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

Genomic rearrangements of the cytokine receptor-like factor 2 (CRLF2) gene,[1,2] which is part of the thymic stromal lymphopoietin receptor (TSLPR), result in overexpression of CRLF2 itself leading to JAK2-mediated activation of STAT5, which regulates cell proliferation, survival, and apoptosis.[3,4,11-13] In this regard, childhood B-cell precursor acute lymphoblastic leukemias (BCP-ALLs), bearing a rearranged CRLF2, display a high rate of relapse.[5-10] Furthermore, *CRLF2* genomic rearrangements are strictly associated with its surface overexpression, rendering this marker suitable for detection by flow cytometry (FCM).[14]

To determine CRLF2 expression in childhood BCP-ALLs, we first assessed TSLPR surface expression. For this purpose, we carried out, at diagnosis, standard multiparametric FCM (Dworzak *et al*., manuscript in preparation) on 421 consecutive diagnostic bone marrow (BM) samples from BCP-ALL children (256 males and 165 females), enrolled in six centers of the AIEOP-BFM-ALL-2009 trial between December 2010 and June 2013. Our gating strategy used to measure TSLPR surface expression (*Online Supplementary Figure S1*) allowed us to distinguish three blast subpopulations according to the intensity of TSLPR staining: the first one was defined as negative (i.e. positivity <10%), the second one was moderately positive (i.e. positivity ≥ 10% to <50%), and the third one was strongly positive (i.e. positivity ≥ 50%). We found 383 (91.2%) negative samples, 8 (1.9%) moderately positive, and 29 (6.9%) strongly positive. Inter-center distribution of patients' subgroups is shown in Table 1. Representative examples are reported in Figure 1A-C.

We then studied the immunophenotypic profile of TSLPR among the 86 patients enrolled in Center 1 during initial screening. Fine tuning of fluorescence distribution of 79 of 86 patients that had been previously found negative for TSLPR (i.e. positivity <10%) (Table 1) allowed us to further distinguish three different expression patterns: 1) TSLPR-stained blasts overlapping with control fluorescence (n. 72, mean positivity 0.52%±0.52%, range 0.0%-2.2%); 2) a second population of TSLPR-stained blasts clearly shifted to the right (n=5, mean % positivity 2.72%±0.16%, range 2.5%-2.9%), which was identical to the TSLPR moderate pattern we observed previously in the diagnostic screening apart from TSLPR positivity being less than 10%; 3) a third pattern showing two clearly distinct blast populations: a larger one, TSLPR fully negative, and a smaller one positive, shifted to the right (n=2, positivity was 1% and 3.5%, respectively). Hereinafter, we will refer to these three patterns as fully negative, moderately positive (<10%), and partially positive, respectively. Representative examples are shown in Figure 1D-F. Interestingly, one TSLPR moderately positive (<10%), and 2 TSLPR partially positive patients (UPNs 016, 013, and 039, respectively) showed low levels of *P2RY8-CRLF2* expression (F.C. < 0.50), suggesting the presence of a minor CRLF2 sub clone (*Online Supplementary Table S2*).

Next, CRLF2 transcripts levels, *CRLF2* aberrations (*P2RY8-CRLF2, IGH@-CRLF2, CRLF2* F232C), and *JAK2* and *IL7R* mutations were analyzed in 86 of our BCP-ALL samples collected in Center 1 as described previously.[9] We detected CRLF2 overexpression in 9.3% of BCP-ALL patients. Seventy-nine of these patients (91.8%) were negative for surface TSLPR expression as assessed by both FCM (<10%) and RQ-PCR (<20 FC), while only 7 (8.1%) were concordantly positive. Intriguingly, one patient (UPN 084) showed overexpression of CRLF2 (FC 33.2), whereas TSLPR expression level was undetectable. However, this patient did not display *P2RY8-CRLF2* gene fusion. Two of the 7 patients with CRLF2 overexpression (UPN 30 and UPN 62), as assessed by both techniques, were negative for *P2RY8-CRLF2* fusion and *IGH@-CRLF2* translocation. Conversely, 5 non-over-expressed cases showed barely detectable levels of *P2RY8-CRLF2* gene fusion. Thus, while our results seem to indicate a lack of correlation between genomic rearrangement and CRLF2 overexpression, as assessed by PCR, they clearly show that CRLF2-overexpressing BCP-ALLs are characterized by a strong positivity for TSLPR when analyzed by FCM.

