The immunomodulatory molecule TIGIT is expressed by chronic lymphocytic leukemia cells and contributes to anergy

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Supplemental Material

The TIGIT/CD226/CD155 immunomodulatory axis is deregulated in CLL and contributes to B-cell anergy

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Supplemental methods

RNA extraction and qRT-PCR

RNA was extracted as described[1]. qRT-PCR was performed using the CFX384 instrument and analyzed with the CFX Maestro Software (Biorad). Primers for *TIGIT* (Hs00545087_m1), *CD226* (Hs00170832_m1), *CD155* (PVR, Hs00197846_m1), *IL10* (Hs00961622_m1) and *B2M* (Hs00984230_m1) were all from Thermofisher. The data were analyzed with the $2^{-\Delta\Delta Ct}$ method, to calculate the relative expression of the gene under analysis. For each gene, expression levels were computed as the difference (ΔCT) between the target gene CT and *B2M* CT. Values were normalized over those of Control RNA (Life Technologies, Thermofisher) (ΔΔCT), added in each experiment for calibration purposes, and expressed in linear or in logarithmic scale.

Multispectral IHC, confocal microscopy and image analysis

IHC studies were performed on CLL LN tissues (Figure 1A n=6; Figure 5B, n= 3 UM-CLL; n=3 M-CLL) and reactive LN controls (Figure 1A n=4), as described[2]. The clinical characteristics of patients with mutated and unmutated IGHVs are presented in supplemental Table 1. Briefly, 4-5 µm sections prepared from FFPE human tissues were deparaffinized prior to antigen retrieval in citrate buffer. After blocking (5% donkey serum), primary antibodies were incubated overnight at 4 °C. The primary antibodies used for this study are the following: goat anti-human CD20 (ab194970, Abcam), rat anti-human ki67 (ab156956, Abcam), rabbit anti-human TIGIT (A700-047, BETHYL), rabbit anti-human CD226 (A700-063, BETHYL). Following primary antibody incubation and washing steps (0.05% Triton PBS), secondary antibody (donkey) staining was performed for 1 h at room temperature. All secondary antibodies were obtained from Jackson Immunoresearch: DyLightTM 405 AffiniPure Donkey anti-goat, Alexa Fluor® 488 AffiniPure Donkey anti-rabbit, Alexa Fluor® 647 AffiniPure Donkey anti-rat. The specificity of staining was optimized and controlled by using appropriate dilutions of primary or secondary staining alone. Slides were sealed with coverslips using mounting solution FluorSaveTM reagent (Merck Millipore) and imaged within two days. Medial optical section images were captured with an A1R confocal microscope using a 20X objective with NIS-elements imaging software (Nikon). Detectors were set to detect an optimal signal below saturation limits. Fluorescence was acquired sequentially to prevent passage of

fluorescence from other channels (DU4 sequential acquisition). Image sets to be compared were acquired during the same session.

The percentage of LN $CD20^+$ CLL cells expressing TIGIT or CD226 in M or UM CLL cases was measured with the Imaris image analysis software v.9.7.2 (Bitplane). The surface tool was used to threshold the fluorescent signals and create two surfaces: i) $CD20^+$ cells (blue fluorescent channel) and ii) $CD20^+TIGIT^+$ / $CD20^+CD226^+$ (green fluorescent channel) double-positive cells (colocalization channel). For the B cell proliferation analysis (Ki67 expression), the area (μm^2) of the colocalization signal of $CD20^+TIGIT^+$ with Ki67 and $CD20^+CD226^+$ with Ki67 was calculated. Three-five confocal images from representative areas were analyzed per patient tissue sample.

Phosflow assay

Phosflow assay to evaluate BTK phosphorylation upon BCR stimulation was performed as described[3]. Briefly, CLL cells (10^6) were thawed and plated over night to let them recover from anergy due to *in vivo* chronic BCR stimulation. After that, 10^6 cells were stimulated with 5 μg/ml anti-IgM (αIgM, Southern Biotech) for 5 minutes at 37 °C. Where indicated, cells were pre-treated (1 hour, on ice) with 5 μg/ml rhTIGIT-Fc or with αTIGIT or αCD226 blocking monoclonal antibodies (5 μg/10^6 cells, 30 minutes on ice), followed by 1 hour with rhCD155-Fc (5 μg/ml), before αIgM stimulation.

CpG/IL-15 stimulation

Stimulation of CLL cells proliferation with CpG/IL-15 was performed as described[4]. Briefly, $5x10^5$ CLL cells were cultured in a 96-well plate in RPMI 10% FCS and stimulated with 0.2 μM CpG ODN2006 and 15ng/ml of IL-15 for 6 days (replenished every 2 days). After stimulation, cell were partly used for surface staining of TIGIT and CD226, performed as previously described, and partly fixed and permeabilized with ice-cold 70% ethanol to proceed with intracellular staining of Ki67, following manufacturer's instructions. Details for reagents used are listed in Supplemental table 4.

IL-10 production

To assess IL-10 production, CLL cells were stimulated and stained as previously described[5]. Briefly, 10^6 cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50ng/ml) and ionomycin (1μg/ml) for 5 hours at 37 °C in the presence of a protein transport inhibitor cocktail (Invitrogen Ebioscience, Thermofisher) to prevent IL-10 secretion by CLL cells. Cells were washed and surface staining with anti-CD5 BB515 and anti-CD19 PECy7 (both from BD Biosciences) was performed. After that, cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) before staining with anti-IL10 APC (BD Biosciences).

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Supplemental Table1. CLL sample cohort. AGE= age at diagnosis; WBC= white blood cells (x10^3/μl); M=mutated; UM=unmutated; tri=trisomy; del=deletion.

SAMPLE ID	SEX	AGE	WBC	RAI	*IGHV*	FISH	*NOTCH1* mutations	CD5/CD19 (%)	$CD38^+$ (%)	$CD49d^+$ (%)	Notes
CLL #1	M	67	38,5	I	M	tri12; del11; del17	No	96,3	75,8	90,7	
CLL #2	F	51	17,7	I	UM	del13	No	92,5	35,9	34,9	*TP53* mut
CLL #3	M	61	63,3	I	M	del13	No	94,1	83,9	37,6	
CLL #4	M	79	24	0	M	del13	No	90,6	54,7	49,7	
CLL #5	F	56	17,2	0	M	del13	No	97,3	67,5	64,7	
CLL #6	M	65	61,3	II	M	del13	No	95,2	57,7	53,2	
CLL #7	M		4,7	0	UM	del11	No	98,4	75	48,4	
CLL #8	M		8,6	I	UM	del13	No	92	30,9	43,2	
CLL #9	F	61	16,9	0	UM	tri12	No	92,9	46,9	50,4	
CLL #10	M		5,4	I	M	tri12	No	98,9	55,8	76,4	
CLL #11	F	47	12,8	0	M	del13	No	87,3	49,6	42,7	
CLL #12	F	53	4,5	0	UM	tri12	Yes	59,5	49	59,4	
CLL #13	M	81		II	UM	normal	Yes	95,2	86,6	53,3	
CLL #14	M	69	18,7	II	M	del13	No	92,8	53,4	46,4	
CLL #15	F	65	480	0	UM	del13	Yes	96,6	51,5	79,5	
CLL #16	M	47	46,4	II	M	del13	No	92,8	48,4	61,9	
CLL #17	M	53	30,1	0	UM	del13	No	91,7	42,1	61,4	
CLL #18	M	55	14,9	0	UM	del13	No	96,1	62,6	55,8	
CLL #19	F		7,4	0	M	del13	No	74,7	41,5	34,9	
CLL #20	M		182,7	IV	UM	tri12; del17	No	93,6	45,7	68,6	
CLL #21	F	50	72,1	IV	M	tri12	No	92,8	91,9	93,6	
CLL #22	F	63	32,7	IV	UM	tri12	Yes	90,4	36,8	24,2	
CLL #23	M	49	56,5	II	UM	tri12	No	89,1	51,4	92,7	*TP53* mut
CLL #24	M	47	121,4	II	UM	tri12	Yes	94,7	25,3	50,4	
CLL #25	F		14,8	I	M	del13	No	94,5	31,9	15,7	

