Fine tuning of surface CRLF2 expression and its associated signaling profile in childhood B-cell precursor acute lymphoblastic leukemia

Cristina Bugarin,[1] Jolanda Sarno,[1] Chiara Palmi,[1] Angela Maria Savino,[1] Geertruy te Kronnie,[2] Michael Dworzak,[3] Angela Shumich,[3] Barbara Buldini,[2] Oscar Maglia,[1] Simona Sala,[1] Ilaria Bronzini,[2] Jean-Pierre Bourquin,[4] Ester Mejstrikova,[5] Ondrej Hrusak,[5] Drorit Luria,[6] Giuseppe Basso,[2] Shai Izraeli,[6] Andrea Biondi,[1,7] Giovanni Cazzaniga[1] and Giuseppe Gaipa;[1] on behalf of the I-BFM study group

[1]Pediatric Clinic, University of Milano Bicocca, M. Tettamanti Research Center, Monza, Italy; [2]Lab. Hemato-Oncology, University of Padua, Italy; [3]Children's Cancer Research Institute and St. Anna Children's Hospital, Dept. of Pediatrics, Medical University of Vienna, Austria; [4]University of Zurich, Switzerland; [5]Childhood Leukaemia Investigation Prague, Department of Paediatric Haematology and Oncology, Charles University Prague, 2nd Medical School, Czech Republic; [6]Pediatric Hematology Oncology, Schneider Children's Medical Center of Israel; and [7]Department of Pediatrics, Hospital San Gerardo/Fondazione MBBM, Monza, Italy

Correspondence: abiondi.unimib@gmail.com/andrea.biondi@unimib.it
doi:10.3324/haematol.2014.114447

Supplemental methods

Phospho flow cytometry

MUTZ5 cells harboring IGH@-CRLF2 translocation and JAK2 R683G mutation, MHH-CALL4 displaying IGH@-CRLF2 translocation and JAK2 I682F mutation, or primary thawed cells were subject to phospho flow cytometric assay. For this purpose, cells were starved in X-vivo medium and rested at 37°C for 16 hours or 1 hour. Then, cells were stimulated with rh-TSLP (10 ng/mL) for 30 minutes at 37°C to allow signal transduction, and treated according to an established internal protocol. Starved cells were fixed with paraformaldehyde (1.5%) and permeabilized with 90% ice-cold methanol and then incubated with anti-phospho-protein-directed MoAbs (or isotype matched IgG) and surface antigen-directed MoAbs. Characteristics of MoAbs and staining combinations are described in Supplemental Table 1. Cells were acquired on a FACSaria™ flow cytometer (BD) equipped with 488-nm, 633-nm and 405-nm lasers. Data were collected (at least 100000 events per tube) and analyzed using DIVA™ software (BD). Positivity threshold for phosphoprotein expression was established by the use of isotype IgG-negative control (Supplemental Figure 2 panel A). Basal levels of each phosphoprotein were then calculated as percentage (%) of phosphoprotein positive (p-positive) cells in unstimulated conditions (Supplemental Figure 2 panel B). Response to each cytokine (rhTSLP) was calculated by subtracting the % of p-positive cells in the basal state from that obtained upon exposure to cytokine (Supplemental Figure 2 panel C).

Quantitative expression of CRLF2

CRLF2 transcript levels on diagnostic samples were analyzed using TaqMan Gene Expression Assay Hs00913509_s1 (Applied Biosystems, Foster City,CA,USA) following the manufacturer's instructions. The presence and level of the fusion transcript P2RY8-CRLF2 was analyzed by Universal Probe Library System (UPL) (Roche Diagnostic, Basel, Switzerland) as well as the housekeeping GUS gene transcript, tested as internal control. Optimal primers and probe for P2RY8-CRLF2 and GUS amplification were selected using the Roche ProbeFinder software (https://www.roche-appliedscience.com/sis/rtpcr/upl). In particular, for P2RY8-CRLF2 amplification we used primers designed in the first exon of P2RY8 (5'-gctacttctgccgctgctt-3') and in the first exon of CRLF2 (5'-gcagaaagacggcagctc-3') with the UPL probe n. 28 (Roche UPL cat. n. 04687604001). Each cDNA sample (20 ng RNA equivalent) was tested in duplicate (Ct range between replicates <1.5). The

