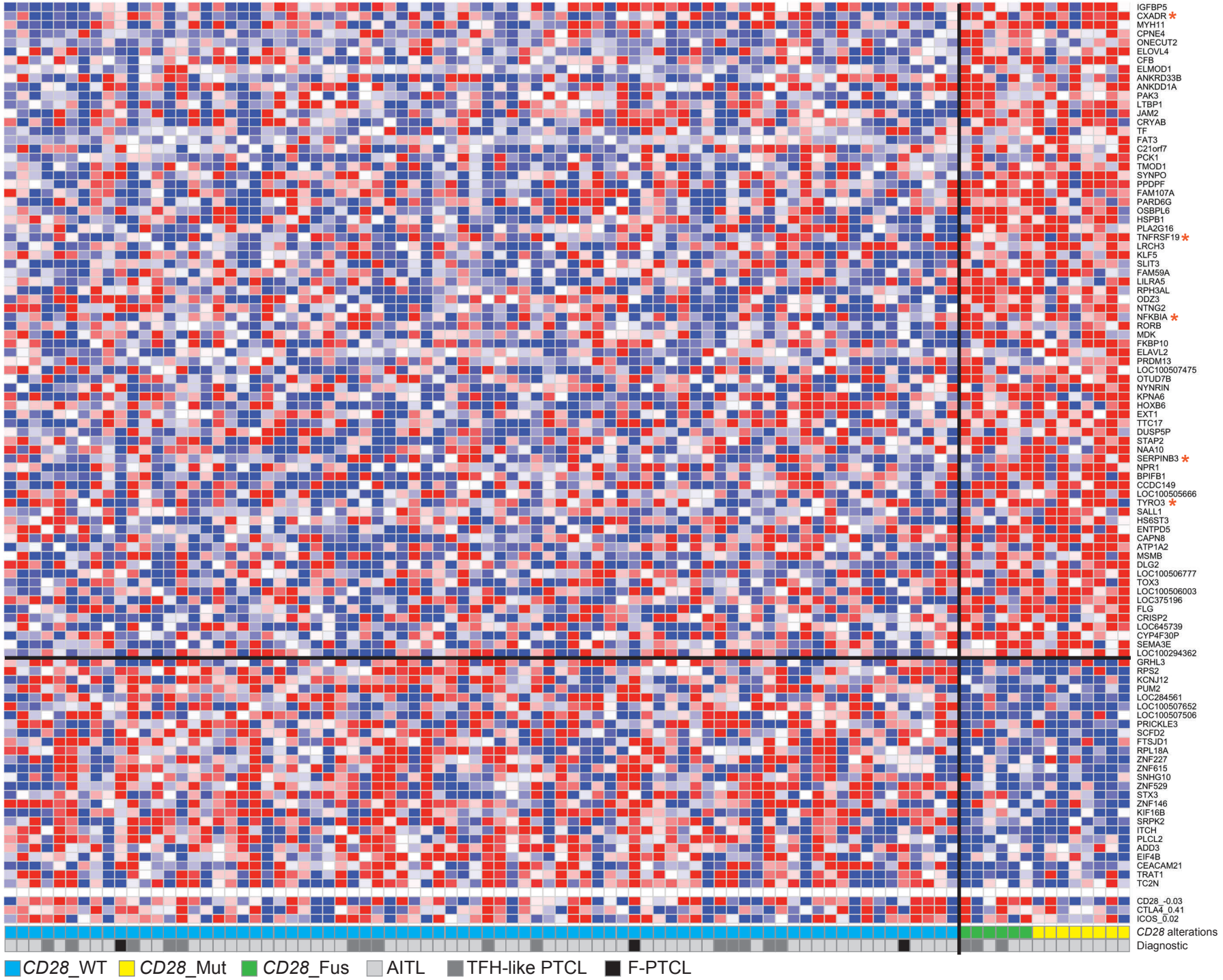
Supplementary Table S2. List of genes signatures differentially expressed comparing by GSEA analysis GEP profiles of samples according to the mutational status for *CD28* (mutated/fusion vs wild type). NES: Normalized Enrichment Score; FDR: False Discovery Rate.