SAMPLE ID	SEX	AGE	WBC	RAI	*IGHV*	FISH	*NOTCH1* mutations	CD5/CD19 (%)	CD38^{+} (%)	CD49d^{+} (%)	Notes
CLL #26	F	65	43,4	I	M	normal	No	90,2	31,5	26,9	
CLL #27	F	65	69	II	M	normal	No	94,7	27,7	17,4	
CLL #28	M	67	16	0	M	del13	No	86,4	24,6	18	
CLL #29	M	79	14,8	III	UM	del11	No	95,7	66,5	29,4	
CLL #30	F	73	92	III	UM	tri12	No	82,5	84,1	97,6	
CLL #31	F		96,8	II	M	del13	No	88,8	37,3	30,2	
CLL #32	M	35	23	I	M	normal	No	79,4	30,5	14,3	
CLL #33	M	67	14,1	II	UM	tri12	Yes	92,1	82,7	61,6	
CLL #34	M	78	22,1	IV	UM	normal	No	96,8	40,9	54,2	
CLL #35	F	70	13	0	UM	normal	No	93,3	32,5	19,8	
CLL #36	M		8,7	II	UM	del13	No	69,7	73,7	95,3	
CLL #37	M	54		I	M	normal	No	96	51,3	87,8	
CLL #38	F	60	18	0	UM	normal	No	82,4	37,5	24,2	
CLL #39	M		13,3	I	UM	normal	No	91,4	9,1	1,1	
CLL #40	M	35		0	M	normal	No	93,2	41,6	37,5	
CLL #41	M	62	58	I	M	normal	No	95,8	34,8	26,7	
CLL #42	F	64	99,3	0	UM	del17	Yes	91,4	36,6	47,3	
CLL #43	M	50	23,1	II	M	tri12	No	81,9	29	73,2	
CLL #44	M	72	21,1	0	M	del13	No	72,7	20,4	10,4	
CLL #45	M		128	IV	M	del13	No	93,5	26,7	28,3	*TP53* mut
CLL #46	M	42	19,5	I	M	del13	No	84,4	18,3	17,9	
CLL #47	M	59	33	I	M	del17; del13	No	**99,6**	59,4	43,5	
CLL #48	M	51	175,1	II	M	normal	No	83,1	15,1	10,4	
CLL #49	F		32	I	UM	del13	No	90,1	22,6	31,3	*TP53* mut
CLL #50	M	64	31,1	I	UM	del13	No	84	19,5	16	
CLL #51				II	UM	del13	No	70,1	23,6	18,2	
CLL #52	M	73	38,2	0	M	normal	No	96,4	15	10,8	

SAMPLE ID	SEX	AGE	WBC	RAI	*IGHV*	FISH	*NOTCH1* mutations	CD5/CD19 (%)	CD38^{+} (%)	CD49d^{+} (%)	Notes
CLL #53	M	59	82,1	I	M	del13	No	87,5	19,3	19,1	
CLL #54	F	58	2,3	I	UM	del13	No	82,7	32,6	86,5	*TP53* mut
CLL #55	M	74	5,3	I	UM	del13	No	89,6	9,9	8,63	
CLL #56	M	66	1,7	I	UM	del13	No	92,4	44,6	14,7	
CLL #57	M	54	48,9	I	M	del13	No	90,5	22,6	67,9	*TP53* mut
CLL #58	F	77	3,5	0	M	normal	Yes	86,3	42,7	96,5	
CLL #59	F	80	1,5	0	UM	normal	Yes	96,1	3,2	13,7	
CLL #60	M	66		I	M	del13	No	93,1	0,2	8,21	
CLL #61	F	52	1	I	M	del13	No	84,5	0,1	8,11	
CLL #62	M	71	2	I	UM	del13	No	83	26,6	4,3	
CLL #63	F	63	1,6	I	M	del13	No	86,8	0,3	5,7	
CLL #64	F			0	M	normal	No	67,7	2,3	17,4	
CLL #65	M	82	3,2	0	UM	normal	No	76,8	2,5	37,8	
CLL #66	F	65	38	I	M	tri12	Yes	92,2	0,2	8,5	
CLL #67	M	63	80,2	II	M	normal	No	96,2	0,1	54,9	
CLL #68	M	53	15,7	I	M	del13	No	82,3	0,2	55,9	
CLL #69	F	82	36,3	I	M	del11	No	95	0,1	12	
CLL #70	F	74		I	M	del13	No	95,8	28,5	29,6	
CLL #71	F	50	28,8	I	UM	del13	tbd	91,2	0,2	28,6	
CLL #72	F	69	2,1	I	UM	del13	No	97,1	1	58,6	
CLL #73	F	69	2,3	I	M	del13	No	81,8	16	9,6	
CLL #74	M	62	1,1	0	UM	tri12	No	91,1	8	88,2	
CLL #75	M	55	3,3	I	M	del13	No	82,5	1	28,7	
CLL #76	M	52	21	0	UM	del13	No	98,3	78	23,7	
CLL #77	F	54	1,7	0	M	del13	No	80,5	17	31,4	
CLL #78	M	59	1,9	I	M	del13	No	84,2	1	20,5	
CLL #79	F		2	0	UM	del11	No	95,6	10	13,9	