amplification reaction was performed on the 7900HT FAST Real Time PCR System instrument (Applied Biosystems) for CRLF2 expression and on the Light Cycler 480 (Roche) for P2RY8-CRLF2 with the following protocol: initial step at 95°C for 10 min, then 50 cycles at 95°C for 15s and at 60°C for 1 min. Relative gene expression (indicated as fold change) was quantified by the 2-DDCt method (22). The DDCts for CRLF2 expression were calculated by subtracting the median of the DCt of a published cohort of 464 BCP-ALL patients enrolled in Italy in the AIEOP-BFM ALL2000 study from February 2003 to July 2005 (22) to the DCt of each sample. The DDCts for P2RY8-CRLF2 expression were calculated by subtracting the DCt of a selected positive patient external to this cohort to the DCt of each sample.

Mutational screening of JAK2, CRLF2 and IL7Rα

Mutational screening of JAK2, CRLF2 and IL7Rα was performed in 82/86 consecutive patients and in 15/15 patients (13/15 for JAK2) selected retrospectively from Monza's cell bank for phospho flow analysis. High Resolution Melting (HRM) analysis was applied to identify JAK2 mutations in exon 16 using HRM Master (Roche Diagnostics) as previously described (21). Sequencing of CRLF2 exon 6 and IL7R exons 5 and 6 was performed by Sanger sequencing of PCR products from patients DNA after whole genome amplification using GenomePhi V2 DNA Amplification Kit (GE Healthcare Life Science); we designed the following primers for CRLF2-F (5'-AGGGAGACTGGTTAGGGATGA-3'), CRLF2-R (5'-TGGGCATTGTATGGAAACTG -3') and for IL7R exon 5 IL7R-F (5'-GCAACACCTCTTTTCCATC-3') and IL7R-R (5'-GGGAACAAAAACTCTACCACCA-3') and exon 6 IL7R-F(5'-TGCATGGCTACTGAATGCTC-3') and IL7R-R (5'-CCCACACAATCACCCTCTTT- 3').

Statistical analysis

Statistical significance of phosphoprotein levels among groups of patients with different TSLPR pattern (strongly positive, moderately positive, and fully negative) was determined by one-way ANOVA analysis of variance followed by post hoc Bonferroni's multiple comparison test. P values less than 0.05 were considered statistically significant. All data were presented as mean ± standard deviation (SD). GraphPad PrismV5.0 (GraphPadSoftware, San Diego, CA, USA) was used for statistical analysis.* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Supplemental Table 1: Characteristics of selected antibodies and staining combinations for TSLPR immunophenotypic screening and phosphoflow cytometry assay.

Selected Antibodies					
Reactivity	**Clone**	**Fluorochrome**	**Source**	**Ig class**	**Use**
TSLPR	1D3	PE	Biolegend	mouse IgG2aλ	10µl/1x10^{6}cells
TSLPR	1B4	PE	Biolegend	Mouse IgG1K	10µl/1x10^{6}cells
CD19	SJ25C1	APC	BD	mouse IgG1 k	3µl/1x10^{6}cells
CD10	HI10a	PE	Biolegend	mouse IgG1 k	2µl/1x10^{6}cells
CD10	HI10a	PE-CY7	BD	mouse IgG1 k	2µl/1x10^{6}cells
CD45	2D1	PerCP	BD	mouse IgG1 k	2µl/1x10^{6}cells
CD7	8H8	ECD	Beckman Coulter	mouse IgG2a	1µl/1x10^{6}cells
IgG1 k Isotype control	MOPC-21	Alexa Fluor 488	BD	mouse IgG1 k	10µl/1x10^{6}cells
IgG1 k Isotype control	MOPC-21	Alexa Fluor 647	BD	mouse IgG1 k	10µl/1x10^{6}cells
p-Stat5 (Y694)	47	Alexa Fluor 488	BD	mouse IgG1	10µl/1x10^{6}cells
p-S6 (pS235/pS236)	N7-548	Alexa Fluor 647	BD	mouse IgG1 k	10µl/1x10^{6}cells
IgG Isotype control	236B4	Alexa Fluor 488	Cell Signaling	rabbit IgG	2µl/1x10^{6}cells
IgG Isotype control	DA1E	Alexa Fluor 647	Cell Signaling	rabbit IgG	2µl/1x10^{6}cells
p-4E-BP1 (Thr37/46)	236B4	Alexa Fluor 488	Cell Signaling	rabbit IgG	2µl/1x10^{6}cells
anti p-AKT (S473)	D9E	Alexa Fluor 647	Cell Signaling	rabbit IgG	8µl/1x10^{6}cells

