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DUX4r, *ZNF384r* and *PAX5*-P80R mutated B-cell precursor acute lymphoblastic leukemia frequently undergo monocytic switch

Michaela Novakova,[1,2,3*] Marketa Zaliova,[1,2,3*] Karel Fiser,[1,2*] Barbora Vakrmanova,[1,2] Lucie Slamova,[1,2,3] Alena Musilova,[1,2] Monika Brüggemann,[4] Matthias Ritgen,[4] Eva Fronkova,[1,2,3] Tomas Kalina,[1,2,3] Jan Stary,[2,3] Lucie Winkowska,[1,2] Peter Svec,[5] Alexandra Kolenova,[5] Jan Stuchly,[1,2] Jan Zuna,[1,2,3] Jan Trka,[1,2,3] Ondrej Hrusak[1,2,3#] and Ester Mejstrikova[1,2,3#]

[1]CLIP - Childhood Leukemia Investigation Prague, Czech Republic; [2]Department of Paediatric Hematology and Oncology, Second Faculty of Medicine, Charles University, Prague, Czech Republic; [3]University Hospital Motol, Prague, Czech Republic; [4]Department of Internal Medicine II, University Hospital Schleswig-Holstein, Kiel, Germany and [5]Comenius University, National Institute of Children's Diseases, Bratislava, Slovakia

*MN, MZ and KF contributed equally as co-first authors.

#OH and EM contributed equally as co-senior authors.

ABSTRACT

Recently, we described B-cell precursor acute lymphoblastic leukemia (BCP-ALL) subtype with an early switch to the monocytic lineage and the loss of the B-cell immunophenotype, including CD19 expression. Thus far, the genetic background has remained unknown. Among 726 children consecutively diagnosed with BCP-ALL, 8% patients experienced a switch detectable by flow cytometry (FC). Using exome and RNA sequencing, the switch was found to positively correlate with three different genetic subtypes: *PAX5*-P80R mutation (five cases with switch of five), rearranged (*DUX4r*) (30 cases of 41) and rearranged (*ZNF384r*) (four cases of ten). Expression profiles or phenotypic patterns correlated with genotypes, but within each genotype no cases who subsequently switched could be indentified. If switching was not taken into account, the B-cell-oriented FC assessment underestimated the minimal residual disease level. For patients with *PAX5*-P80R, a discordance between FC-determined and polymerase chain reaction-determined minimal residual disease was found on day 15, resulting from a rapid loss of the B-cell phenotype. Discordance on day 33 was observed in all the *DUX4r*, *PAX5*-P80R and *ZNF384r* subtypes. Importantly, despite the substantial phenotypic changes, possibly even challenging the appropriateness of BCP-ALL therapy, the monocytic switch was not associated with a higher incidence of relapse and poorer prognosis in patients undergoing standard ALL treatment.

Correspondence:

ESTER MEJSTRIKOVA
ester.mejstrikova@lfmotol.cuni.cz

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Introduction

We recently described a pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) subtype with an early switch towards the monocytic lineage.[1] Such a monocytic switch leads to a variable degree of monocytosis in the early phase of treatment. The switched monocytic cells ("monocytoids") have significantly diminished expression of immature or lymphoid markers (*i.e.*, CD19 and CD34) and upregulated myeloid markers (*i.e.*, CD33 and CD14) while keeping shared immunoglobulin/T-cell receptor (IG/TR) rearrangements with malignant B lymphoblasts. The increase in monocytoid cells was most prominent at days 8 and 15 after the start of therapy, although the proportion differed between the patients. We reported frequent expression of CD2 and a higher prevalence of *ERG* deletions and *IKZF1* gene alterations in this leukemia subtype than in other subtypes.[1] However, at that time, we

were unable to uncover the genetic background. In our previous work, we observed a slower response to initial treatment in patients with a monocytic switch than in patients without it. Despite significant changes in the phenotype towards the monocytic lineage in some of the patients, risk-based ALL treatment remained the treatment of choice.[1]

Recently, new genetic subtypes of ALL, particularly within the "B-other" group, were discovered using RNA sequencing (RNA-seq).[2,3] We investigated whether any of the newly defined subsets had a higher tendency to undergo monocytic switch.