To determine a functional read out of CRLF2 genomic rearrangements, MUTZ5 cells (*IGH@-CRLF2*; JAK2 R683G), MHH-CALL4 cells (*IGH@-CRLF2*; JAK2 I682F), or primary thawed cells were subject to phospho flow cytometric assay (*Online Supplementary Appendix*). Likewise, a total of 41 cryopreserved BCP-ALL samples obtained according to their availability in cell banks [28 were obtained from the consecutive series of Center 1 (total 86) and 15 from a local cell bank] and viability after thawing (cut off ≥80%) were subject to phospho flow assay. Twenty-four BCP-ALL samples were TSLPR fully negative, 5 moderately positive (all of them <10%), and 12 strongly positive.

Next, we sought to determine basal and TSLP-induced pSTAT5 expression in CD45 intermediate/CD10$^+$/CD7$^-$ blasts. The mean level of basal pSTAT5 detected in the three subgroups fully negative, moderately positive, and strongly positive for TSLPR was 0.71%±1.03% (range 0.0%-4.0%), 2.64%±3.64% (range 0.2%-9.0%), and 11.30%±18.31% (range 0.0%-65.6%). Statistical differences were calculated by one-way ANOVA analysis of variance (P=0.0200). As expected, we observed much higher

Table 1. TSLPR reactivity in BCP-ALL blasts at diagnosis analyzed in six different centers.

TSLPR profile	Centers #						Overall
	1 n=86	2 n=28	3 n=128	4 n=79	5 n=54	6 n=46	n=421
Negative	79 (91.8%)	25 (89.3%)	115 (89.9%)	76 (96.2%)	48 (88.8%)	41(89.1%)	384 (91.2%)
Moderately positive	1 (1.2%)	1 (3.6%)	4 (3.1%)	0 (0.0%)	1 (1.9%)	1 (2.2%)	8 (1.9%)
Strongly positive	6 (7.0%)	2 (7.1%)	9 (7.0%)	3 (3.8%)	5 (9.3%)	4 (8.7%)	29 (6.9%)

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phosphorylation of STAT5 in the TSLPR strongly positive samples than the fully negative ones, with a mean of pSTAT5$^+$ cells of 60.79%±12.79% (range 37.0%-83.6%) and 2.95%±3.26% (range 0.2%-11.0%), respectively (P<0.001 by Bonferroni test) (Figure 2A). Furthermore, CRLF2-rearranged MUTZ5 and MHH-CALL4 cells showed aberrant TSLP-induced pSTAT5 compared with CRLF2 wild-type REH cells (*data not shown*). Interestingly, the