SAMPLE ID	SEX	AGE	WBC	RAI	*IGHV*	FISH	*NOTCH1* mutations	CD5/CD19 (%)	CD38^{+} (%)	CD49d^{+} (%)	Notes
CLL #80	M	49	1,8	I	M	del13	No	81,8	16	11,6	
CLL #81	M	60	1,4	0	M	tri12	No	35,7	88	94,6	
CLL #82	M	61	61,7	I	M	del13	No	80,2	1,3	16,9	
CLL #83	F	63	30,6	0	UM	normal	No	90,5	4,3	14,2	
CLL #84	M	48	35,7	I	UM	del13	No	45	86,9	45,4	
CLL #85	F	42	40,1	I	UM	del13	No	90,4	79,3	98,3	
CLL #86	M	54	43,5	I	M	del13	No	95,2	1,8	16,7	
CLL #87	M	56	25,7	0	M	normal	No	91,6		15,6	*TP53* mut
CLL #88	M	63	76,8	IV	UM	del13	No	80,7	1	99,3	*TP53* mut
CLL #89	M	75	260	IV	UM	tri12	No	55	1,1	92,5	
CLL #90	F	64	221,9	II	UM	del13	No	98,2	76,9	29,7	
CLL #91	F	57	107,1	II	M	del13	No	92,2	0,8	0,1	
CLL #92	M	63	261	IV	UM	del11; del17	No	92,2	10,7	63,7	*TP53* mut
CLL #93	F	73	255,9	II	UM	normal	Yes	76,4	37	19	
CLL #94	F	47	41,7	IV	M	del13	No	94,5	1,5	4,6	
CLL #95	F	68	168,6	III	UM	tri12	ND	95,7	59,3	93,1	
CLL #96	M	58	65,4	II	UM	del13	No	93,8	72,9	70,8	
CLL #97	M	69	208	III	UM	del13	No	92,3	1,5	0,1	
CLL #98	M	57	115	IV	UM	del13	No	84,5	15,9	21,2	
CLL #99	M	54	222,4	III	UM	del13	Yes	95,5	85,3	65,3	
CLL #100	M	58	178,7	III	M	del13; del11	Yes	93,1	2,4	0,1	
CLL #101	M	61	278,1	IV	UM	del13; del11	No	96,1	27,9	4,6	*TP53* mut
CLL #102	M	35	80	I	UM	normal	No	95	40,5	19,1	
CLL #103	M	65		I	UM	normal	No	90	43,9	21,7	
CLL #104	M	75		I	UM	normal	Yes	90,7	75,8	14,6	
CLL #105	F	70	32,7	0	UM	del13	No	93,4	46,5	20,3	
CLL #106	F	65	92,8	I	UM	del13	Yes	94,8	40,3	26,8	

SAMPLE ID	SEX	AGE	WBC	RAI	*IGHV*	FISH	*NOTCH1* mutations	CD5/CD19 (%)	CD38^{+} (%)	CD49d^{+} (%)	Notes
CLL #107	M	60	24,8	I	UM	del11	Yes	88,2	43,9	31,6	
CLL #108	M	54	64,3	I	UM	del13	No	99,1	40,3	33,9	
CLL #109	F	56	49,5	0	UM	tri12	Yes	93,9	56,2	54,7	
CLL #110	F	58	31,1	0	UM	del13	No	85,6	36,1	9,9	
CLL #111	F	73	46,4	I	UM	tri12	Yes	72,7	70	93,2	
CLL #112	M	74	57,2	IV	UM	del13	Yes	89,9	45	31,9	
CLL #113	M	68	80,5	II	UM	del17	No	97,6	54,4	91,7	*TP53* mut
CLL #114	M		36,8	II	UM	del13	No	98,6	40	39,2	
CLL #115	F	74	31,6	0	M	del13	No	98,4	53,7	33	

Supplemental Table2. Features of CLL samples examined during the follow up of the disease. AGE= age at diagnosis; WBC= white blood cells (x10^3/μl); M=mutated; UM=unmutated; tri=trisomy; del=deletion.

SAMPLE ID	SEX	AGE	WBC	RAI	*IGHV*	FISH	*NOTCH1* mutations	CD5/CD19 (%)	$CD38^+$ (%)	$CD49d^+$ (%)	Notes
04015	M	46	11,4	IV	UM	del17	No	74,9	85,9	66,4	
17698	M	41	30	IV	ND	del11; del13	Yes	83,4	72,4	80	
04025	M	70	42	III	UM	del11	No	91,6	37,2	66,4	*TP53* mut
04028	M	77	25	IV	ND	del17	No	93,9	44,4	71	*TP53* mut
19275	M	70	8,4	I	M	normal	No	81	84,4	48,2	
4716	F	61	265	IV	UM	tri12	No	92,7	67,2	72,9	
04012	F	71	3,11	IV	UM	del17; del13	Yes	60,3	67,7	74,7	*TP53* mut
04013	M	72	38	II	M	del17; del13	No	94,7	47,9	49	*TP53* mut
6924	M	47	22,9	II	UM	del11; del13	No	84,7	54,8	44,5	
12752	M	81	9,62	I	UM	del13	No	48	71,1	23,8	
04002	F	64	102	I	UM	del17	No	79,2	10,5	4,55	*TP53* mut
22400	M	58	95	IV	UM	del11	No	82,9	61,3	48,9	
22742	M	65	14,5	II	M	del13	No	96,1	45,9	56,9	
MA	M	46	220	II	M	normal	No	97,2	42,2	96,8	

Supplemental Table 3. Features of CLL patients used for histologic analyses on LN biopsies.

SAMPLE ID	SEX	AGE	RAI/BINET	*IGHV*	FISH	$CD38^+$ (%)
CLL #1001	M	76	0/A	M	Normal	85
CLL #1002	M	64	1/A	M	del (13q)	0
CLL #1003	M	50	NK	M	NK	NK
CLL #1004	M	76	2/B	UM	del (13q)	60
CLL #1005	F	75	NK	UM	del (13q)	NK
CLL #1006	F	80	NK	UM	del (13q)	NK