Monocytic switch is accompanied by the gradual loss of CD19. As the determination of minimal residual disease (MRD) by flow cytometry (FC) relies on B-cell markers, switching leads to MRD underestimation. The FC MRD value on day 15 is used for patient stratification in the current pediatric Berlin-Frankfurt-Münster (BFM) BCP-ALL treatment protocols. It is an open question whether FC monitoring of MRD should be adapted for patients with switch.

Recently, anti-CD19 therapy, namely, blinatumomab, was added to pediatric ALL frontline treatment protocols, and a larger proportion of patients will be treated with it in the near future. As patients with switch that includes CD19 loss may be selected for anti-CD19 treatment, knowledge about subtypes prone to switch will be of interest. Moreover, anti-CD19 therapy may result in monocytic switch, as was repeatedly reported, especially for cases of *KMT2A* rearrangement.[4-9]

The aim of this study is to describe the molecular landscape of ALL with monocytic switch in the context of newly discovered genetic subtypes. We aim to describe the switching behavior in distinct genetic subtypes and to analyze the extent of its influence on the FC assessment of MRD. In addition, we analyzed the impact of switching phenomenon on relapse risk.

Methods

This study was approved by the Institutional Review board of the University Hospital Motol and the Second Faculty of Medicine and informed consent was obtained from all patients and their guardians in accordance with the Declaration of Helsinki.

We included 726 BCP-ALL patients (age, 0-18 years) diagnosed in the Czech Republic between 09/2007 and 02/2019. Patients were treated according to the following BFM protocols: ALL-IC-BFM-2002 (n=17); ALL BFM 2000 (n=177); ALL BFM 2009 (n=483); the Interfant 2006 protocol (for children younger than 12 months; n=30); the EsPhALL protocol (for *BCR-ABL1*-positive patients; n=16); and other protocols (n=3; one patient was treated by a modified protocol, one Down syndrome patient with significant comorbidities was treated with a reduced ALL BFM 2009 protocol, and one patient moved abroad after induction therapy). Some of the 726 patients (179 of 726) diagnosed between 09/2007 and 05/2010 were included in a previous study.[1] For selected analyses, we expanded this consecutive cohort with additional 19 patients who were diagnosed before 09/2007 and for whom the switching phenomenon was identified and described retrospectively (n=16)[1] or who were diagnosed abroad (Germany, n=2; and Slovakia, n=1) (*Online Supplementary Figure S1*). All patients were screened for the presence of recurrent fusion genes (*BCR-ABL1*, *KMT2A-AFF1*, *ETV6-RUNX1* and *TCF3-PBX1*). In all patients, standard cytogenetic evaluation and assessment of the DNA index were performed as published previously.[1,10] The B-other subset was defined as BCP-ALL by the absence of all routinely investigated classifying aberrations (*ETV6-RUNX1*, hyperdiploidy, hypodiploidy, *BCR-ABL1*, *KMT2A* rearrangements and *TCF3-PBX1*).

Flow cytometry

The diagnostic phenotype was determined through standard protocols.[11–13] Ambiguous lineage acute leukemia (ALAL) diagnosis was based on the European Group for the Immunological Characterization of Leukemias (EGIL) criteria[11,12,14,15] and/or the World Health Organization[16] classification. A summary of the antibody clones and vendors is presented in the *Online Supplementary Table S1*. In addition, the percentage of the B-monocytoid population defined as $CD19^{pos}CD14^{pos}$ was determined with an eight-color combination of antibodies against CD45, CD14, CD34, CD19, CD33, CD20, CD10 and CD3 at diagnosis (day zero [d0]), day 8 [d+8], day 15 [d+15], and day 33 [d+33] in the bone marrow (BM) and/or peripheral blood (PB) as shown previously.[1] FC-assessed MRD was evaluated using three- or four-color monoclonal antibody combinations in the period between 1998 and 2007[17] and using eight-color combinations starting in 2007.[18,19] The sensitivity (level of quantification and level of detection, LoQ and LoD, respectively) of the FC-assessed MRD was defined by the number of nucleated cells measured in an MRD-specific tube (for a sensitivity of 10^{-3} and 10^{-4}, 20,000 and 200,000 nucleated cells were measured, respectively). Only samples with an appropriate FC MRD sensitivity were included in the analysis: generally, a sensitivity of 10^{-4} was required; for cases with polymerase chain reaction (PCR)-determined MRD $\geq 10^{-2}$, measures of sensitivity that were at least one log value lower than that of the actual PCR-determined MRD value were sufficient. The sample was assessed as FC MRD positive when a cluster of at least 20 events with an aberrant B-cell phenotype was detected.