Staining panel for Immunophenotyping					
Tube 1	TSLPR PE	CD45 PerCP	CD10 PE-CY7	CD19 APC	CD7 ECD

Staining panel for Phosphoflow					
Tube 1	IgG1 k Isotype control Alexa 488	IgG1 k Isotype control Alexa 647	CD10 PE	CD45 PerCP	CD7
Tube 2	p-Stat5 Alexa 488	p-S6 Alexa 647	CD10 PE	CD45 PerCP	CD7

Supplemental Table 2: Phenotypic, molecular, and signaling features in 101 patients according to surface TSLPR expression.

UPN	TSLPR pattern	TSLPR surface expression (%)*	CRLF2 RQ-PCR overexpression**	CRLF2 rearrangement	CRLF2 mutation	JAK2 mutation	IL7Rα mutation	pSTAT5 response	pS6 response	p4EBP1 response	pAKT response
034	Strongly positive	99.9	Yes (132.9)	P2RY8-CRLF2	wt	neg	wt	83.6%	33.3%	36.2%	23.2%
059		99.9	Yes (108.8)	P2RY8-CRLF2	wt°	neg	wt	67.8%	68,8%	14,7%	18,7%
099		99.1	Yes (127.2)	P2RY8-CRLF2	wt	neg	wt	47.1%	34,4%	5,5%	0,0%
096		98.7	Yes (1032.7)	IGH@CRLF2	wt	R683G	wt	37.0%	41,6%	3,9%	40,4%
090		95.7	Yes (44.0)	P2RY8-CRLF2	wt	neg	wt	70.4%	n.t.	n.t.	n.t.
091		96.0	Yes (300.0)	P2RY8-CRLF2	wt	neg	wt	61.8%	n.t.	n.t.	n.t.
031		96.3	Yes (102.8)	P2RY8-CRLF2	wt	neg	S185C, T244I	66.0%	79,3%	3,5%	44,3%
032		95.2	Yes (397.6)	P2RY8-CRLF2	wt	L681-I682 insEA	wt[#]	67.3%	28,6%	3,4%	0,0%
097		95.1	Yes (102.2)	P2RY8-CRLF2	wt	neg	wt	49.6%	19,4%	-4,2%	0,0%
062		85.0	Yes (22.1)	wt	wt	neg	wt[#]	66.5%	9,7%	5,4%	0,0%
095		81.8	Yes (201.3)	P2RY8-CRLF2	wt	neg	wt	63.7%	9,4%	11.9%	3,8%
087		78.2	Yes (30.0)	P2RY8-CRLF2	wt	neg	wt	48.7%	n.t.	n.t.	n.t.
030		71.2	Yes (207.9)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
013	Partially positive	3.5	No (2.49)	P2RY8-CRLF2 low[§]	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
039		1.0	No (3.3)	P2RY8-CRLF2 low[§]	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
073	Moderately positive (≥ 10%)	11.3	Yes (27.7)	wt (IGH@CRLF2 n.t.)	wt	neg	wt[#]	n.a.	n.a.	n.a.	n.a.
047	Moderately positive (<10%)	2.9	No (6.2)	wt	wt	neg	wt[#]	34.3%	25.4%	0.4%	1.9%
016		2.8	No (1.4)	P2RY8-CRLF2 low[§]	wt	neg	wt[#]	25.5%	1.0%	n.t.	n.t.
078		2.8	No (4.1)	wt	wt	neg	wt	16.0%	3.5%	10.7%	0.5%
067		2.6	No (12.0)	wt	wt	neg	wt[#]	19.0%	6.1%	1.7%	0.4%
037		2.5	No (2.4)	wt	wt	neg	wt[#]	17.0%	0.5%	0.0%	0.0%
004	Fully negative	2.2	No (1.8)	wt	wt	neg	wt	3.5%	0.0%	n.t.	n.t.
072		2.2	No (4.0)	wt	wt	neg	wt	1.2%	1.5%	0.3%	-0.5%
035		1.6	No (2.0)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
008		1.6	No (3.5)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
028		1.5	No (2.3)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
055		1.5	No (0.6)	wt	wt°	neg	wt	0.7%	1.0%	n.t.	n.t.
049		1.5	No (9.3)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
080		1.5	No (0.4)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
069		1.4	No (1.2)	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
094		1.2	No (4.0)	wt	wt	neg	wt	1.5%	-4.7%	0.2%	0.0%
061		1.1	No (0.1)	wt	wt	neg	wt[#]	0.4%	1.8%	3.5%	0.0%
079		1.0	No (0.7)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
045		0.9	No (1.2)	wt	wt°	neg	wt[#]	3.0%	0.6%	n.t.	n.t.
086		0.9	No (0.1)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.