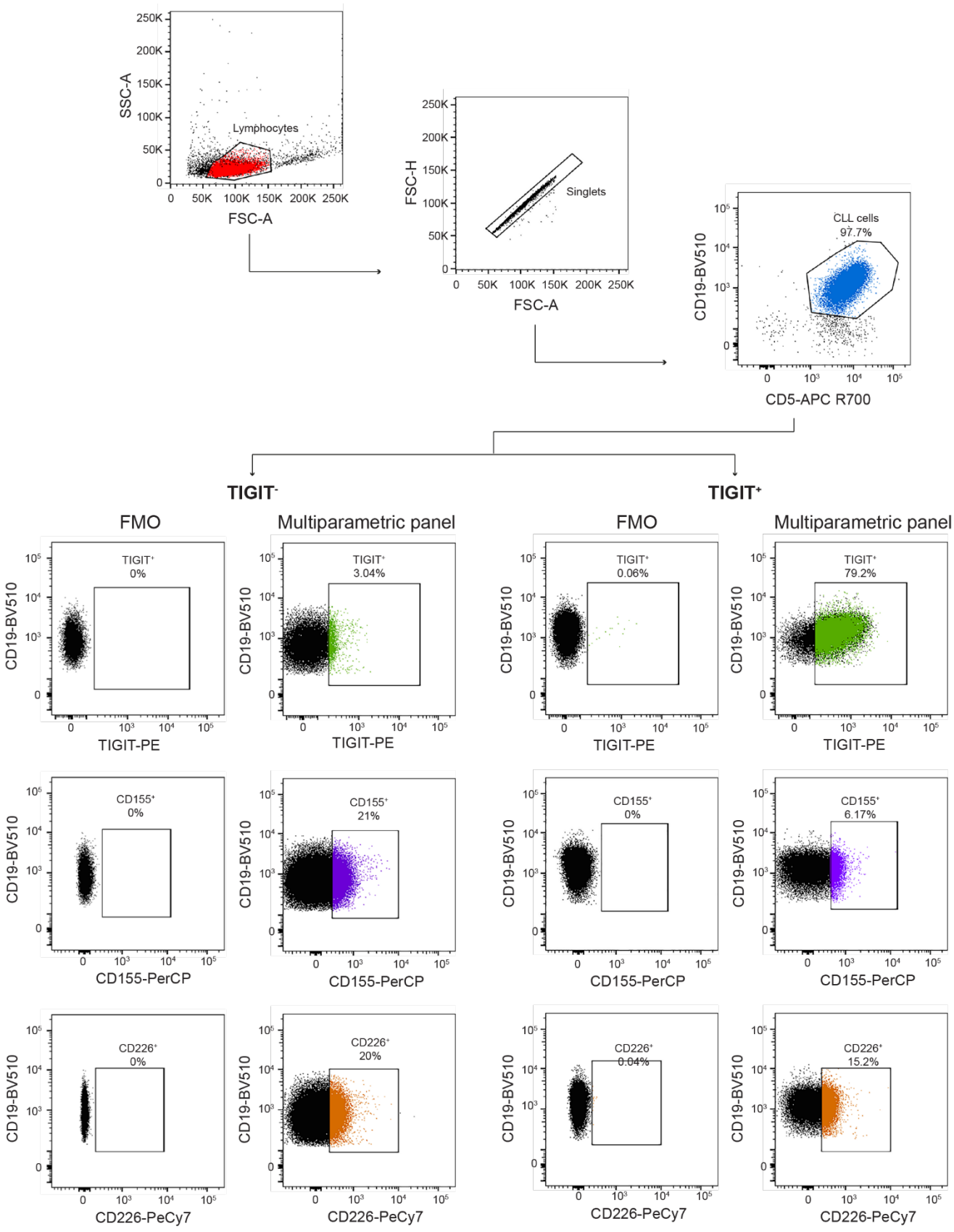
*NK=not known

Supplemental Table 4. List of antibodies and reagents.

Reagent	Manufacturer
APC-R700 Mouse anti-Human CD5	BD Biosciences, Milan, Italy
BB515 Mouse anti-Human CD5	BD Biosciences, Milan, Italy
BV510 Mouse anti-Human CD19	BD Biosciences, Milan, Italy
PE-Cy7 Mouse anti-Human CD19	BD Biosciences, Milan, Italy
APC Mouse anti-Human CD73	BD Biosciences, Milan, Italy
BB515 Mouse anti-Human CD38	BD Biosciences, Milan, Italy
BV605 Mouse anti-Human CD49d	BD Biosciences, Milan, Italy
BV650 Mouse anti-Human CD3	BD Biosciences, Milan, Italy
APC-R700 Mouse anti-Human CD4	BD Biosciences, Milan, Italy
BV510 Mouse anti-Human CD8	BD Biosciences, Milan, Italy
APC-H7 Mouse anti-Human CD14	BD Biosciences, Milan, Italy
PE anti-human BTK (pTyr223)/ITK (pTyr180)	BD Biosciences, Milan, Italy
APC Mouse anti-Human IL-10	BD Biosciences, Milan, Italy
PE Mouse anti-Human IgM	BioLegend, San Diego, CA, USA
PE Mouse anti-Human TIGIT (Clone MBSA43)	Invitrogen Ebioscience, Thermofisher, Milan, Italy
PerCP-eFluor 710 Mouse anti-Human CD155 (Clone 2H7CD155)	Invitrogen Ebioscience, Thermofisher, Milan, Italy
PE-Vio770 Mouse anti-Human CD226 (DNAM1) (Clone DX11)	Miltenyi Biotec, Bologna, Italy
APC Anti-Human and -Mouse Ki-67	Miltenyi Biotec, Bologna, Italy
Recombinant Human TIGIT Fc Chimera Protein	R&D System, Bio-Techne SRL, Milan,Italy
Recombinant Human CD155/PVR Fc Chimera Protein	R&D System, Bio-Techne SRL, Milan,Italy
TIGIT Monoclonal Antibody (MBSA43), Functional Grade	Invitrogen Ebioscience, Thermofisher, Milan, Italy
CD226 (DNAM-1) Monoclonal Antibody (DX11)	Invitrogen Ebioscience, Thermofisher, Milan, Italy
Goat Anti-Human IgM-unlabeled	Southern Biotech, Birmingham, AL, USA
CpG ODN 2006	InvivoGen, Toulouse, France
Recombinant Human IL-15 Protein	R&D System, Bio-Techne SRL, Milan,Italy

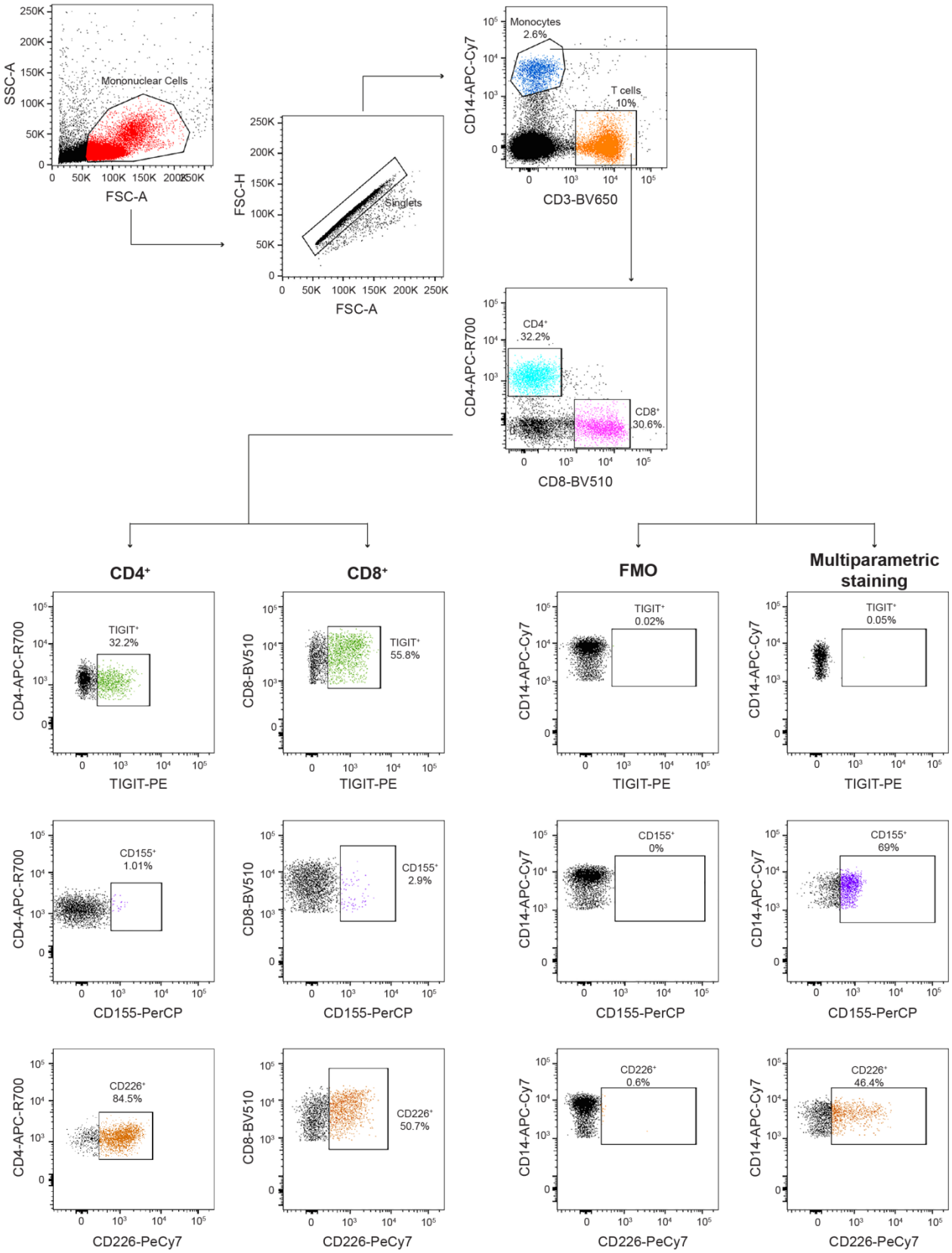
Supplemental Table 5. Flow cytometry multiparametric strategy. FMO= Fluorescence Minus One.