Definition of the switching phenomenon

Based on our previous work,[1] we defined the switching phenomenon as the presence of an intermediate B-monocytoid population, *i.e.*, BCP-ALL blasts with a gradual decrease in CD19 expression accompanied by a gradual increase in the expression of at least one monocytic marker (CD14, CD33, or higher side scatter, SSC) (*Online Supplementary Figure S2*) at any time point between d0 and d+33. We used fluorescence-activated cell sorting (FACS) of the intermediate B-monocytoid ($CD19^{pos}CD14^{pos}$) and monocytoid ($CD19^{neg}CD14^{pos}$) populations to show that the IG/TR rearrangements were identical to those in B lymphoblasts when enough material was available (patients n =37; samples n=70) between d0 and d+33.

Immunoglobulin/T-cell receptor polymerase chain reaction-assessed minimal residual disease

Patient-specific IG/TR assays were developed as described previously.[1,20,21] In patients with two independent IG/TR targets, the higher value was reported. Sensitivity (LoD) and quantitative range (LoQ) for each assay was defined according to the European Study Group on MRD guidelines.[22] A minimum LoD of 10^{-4} was achieved in all patients/timepoints.

Statistics

Fisher's exact test was used for comparing categorical variables, and the Mann-Whitney test was used for continuous variables. Other tests used are explicitly indicated in the text. The results were considered significant when *P*-values were less than 0.05. Statistical analyses were performed using GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA), R[23] and StatView version 5.0 (StatView Software, Cary, NC, USA).

Data analysis of genetic and immunophenotypic data

RNA-seq data (patients with switch, n=73; patients without switch, n=124), whole-exome sequencing (WES) data (patients with switch, n=30; patients without switch, n=70), single-nucleotide polymorphism (SNP) data (patients with switch, n=59; patients without switch, n=108) and ERG deletion data were analyzed in diagnostic samples as published previously.[24–26] Diagnostic samples were sorted if blasts comprised fewer than 80% of mononuclear cells. Purity of sorted populations was at least 90%. Patients in the B-other group without the following aberrations were assigned to the B-other rest subgroup: *DUX4*, *ZNF384*, *MEF2D* and *NUTM1* rearrangements; *BCR-ABL1*-like and *ETV6-RUNX1*-like expression profiles; and iAMP21, *PAX5*-P80R and *IKZF1*-N159Y mutations.

In order to analyze RNA-seq and immunophenotypic data, uniform manifold approximation and projection (UMAP)[27] was used as the dimensionality reduction algorithm. Hierarchical clustering analysis (HCA) was performed using Euclidean distance and Ward's linkage.

Results

Incidence and features of patients with monocytic switch

Prospectively, we identified 61 patients with monocytic switch using the criteria described above (Table 1), which corresponded to 8% of patients.

No sex difference occurred in the monocytic switch (69% females *vs*. 56% males, not significant [n.s.]) but the monocytic switch was associated with older age at diagnosis (median 7.8 *vs*. 4.5 years, respectively, *P*<0.001) and a higher initial white blood cell (WBC) count (median 10,750/μL *vs*. 6,670/μL, *P*=0.038), lower hemoglobin level (median 7.8 g/dL *vs*. 8.8 g/dL, *P*=0.0046), higher platelet count (median 74,000/μL vs. 58,000/μL, *P*=0.048) and a higher proportion of blasts in PB (59% *vs*. 32%, *P*=0.0039), while the proportion in BM did not differ (91.2% *vs*. 90%, n.s.).