Supplemental Table 2 (Continued)

082	Fully negative	0.9	No (3.6)	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
071		0.8	No (6.1)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
052		0.8	No (0.7)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
041		0.7	No (2.6)	P2RY8-CRLF2 low[§]	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
036		0.7	No (0.3)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
048		0.7	No (18.8)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
019		0.6	No (4.1)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
101		0.6	No (0.2)	wt	wt	neg	wt[#]	0.2%	-5.9%	0.5%	-1.0%
066		0.6	No (3.2)	wt	wt	neg	wt	11.0%	0.1%	-7.9%	0.0%
046		0.6	No (2.3)	P2RY8-CRLF2 low[§]	wt	neg	wt	4.0%	n.t.	n.t.	n.t.
042		0.6	No (0.3)	wt	wt	neg	wt[#]	0.3%	0.0%	n.t.	n.t.
077		0.6	No (5.1)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
002		0.6	No (0.5)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
010		0.6	No (0.9)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
001		0.5	No (0.2)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
076		0.5	No (3.7)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
060		0.5	No (0.2)	wt	wt	neg	wt	0.2%	-5.1%	0.8%	0.0%
081		0.5	No (0.2)	wt	wt	neg	wt[#]	0.4%	2.5%	1.9%	0.0%
098		0.5	No (1.1)	wt	wt	neg	wt[#]	0.6%	0.0%	-4.7%	0.0%
038		0.5	No (0.6)	wt	wt	neg	wt	4.7%	0.7%	0.0%	0.0%
050		0.5	No (0.0)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
025		0.5	No (0.7)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
100		0.4	No (0.1)	wt	wt°	neg	wt[#]	2.5%	0.0%	-0.9%	-2.8%
068		0.4	No (2.6)	wt	wt°	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
093		0.4	No (5.4)	wt	wt	n.a.	wt	0.8%	-13.1%	2.3%	0.0%
092		0.4	No (5.9)	wt	wt	neg	wt[#]	3.3%	4.4%	-5.9%	0.0%
088		0.4	No (0.5)	wt	wt	n.a.	wt	6.1%	-5.0%	0.0%	0.0%
051		0.4	No (0.0)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
089		0.3	No (3.3)	wt	wt	neg	wt	1.0%	n.t.	n.t.	n.t.
075		0.3	No (2.1)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
070		0.3	No (1.6)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
063		0.3	No (0.0)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
058		0.3	No (0.9)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
018		0.3	No (0.2)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
003		0.3	No (0.1)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
023		0.2	No (2.8)	wt	wt	neg	wt	9.1%	0.9%	0.0%	0.0%
022		0.2	No (1.2)	wt	wt°	neg	wt[#]	10.8%	0.0%	0.0%	0.0%
084		0.2	Yes (33.2)	wt	wt	neg	wt	1.4%	0.0%	n.t.	n.t.
026		0.2	No (0.1)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
065		0.2	No (4.1)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
064		0.2	No (0.2)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
005		0.2	No (6.5)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.

Supplemental Table 2 (Continued)

054	Fully negative	0.2	No (0.7)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
040		0.2	No (0.1)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
033		0.2	No (0.2)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
085		0.1	No (0.1)	wt	wt	neg	wt	3.4%	0.7%	0.6%	n.t.
044		0.1	No (0.5)	wt	wt	neg	wt[#]	0.9%	0.2%	n.t.	n.t.
083		0.1	No (0.1)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
074		0.1	No (0.4)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
057		0.1	No (0.1)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
056		0.1	No (0.1)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
053		0.1	No (0.0)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
043		0.1	No (0.2)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
029		0.1	No (0.1)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
027		0.1	No (0.7)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
021		0.1	No (0.1)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
020		0.1	No (1.5)	wt	wt°	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
011		0.1	No (0.8)	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
024		0.1	No (0.5)	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
017		0.1	No (0.3)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
014		0.1	No (0.3)	wt	wt°	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
009		0.1	No (15.4)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.
007		0.1	No (0.0)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
015		0.0	No (1.7)	wt	wt°	neg	wt	n.t.	n.t.	n.t.	n.t.
012		0.0	No (0.2)	wt	wt	neg	wt	n.t.	n.t.	n.t.	n.t.
006		0.0	No (0.2)	wt	wt	neg	wt[#]	n.t.	n.t.	n.t.	n.t.

n.t.= not tested; wt = wild type; n.a. = not available

* patients are listed by descendent order of values

** CRLF2 was considered over expressed in patients with levels of gene expression 20 times higher than the median of the considered cohort as described in Palmi *et al* (see ref.9)].