Mix ID	Marker	Fluorochrome
FMO B_1	CD5	APC-R-700
	CD19	BV510
	IgM	PE
FMO B_2	CD5	APC-R-700
	CD19	BV510
	CD73	APC
	CD38	BB515
	CD49d	BV605
TIGIT panel B	CD5	APC-R-700
	CD19	BV510
	CD73	APC
	CD38	BB515
	CD49d	BV605
	TIGIT	PE
	CD155	PerCP-Cy5.5
	CD226	PE-Cy7
FMO T/Mono	CD3	BV650
	CD4	APC-R-700
	CD8	BV510
	CD14	APC-H7
TIGIT panel T/Mono	CD3	BV650
	CD4	APC-R-700
	CD8	BV510
	CD14	APC-H7
	TIGIT	PE
	CD155	PerCP-Cy5.5
	CD226	PE-Cy7



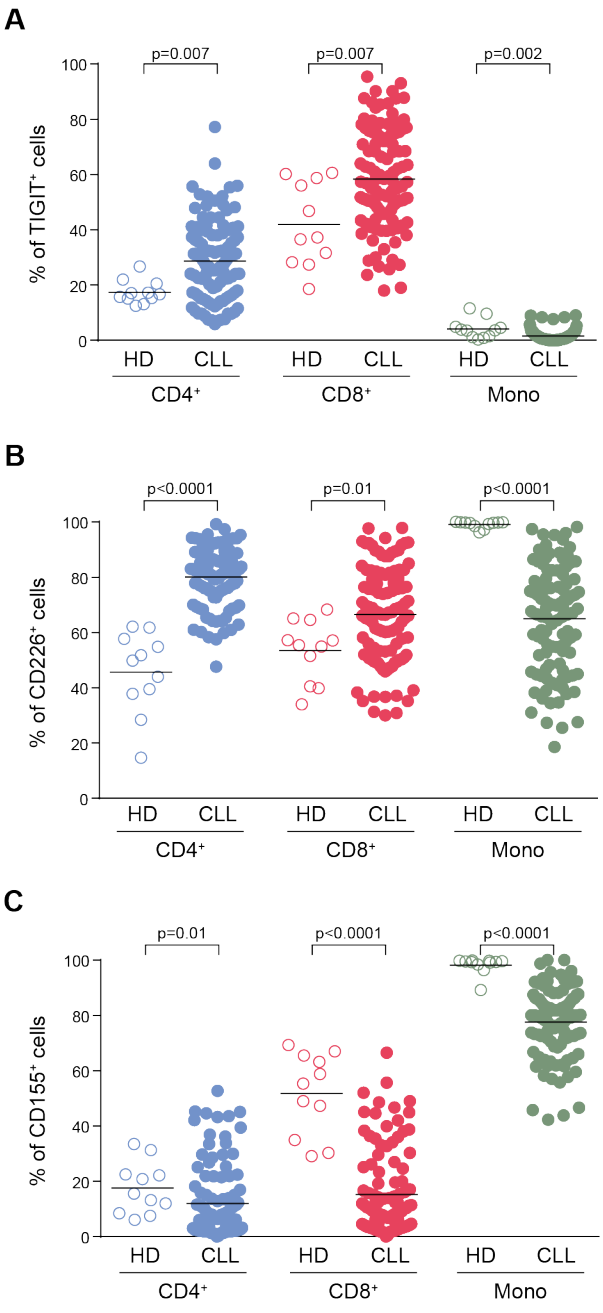
Supplemental Figure 1

Supplemental Figure 1. Flow cytometry gating strategy for B cells. Lymphocytes were morphologically gated and singlets were examined. B cells were isolated as $CD19^+/CD5^+$ cells (CLL cells) or $CD19^+$ cells (normal B lymphocytes). The gates for $TIGIT^+$, $CD226^+$ and $CD155^+$ cells were set based on the Fluorescence Minus One (FMO) to take into account the presence of multiple fluorochromes.