We confirmed the presence of patient-specific IG/TR rearrangements in the sorted monocytoid cells in 33 of 37 patients in whom the sorting was successful at various time points between d0 and d+33 (16 of 19 positive at d0; 23 of 27 positive between d+1 and d+14; 14 of 15 positive at d+15; eight of nine positive between d+16 and d+33). In the morphological examination of some patients, an increase in monocytic cells with variable morphology (monoblasts, promonocytes, and mature monocytes) was very clear. In three patients, at d+8 (DUX4r, n=2; and ZNF384r, n=1), we observed over 10,000 monocytes/μL in the PB samples (*Online Supplementary Figure S3A* and *B*).

Monocytic switch is most frequent in the *PAX5*-P80R, *DUX4r*, and *ZNF384r* genetic subtypes

In order to study the relationship between monocytic switch and genetic background, routine (cyto)genetic investigations were supplemented with a retrospective analysis using RNA-seq data, enabling a more detailed genomic characterization of the ALL patients. Patients with monocytic switch were unequally distributed across the ALL subtypes in the unselected consecutive cohort (chi square test *P*<0.0001; Table 1); they were significantly enriched in the *PAX5*-P80R-, *DUX4r*- and *ZNF384r*- -positive ALL subtypes

Table 1. Distribution of cases with monocytic switch in an unselected cohort of B-cell precursor acute lymphoblastic leukemia patients (n=726) stratified into genetic/biological subtypes.

BCP-ALL subtypes		Monocytic switch		*P*
		No n (%)	Yes n (%)	
HHD[1]		266 (97)	8 (3)	*P*<0.0001
ETV6-RUNX1		180 (99)	1 (1)	*P*<0.0001
KMT2Ar		29 (88)	4 (12)	ns
TCF3-PBX1		27 (100)	0 (0)	ns
BCR-ABL1		13 (76)	4 (24)	ns
Hypodiploidy[2]		8 (89)	1 (11)	ns
B-other[3]	B-other rest[4]	49 (94)	3 (6)	ns
	DUX4r	11 (27)	30 (73)	*P*<0.0001
	BCR-ABL1-like	18 (95)	1 (5)	ns
	ZNF384r	6 (60)	4 (40)	*P*=0.0022
	ETV6-RUNX1-like	10 (100)	0 (0)	ns
	iAMP21	6 (100)	0 (0)	ns
	PAX5-P80R	0 (0)	5 (100)	*P*<0.0001
	NUTM1r	4 (100)	0 (0)	ns
	MEF2Dr	3 (100)	0 (0)	ns
	IKZF1-N159Y	1 (100)	0 (0)	ns
	Unknown[5]	34 (100)	0 (0)	ns
Total		665 (92)	61 (8)	

[1]High hyperdiploidy with >50 chromosomes; [2]<44 chromosomes; [3]BCP-ALL negative for high hyperdiploid cases (*HHD*), *ETV6-RUNX1*, *KMT2Ar*, *TCF3-PBX1*, *BCR-ABL1* and hypodiploidy; [4]B-other analyzed by RNA sequencing (RNA-seq) and not belonging to any of the established subtypes; [5]B-other not analyzed by RNA-seq (this subset is biased towards nonswitching cases because RNA-seq was performed in samples from all patients with monocytic switch, without identified genetic aberrations using polymerase chain reaction [PCR] and/or cytogenetics); [6]*P*-value of the Fisher's exact test on a comparison of the frequency of cases with monocytic switch in individual subsets vs. the frequency of switch among all the remaining cases. Multiple testing correction was done using Benjamini-Hochberg procedure. ns: no statistically significant difference; BCP-ALL: B-cell precursor acute lymphoblastic leukemia.