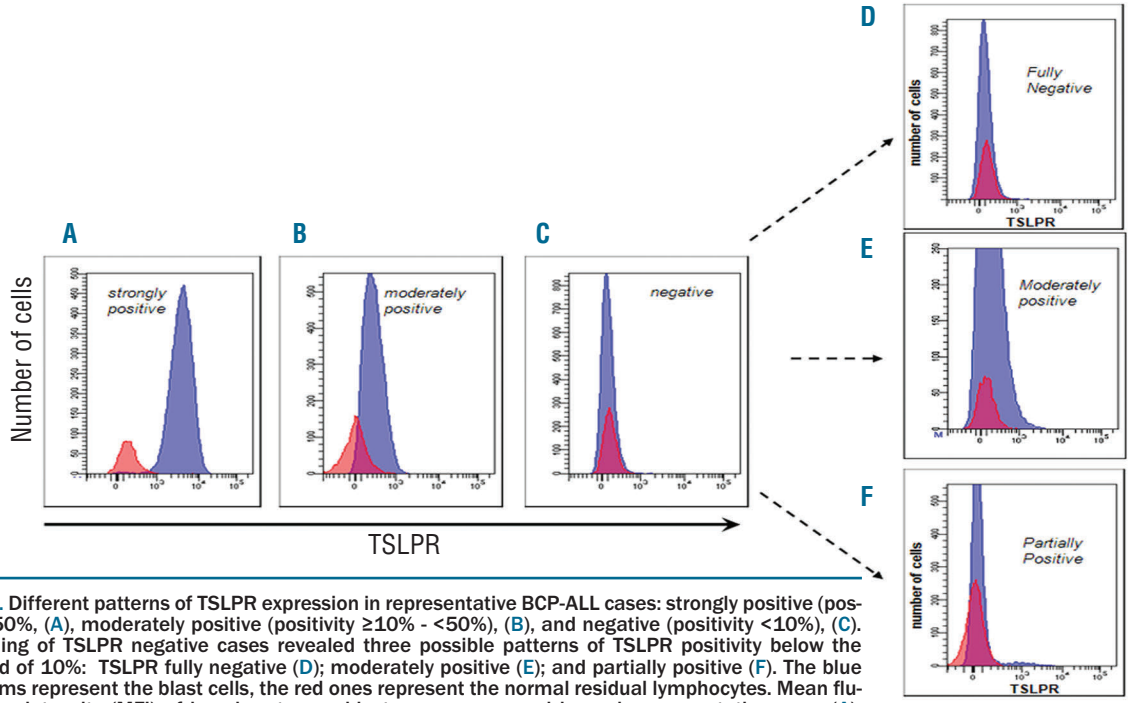

Figure 1. Different patterns of TSLPR expression in representative BCP-ALL cases: strongly positive (positivity ≥50%, (A), moderately positive (positivity ≥10% - <50%), (B), and negative (positivity <10%), (C). Fine tuning of TSLPR negative cases revealed three possible patterns of TSLPR positivity below the threshold of 10%: TSLPR fully negative (D); moderately positive (E); and partially positive (F). The blue histograms represent the blast cells, the red ones represent the normal residual lymphocytes. Mean fluorescence intensity (MFI) of lymphocytes *vs.* blasts were measured in each representative case: (A): 174.0 *vs.* 3.899; (B): 149.0 *vs.* 333; (C) and panel (D) (same representative patient): 93.0 *vs.* 97.0; (E): 88.0 *vs.* 175.0; (F): 48.0 *vs.* 1001.0.

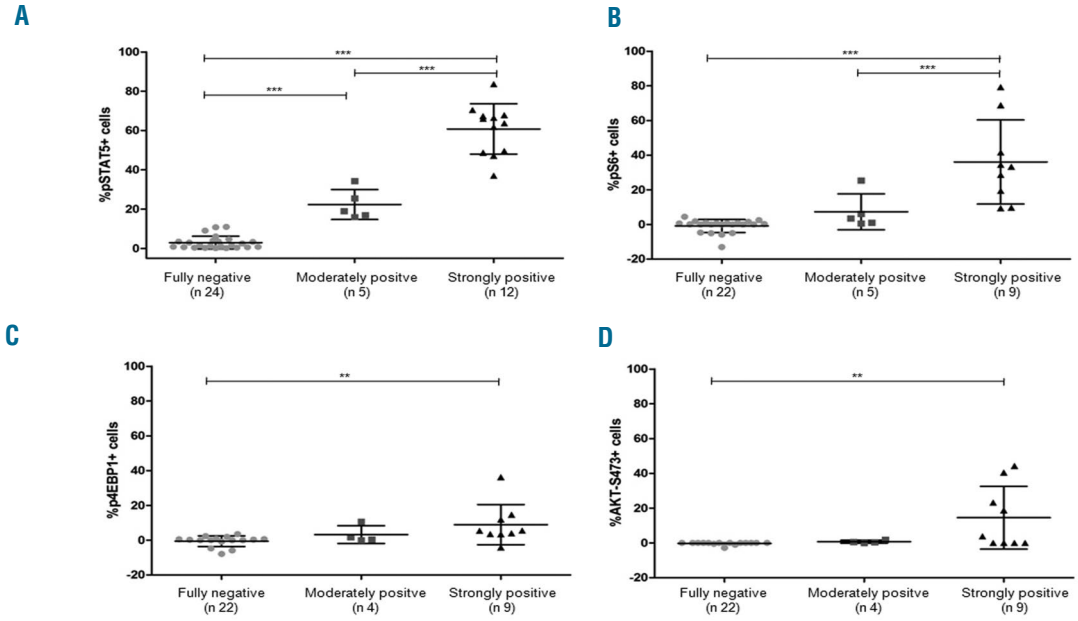

Figure 2. TSLP-induced phosphoprotein responses in BCP-ALL patients according to TSLPR expression (fully negative, moderately positive, or strongly positive). Distribution of positive cells is represented as whiskers plot of 5th and 95th percentile with means and standard deviations. Statistical significance among groups was determined by one-way ANOVA analysis of variance followed by *post hoc* Bonferroni's multiple comparison test (*P<0.05, **P<0.01, ***P<0.001). (A) Shows pSTAT5 response (n 41); (B), (C), and (D) show TSLP-induced pS6, p4EBP1 and pAKT expression (n 36, 35, and 35, respectively). All groups were compared, but only those with statistical significance are indicated by stars and horizontal bars. Data were normalized to the basal phosphorylation level of each phosphoprotein.