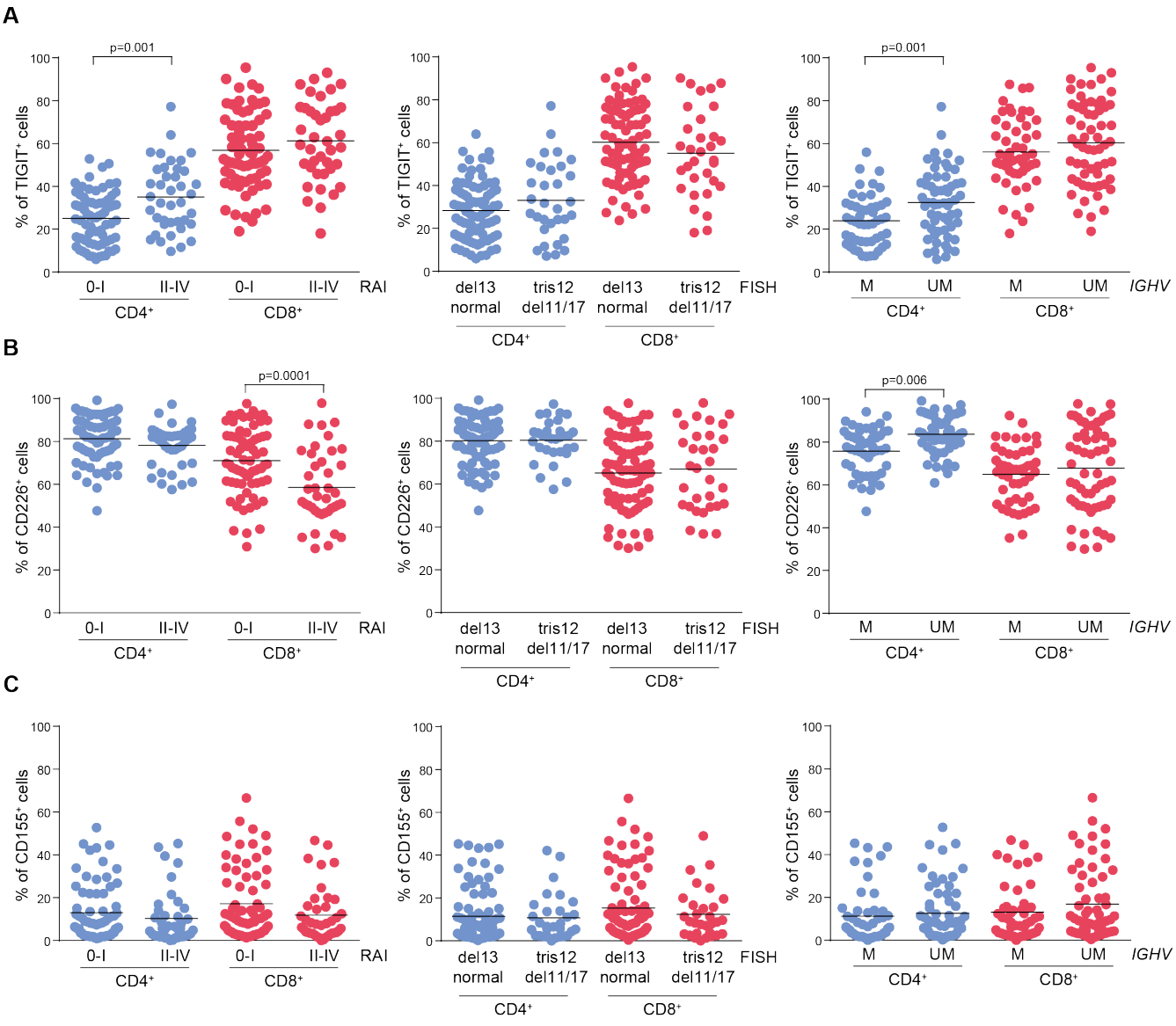
Supplemental Figure 2

Supplemental Figure 2. Flow cytometry gating strategy for T cells and monocytes. Mononuclear cells were morphologically gated and singlets were examined. T cells were defined as $CD3^+$ cells and further divided in $CD4^+$ and $CD8^+$ T lymphocytes, monocytes were defined as $CD14^+$ cells. For each subpopulation, gates for $TIGIT^+$, $CD226^+$ and $CD155^+$ cells were set based on the FMO (not shown for $CD4^+$ and $CD8^+$).



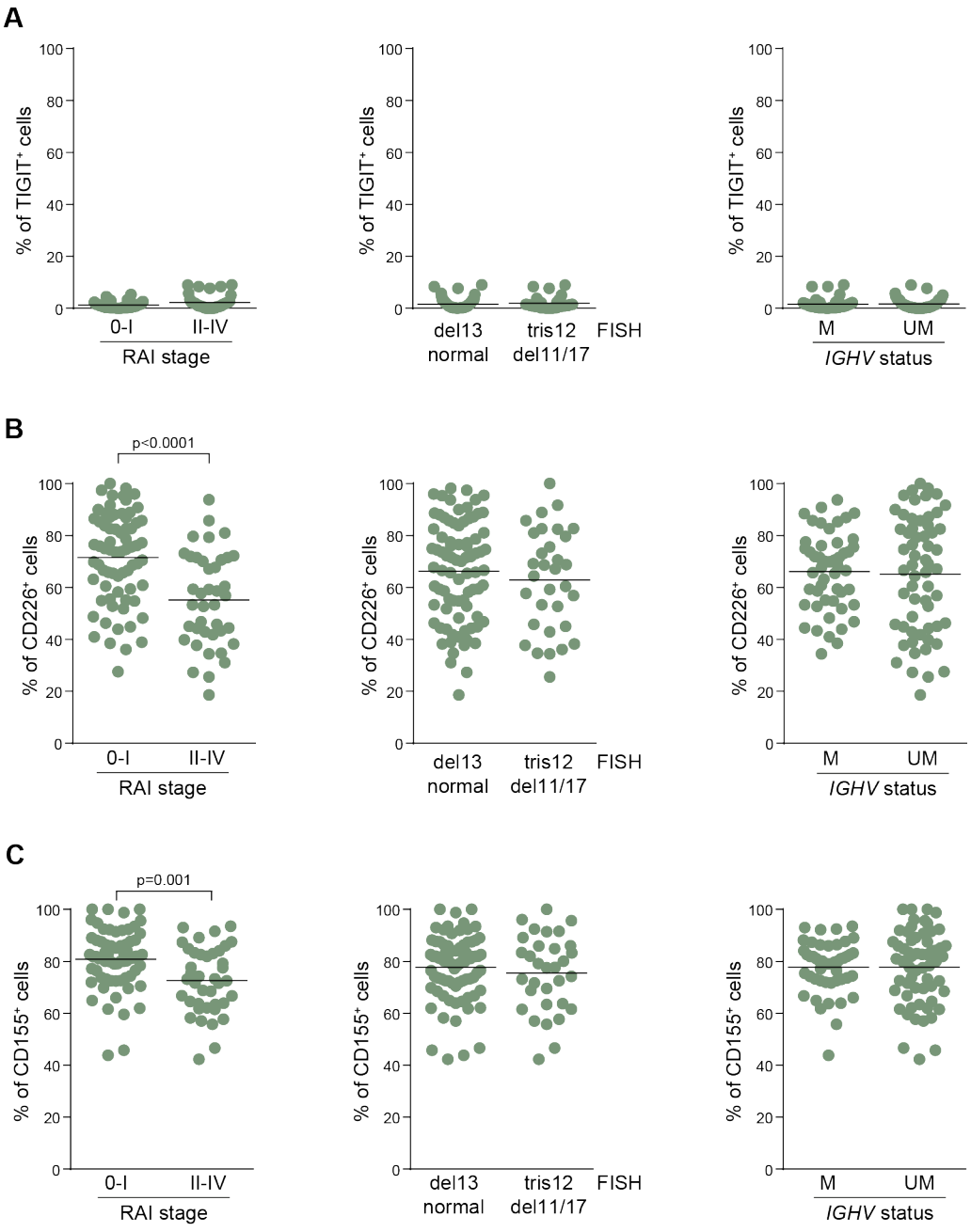
Supplemental Figure 3

Supplemental Figure 3. Expression of TIGIT, CD226 and CD155 on PBMC subpopulations. Flow cytometry analysis of TIGIT, CD226 and CD155 expression on CD4+ or CD8+ T lymphocytes and monocytes collected from CLL patients and healthy donors (HD).