but were significantly less frequent among high-hyperdiploid subtypes and extremely rare in *ETV6*-RUNX1-positive ALL. In addition to 61 patients with monocytic switch in the consecutive cohort, we identified another 19 patients with monocytic switch who belonged to the *DUX4r* (n=15), *PAX5*-P80R (n=2), *ZNF384r* (n=1) and high-hyperdiploid (n=1) ALL subtypes. Nevertheless, except for the *DUX4r* ALL subtype (representing the subgroup with the highest prevalence of switch, with nearly half of all patients having monocytic switch [30 of 61]), the number of patients was too low (and/or the genomic data were too limited) to study the impact of the broader genomic context on monocytic switch occurrence within the individual subtypes. In the *DUX4r* ALL subtype group, we did not find any association between monocytic switch and the most frequent secondary genetic aberrations (deletions of *ERG*, *CDKN2A/B*, *IKZF1* or *PAX5* or mutations in the *NRAS* or *KMT2D* genes; *Online Supplementary Table S2*).

The pattern of monocytic switch correlated with the genotype (Figure 1). Patients with the *DUX4r* ALL subtype presented with predominant CD19 positive ($CD19^{pos}$) B-precursor blasts at diagnosis and typically maintained these cells, while the phenotype gradually became more monocytic on d+8 and d+15. Among the 25 patients with switching phenomenon identified already at diagnosis, only two patients were of the *DUX4r* subtype (Fisher's exact test P=0.0002). Patients with the *PAX5*-P80R mutation also presented with $CD19^{pos}$ B precursor blasts, but after d+8, the B-cell markers had typically disappeared concomitantly during the switch. Patients with the *ZNF384* fusion often co-expressed B-precursor and monocytic markers at diagnosis or presented with bilineal disease with separate monocytoid population of blasts, and the monocytoid population often became more prominent during chemotherapy.

The amplitude of monocytic switch (determined as the maximum number of intermediate B-monocytoid cells) did not differ among the different genetic subtypes (*Online Supplementary Figure S3C*, Kruskal-Wallis test, n.s.).

Differences in the diagnostic gene expression signature associated with a subsequent monocytic switch are driven by genotype

We used RNA-seq to assess changes in gene expression that were related to monocytic switch at the time of diagnosis. We analyzed 197 diagnostic transcriptomes and determined the genes that were differentially expressed among patients with (n=73) and without monocytic switch (n=124). Among the 50 top-ranking genes (only protein-encoding genes with absolute fold changes ≥2.5 were considered; *Online Supplementary Table S3*), we found the pro-