group of 5 patients who were TSLPR moderately positive (<10%) showed enhanced pSTAT5 response with a mean of 22.36%±.63% (range 16.0%-34.3%), significantly higher than TSLPR fully negative patients (P<0.001 by Bonferroni test) (Figure 2A).

We also studied TSLP-induced signaling through the PI3K/AKT/mTOR pathway (S6, 4EBP1 and AKT) in 36 out of 41 BCP-ALL patients [9 TSLPR strongly positive, 5 moderately positive (i.e. <10%), and 22 fully negative]. TSLP stimulation led to a significant increase in phosphorylation levels of S6, 4EBP1, and AKT in TSLPR strongly positive samples as compared to both the fully negative and moderately positive cases (one-way ANOVA P<0.0001, P=0.0119, and P=0.0065, respectively), in good agreement with Tasian *et al*.[14] Differences between groups are detailed in Figure 2B-D. Contrary to reports by Tasian *et al*., in our samples, we observed no significant difference in basal phosphorylation of S6, 4EBP1, and AKT-S473 that could be ascribed to differences in TSLPR expression levels.

Strikingly, neither TSLPR fully negative nor TSLPR moderately positive cases showed mutations in *JAK2, CRLF2*, or *IL7RA*. However, the observation of enhanced level of basal pSTAT5 in TSLPR moderately positive as compared to the fully negative patients may indicate the presence of a CRLF2 rearranged sub clone below the level of detection in this latter subgroup of patients. In favor of this hypothesis, TSLPR strongly positive patients displayed an heterogeneous mutational profile: 10 of 12 carried *P2RY8-CRLF2* rearrangement, one of these also carrying a mutation in JAK insertion L681-I682 insEA and another one carrying the IL7RA mutation S185C; 1 of 12 displayed *IGH@-CRLF2* translocation and *JAK2* point mutation R683G; 1 of 12 was wild type also for *P2RY8-CRLF2* and *IGH@-CRLF2* rearrangements. SNP at codon 244 (rs151218732) of *CRLF2* as well as SNP at codon 244 (rs6897932) of IL7RA were randomly distributed independently of TSLPR overexpression. A summary of phenotypic, molecular and signaling features of the analyzed patients is provided in *Online Supplementary Table S2*.

To the best of our knowledge, this is the first report showing BCP-ALL patients moderately positive for TSLPR characterized by aberrant pSTAT5 and pS6 expression. We are currently investigating whether this signature refers to the presence of minor clones or is due to additional mechanisms driving aberrant JAK/STAT and PI3K/mTOR signal transduction.

In this regard, Tasian *et al*. has pointed to a potential diagnostic value of TSLP-mediated phosphosignaling in patients moderately positive for TSLPR staining (i.e. TSLPR-dim) as it would be a *bona fide* functional read out of the CRLF2 status. However, they did not provide any evidence of TSLPR-dim patients. In our study, we demonstrate the existence of CRLF2 moderately positive patients characterized by an activated phosphosignaling cascade. Thus, it is possible that Tasian *et al*. failed to identify TSLPR moderately positive patients because TSLPR expression was assessed after fixation and permeabilization, a procedure that is known to mask the presence of several surface antigens.

In summary, screening of TSLPR expression in BCP-ALL patients can be successfully achieved using standardized FCM protocols. FCM and PCR are highly concordant in detecting both CRLF2 over-expressed and non-over-expressed patients. However, patients characterized by a moderately or partially positive TSLPR expression associated with aberrant pSTAT5 and pS6 expression could only be detected by FCM analysis. Thus, our findings might prove useful in refining future diagnostic screening of ALL patients and help develop novel CRLF2 inhibitor-based therapies. In this regard, it is important to point out that approximately 50% of ALL patients with a Ph-like gene expression profile, which is associated with a poor outcome, have CRLF2 rearrangements.[15]

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