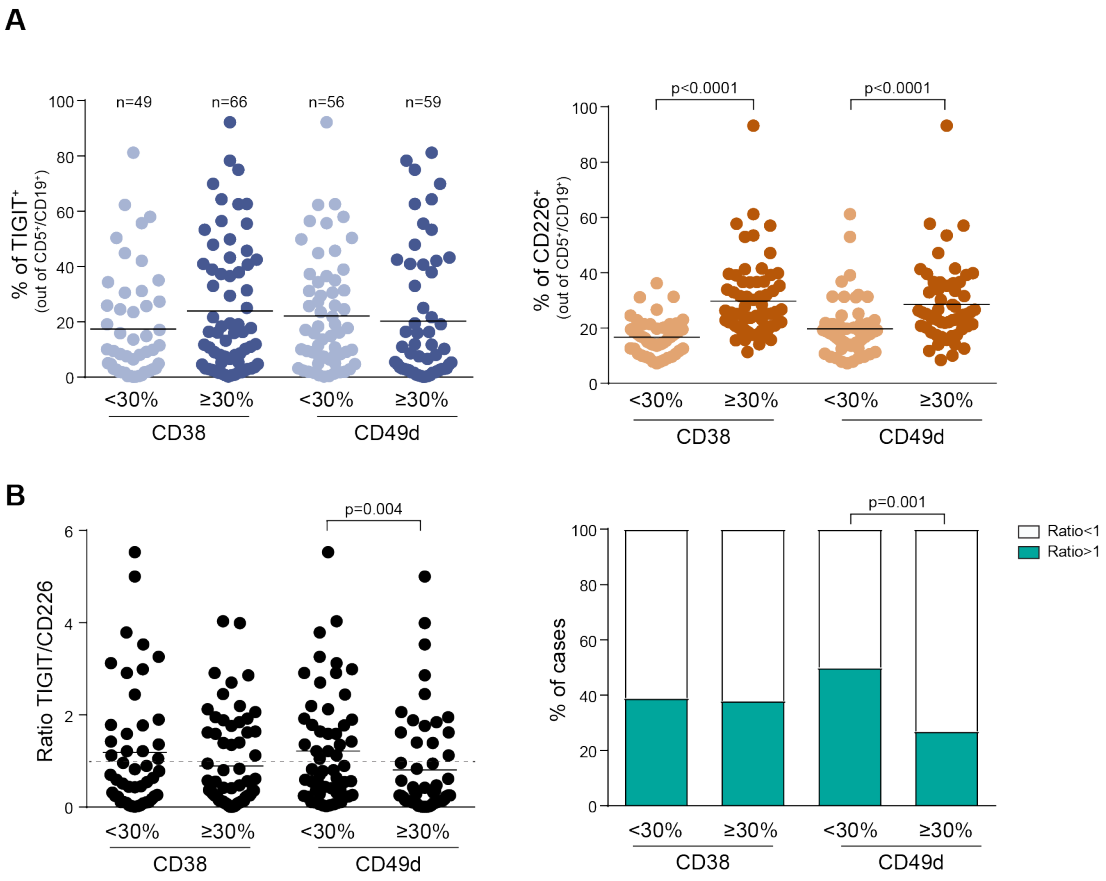
Supplemental Figure 4

Supplemental Figure 4. TIGIT axis in T lymphocytes from CLL patients. A. Flow cytometry analysis of surface TIGIT in $CD4^+$ and $CD8^+$ cells collected from CLL samples, divided according to prognostic markers (left: RAI; middle: cytogenetics; right: *IGHV* mutation). **B.** Flow cytometry analysis of surface CD226 in $CD4^+$ and $CD8^+$ cells collected from CLL samples, divided according to prognostic markers (left: RAI; middle: cytogenetics; right: *IGHV* mutation). **C.** Flow cytometry analysis of surface CD155 in $CD4^+$ and $CD8^+$ cells collected from CLL samples, divided according to prognostic markers (left: RAI; middle: cytogenetics; right: *IGHV* mutation).



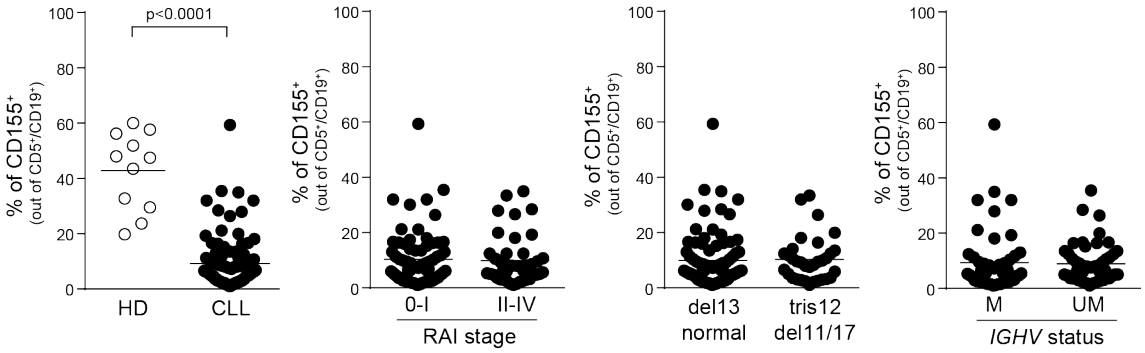
Supplemental Figure 5

Supplemental Figure 5. TIGIT axis in monocytes from CLL patients. A. Flow cytometry analysis of surface TIGIT in monocytes collected from CLL samples, divided according to prognostic markers (left: RAI; middle: cytogenetics; right: *IGHV* mutation). **B.** Flow cytometry analysis of surface CD226 in monocytes collected from CLL samples, divided according to prognostic markers (left: RAI; middle: cytogenetics; right: *IGHV* mutation). **C.** Flow cytometry analysis of surface CD155 in monocytes collected from CLL samples, divided according to prognostic markers (left: RAI; middle: cytogenetics; right: *IGHV* mutation).



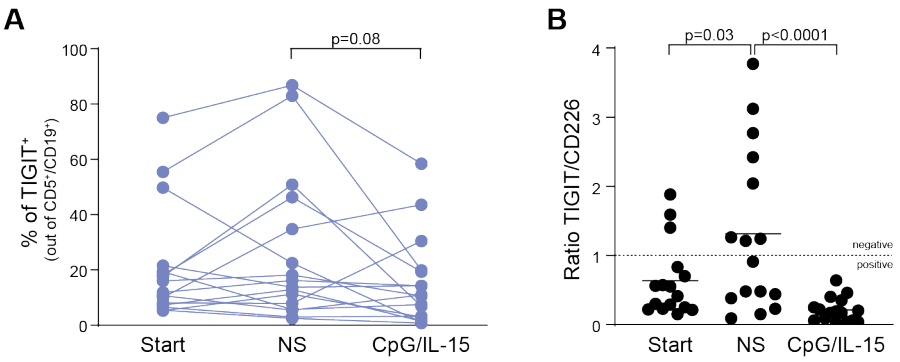
Supplemental Figure 6

Supplemental Figure 6. TIGIT and CD226 expression according to CD38 and CD49d. A. Percentages of TIGIT$^+$ and CD226$^+$ cells in CD38$^+$/CD38$^-$ and CD49d$^+$/CD49d$^-$ CLL samples (top panels); **B.** Dot plot showing TIGIT:CD226 ratio values for all the CLL samples divided according to the expression of CD38 and CD49d (left) and contingency plot indicating the enrichment of samples with ratio ≥1 or <1 in either prognostic category (right). Dashed line at Y=1 indicates the threshold to discriminate between negative signaling (TIGIT:CD226 ratio>1, prevalence of TIGIT) and positive signaling (TIGIT:CD226 ratio<1, prevalence of CD226).