[§] P2RY8-CRLF2 Fold Change < 0.50

[#]These patients showed a T244I polymorphism

[°]These patients showed a V244M polymorphism

note: Among patients tested for p-STAT5, UPNs from 1 to 86 are patients from the prospective series of 421, UPNs from 87 to 101 are patients selected retrospectively from the cell bank

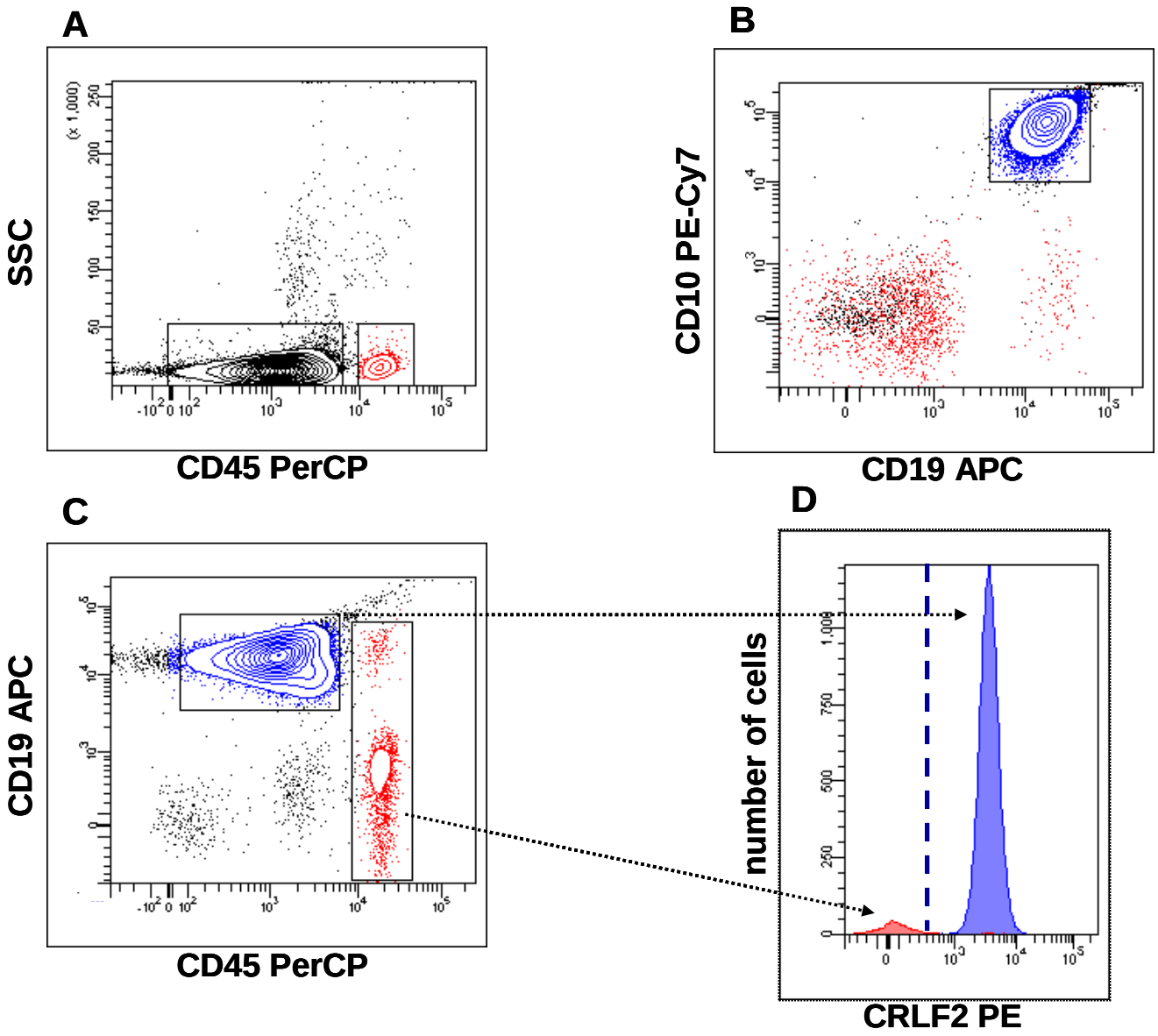
Legend to supplemental Figures

Supplemental Figure 1. Gating strategy employed to measure TSLPR surface expression in blast cells. Immature cells (black) were distinguished from mature lymphocytes (red) in SCC/CD45 dual dot plot (panel A). Within immature cells leukemic blasts (blue) are distinguished by CD19+/CD10+/CD45 intermediate immunophenotype (panel B). Based on CD19/CD45 expression (panel C), TSLPR expression is assessed as % of positive cells by setting the histogram marker exactly at the right end of mature lymphocytes peak (panel D). In all samples prevalence of mature lymphocytes was always ≥ 1.5%. Staining to measure TSLPR surface expression was performed using the combination: CRLF2PE/CD45PerCP/CD19APC/CD10PE-cy7/CD7ECD.

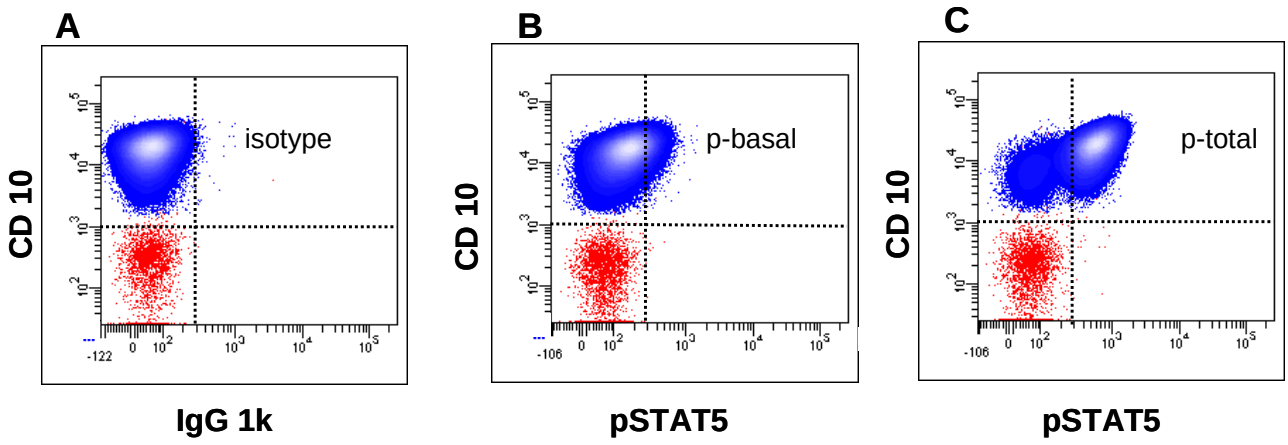
Supplemental Figure 2. Representative phosphoflow analysis in leukemic blast population (blue) gated on CD45-intermediate/SSC-low/CD10+ cells to measure signalling response after cytokine stimulation. Positivity threshold was established by an isotype IgG phospho-specific antibody (panel A). Basal and total levels of p-proteins were calculated as % of positive cells in unstimulated (panel B) and stimulated (panel C) conditions, respectively. p-induced response was then calculated by the indicated formula.

Supplemental Figure 3. TSLP-induced pSTAT5 response in three representative BCP-ALL patients. TSLP-induced pSTAT5 response (blue line) is compared to basal state (green line) and isotype control (grey line) by overlaying histograms. Hyperactive responses induced in a TSLPR strongly positive and in a TSLPR moderately positive patient are shown in Panel A and B respectively; Panel C shows a TSLPR fully negative patient with no pSTAT5 response. Of note pSTAT5 fluorescence intensity is shifted in TSLPR moderately positive blasts in a manner similar to that observed in TSLPR strongly positive blasts (MFI 731 *vs* 612), even though the proportion of shifted cells is minor (25.5% *vs* 67.8%).

Supplemental Figure 1



Supplemental Figure 2



p-induced response = % p-total - % p-basal

Supplemental Figure 3