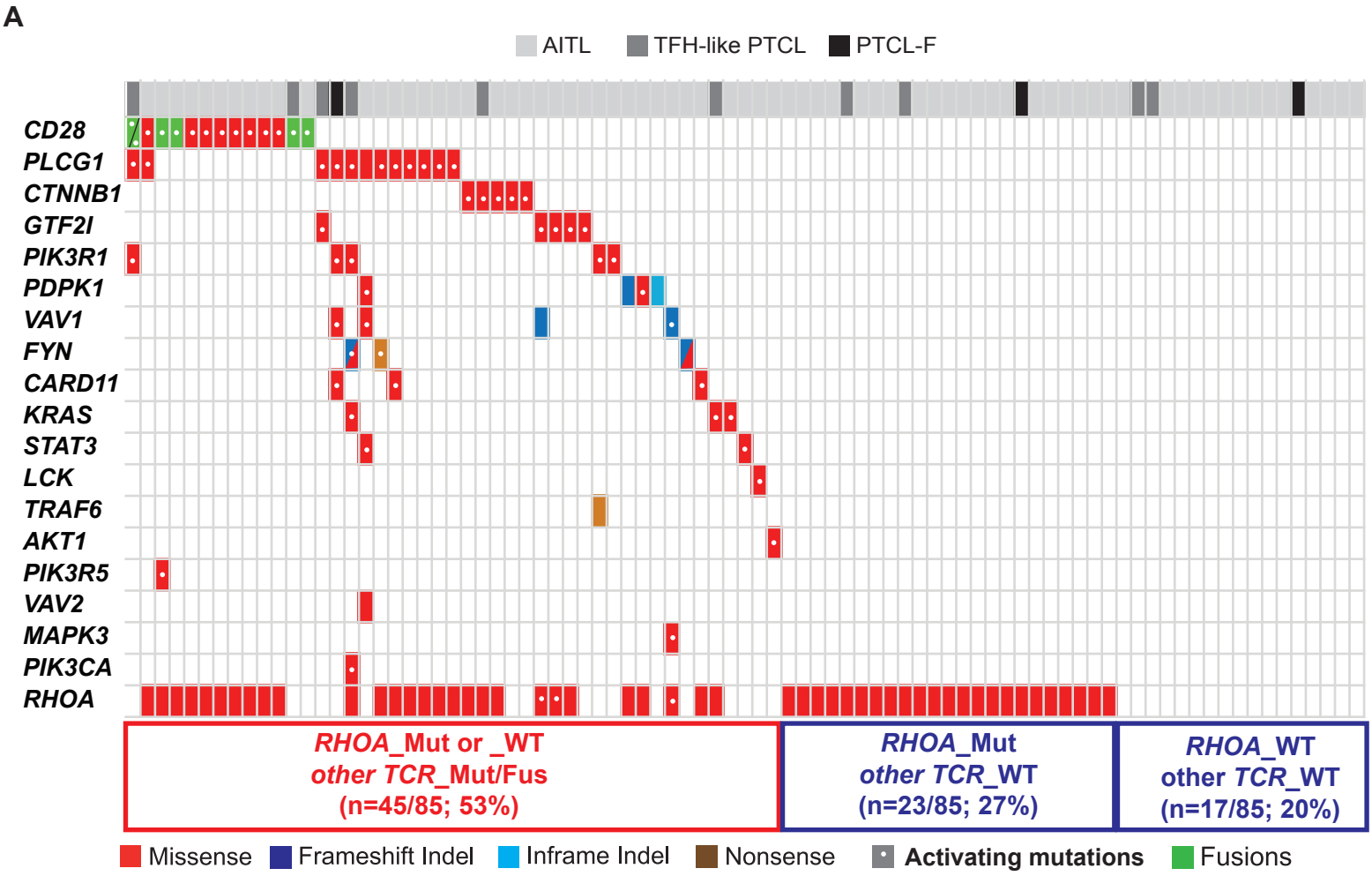
Supplemental Figure S1

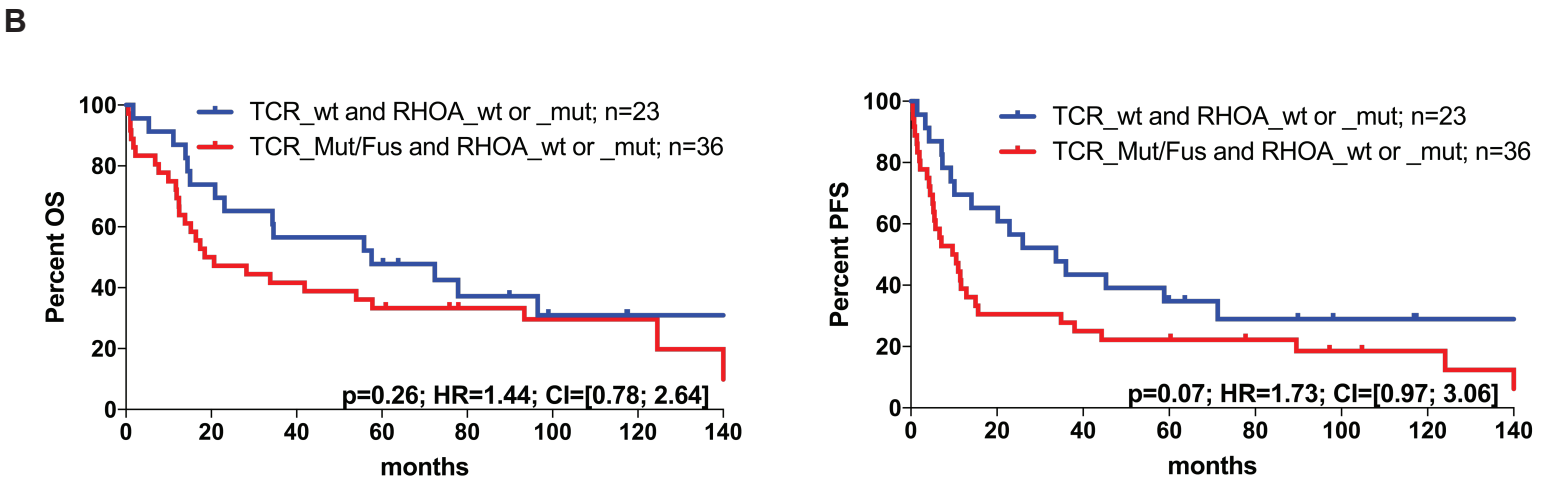


Supplemental Figure S2



Supplementary Figure S3





Supplementary Figure S4

target	5' gene	5' exon	3' gene	3' exon	amplicon length (bp)	Forward primer sequence	Forward primer name	Reverse primer sequence	Reverse primer name
CTLA4(ex1)_*CD28*(ex2)	*CTLA4*	exon 1	*CD28*	exon 2	176	ATGGCTTGCCTTGGATTTCAG	cRT1	CAGCTAAGGTTGACCGCATTGT	cRT2
CTLA4(ex2)_*CD28*(ex4) *	*CTLA4*	exon 2	*CD28*	exon 4	190	ACGGGACTCTACATCTGCAAG	cRT3	GGGCTGGTAATGCTTGCGGGTGGGC	cRT4
CTLA4(ex3)_*CD28*(ex4)	*CTLA4*	exon 3	*CD28*	exon 4	163	GATCCTTGCAGCAGTTAGTTCGGGG	cRT5	GGGCTGGTAATGCTTGCGGGTGGGC	cRT4
ICOS(ex1)_*CD28*(ex2)	*ICOS*	exon 1	*CD28*	exon 2	168	TTTGAACACTGAACGCGAGGAC	cRT6	CAGCTAAGGTTGACCGCATTGT	cRT2
control	*ACTB*	exon 3	*ACTB*	exon 4	198	CCAACCGCGAGAAGATGACC	cRT7	GGTCCAGACGCAGGATGGC	cRT8

* Amplifies also *CTLA4*(ex3)_*CD28*(ex4)

Supplementary table S1. List of RNA-specific PCR primer

Gene sets number	Gene sets names	Functional group	SIZE	NES	FDR q-val
1	GSE21379_WT_VS_SAP_KO_TFH_CD4_TCELL_UP	TFH cells	196	1.8138809	0.008165
2	GSE21380_TFH_VS_GERMINAL_CENTER_TFH_CD4_TCELL_UP		197	1.6204163	0.0475994