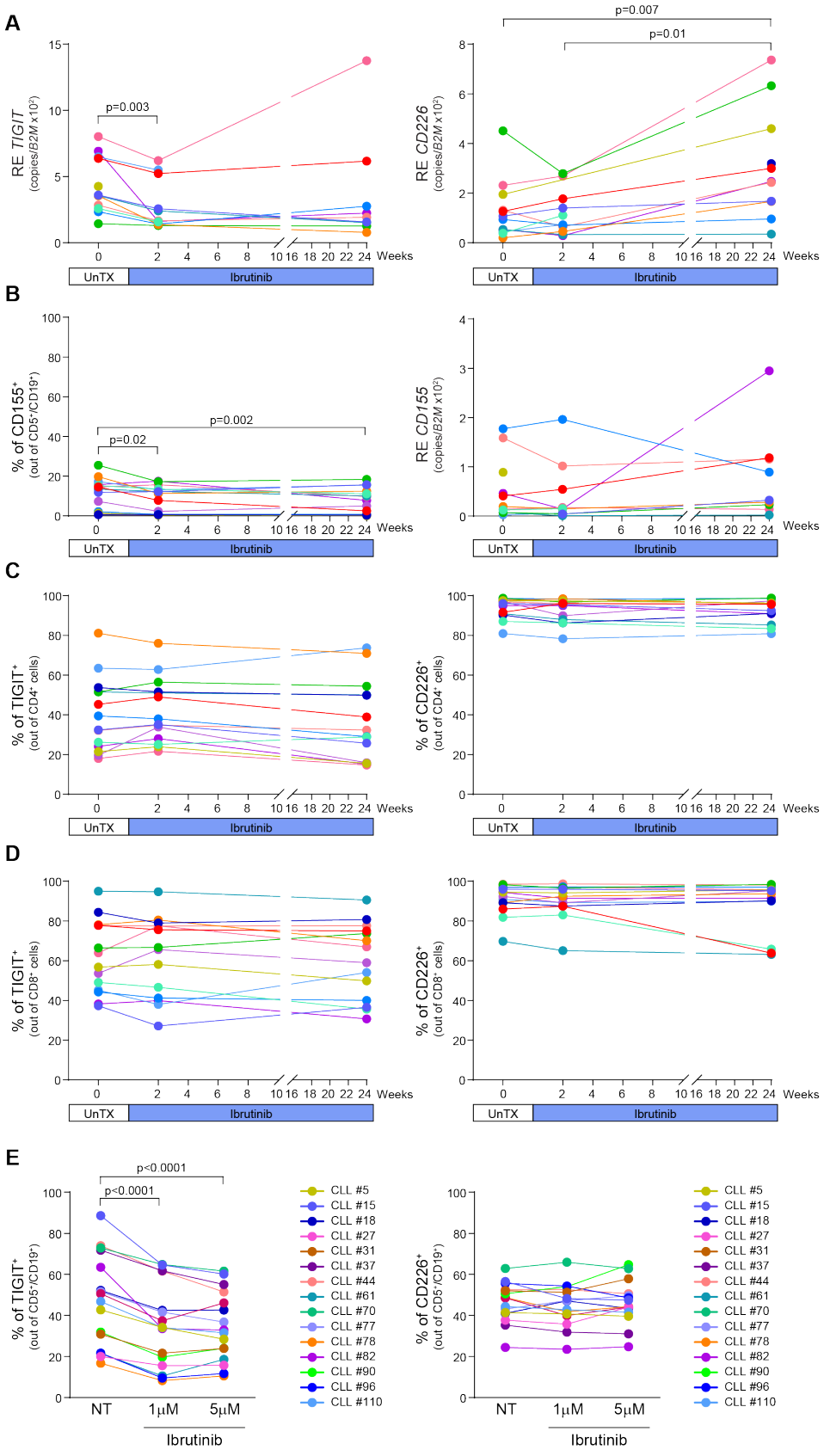
Supplemental Figure 7

Supplemental Figure 7. Expression of CD155. Flow cytometry analysis of surface CD155 expression in CLL samples compared to HD and correlation with CLL prognostic markers.



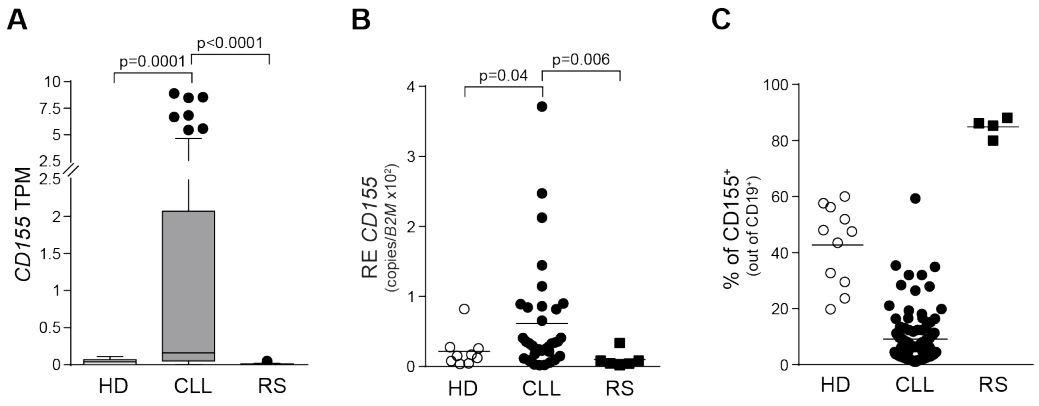
Supplemental Figure 8

Supplemental Figure 8. Modulation of TIGIT axis upon CpG/IL-15 culture. A. Flow cytometry analysis of TIGIT expression at the starting point and after 6 days of culture in the presence or absence of CpG/IL-15. **B.** Analysis of TIGIT:CD226 ratio at the starting point and after 6 days of culture in the presence or absence of CpG/IL-15. Dashed line at Y=1 indicates the threshold to discriminate between negative signaling (TIGIT:CD226 ratio>1, prevalence of TIGIT) and positive signaling (TIGIT:CD226 ratio<1, prevalence of CD226).



Supplemental Figure 9

Supplemental Figure 9. TIGIT, CD226 and CD155 during the follow up. A. qRT-PCR analysis of *TIGIT* and *CD226* expression in CLL samples collected before treatment initiation or after 2 weeks or 24 weeks of ibrutinib therapy. **B.** Flow cytometry (left) and qRT-PCR analysis (right) of CD155 expression in CLL samples collected before treatment initiation or after 2 weeks or 24 weeks of ibrutinib therapy. **C.** Flow cytometry analysis of TIGIT (left) and CD226 (right) on $CD4^+$ T cells collected from CLL samples during the follow up. **D.** Flow cytometry analysis of TIGIT (left) and CD226 (right) on $CD8^+$ T cells collected from CLL samples during the follow up. **E.** Flow cytometry analysis of the percentage of $TIGIT^+$ (left) and $CD226^+$ (right) leukemic cells in CLL samples treated *in vitro* with 1 ad 5 μM ibrutinib for 48 hours.



Supplemental Figure 10

Supplemental Figure 10. CD155 expression in RS. A. *CD155* TPM values in HD, CLL and primary RS samples. **B.** qRT-PCR validation of *CD155* expression in HD and CLL samples and in RS-PDX. **C.** Flow cytometry analysis of surface CD155 in HD and CLL samples and in RS-PDX.