3	GSE40068_BCL6_POS_VS_NEG_CXCR5_POS_TFH_DN		196	-1.8082237	0.0143964
4	GC_T_HELPER_UP_KIM		59	-1.8644149	0.0072206
5	KRAS_UP	cell activation	82	2.1264534	0
6	IFN_PMBC_4X_UP		80	1.9182855	0.0034189
7	HRAS_OVEREXPRESSION_2X_DOWN		70	1.9180064	0.0029305
8	HIF1ALPHA_1.5X_UP		160	1.9162891	0.0026755
9	CD8_T_EFFECTORUP_MEMORYIM_NAIVEDN		28	1.8591489	0.0047524
10	HIF1ALPHA_2X_UP		64	1.8222	0.0080304
11	IFN_PMBC_2X_UP		135	1.8005989	0.0099292
12	BLOOD_MODULE-3.1_INTERFERON_INDUCIBLE		89	1.7473357	0.0179772
13	HRAS_OVEREXPRESSION_4X_UP		94	1.7421672	0.0172706
14	IFN_META		305	1.7389337	0.0168412
15	GC_B_CELL_BLLOW_DLBCLHIGH		47	1.6516587	0.0384158
16	NOTCH_T-ALL_DOWN_PALOMERO		14	-1.7883854	0.0163043
17	MYC_RNAI_OCILY3		54	-1.8265897	0.0124387
18	PROLIFERATION_NODE1618	Proliferation	58	1.901234	0.0030881
19	SREBP1A&2_UP_SCAP_DEP	Metabolism	32	1.743044	0.0180254
20	REACTOME_APOPTOSIS_INDUCED_DNA_FRAGMENTATION	Apoptosis	13	1.6362503	0.0416291

Supplementary table S2. List of genes signatures differentially expressed comparing by GSEA analysis GEP profiles of samples according to the mutational status for CD28 (mutated/fusion vs wild type). NES stands for Normalyzed Enrichment Score. FDR stands for False Discovery Rate