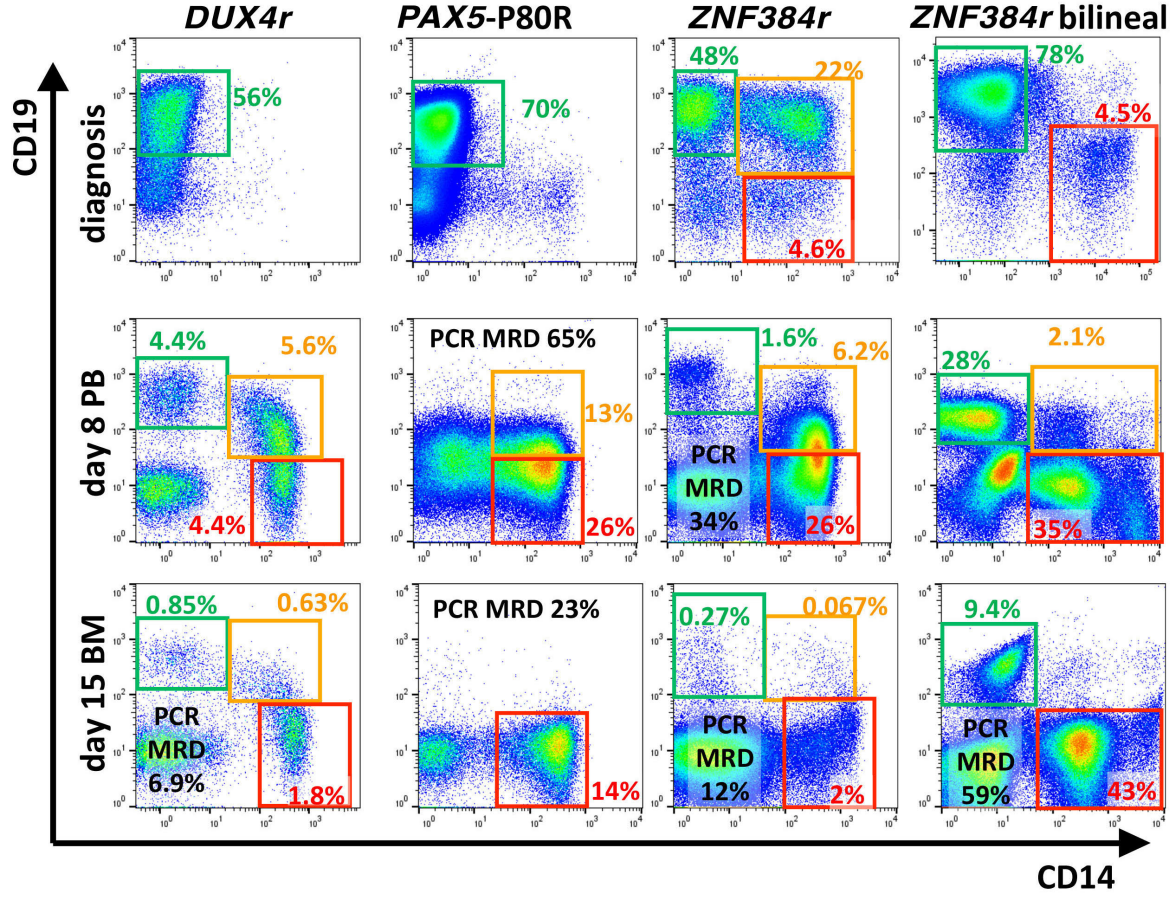

Figure 1. Monocytic switch appearance. In cytometric plots, mononuclear cells after exclusion of doublets and nonmalignant T cells and B cells (if available) are shown. Green, orange and red rectangles highlight the preswitched B-precursor blasts, B-monocytoid intermediate cells and fully switched monocytoids, respectively. Examples of patients with the respective genotypes are shown at day zero (d0) (bone marrow [BM]), d+8 (peripheral blood [PB]) and d+15 (BM). The percentage of each population is shown (of all nuclear cells) in the corresponding color. Polymerase chain reaction (PCR)-determined minimal residual disease (MRD) values in percentages are shown in black. We observed *DUX4r* monocytic switch pattern in 43 of 45 patients, *PAX5*-P80R pattern in seven of seven patients and *ZNF384r* pattern in three of five patients within the respective genotypes in all patients analyzed (n=745).

liferation-activating gene *CRLF2* (lower in patients with monocytic switch than in those without monocytic switch), the cell cycle regulator *CCNA1* (cyclin A1, higher in patients with monocytic switch) and androgen receptor (AR, higher in patients with monocytic switch), as well as six genes with a CD marker designation: *CD371*, *CD301*, *CD1E*, *CD125* (all higher in patients with monocytic switch), *CD158K* and *CD20* (both lower in patients with monocytic switch). Consistent with our previously published data, patients with switch had increased *CEBPA* expression ($P<0.0001$), which was also the case when only *DUX4r* patients were analyzed ($P<0.0001$).

We asked whether these changes were primarily driven by monocytic switch itself or by the underlying genotype. Both the unsupervised hierarchical clustering and the UMAP analyses showed that the patients primarily clustered according to genotype, including the *DUX4r*, *PAX5*-P80R and *ZNF384r* subtypes, which were enriched for monocytic switch, and whether the patients eventually experienced monocytic switch seemed to be secondary (Figure 2A; *Online Supplementary Figure S4*).

As genotype-associated transcriptome differences may override monocytic switch-associated differences, we repeated the analysis in the most prevalent (accounting for >60% of the patients with switch) genotypic subset (*DUX4r*). The *Online Supplementary Figure S5* shows that the clustering of the switching patients was not clear. Among the top-ranking most differentially expressed genes between switching (n=44) and nonswitching (n=11) patients were the previously described *CEBPA*,[1] hematopoietic regulator *FLT3* and Toll-like receptor *TLR10*, which were all higher in patients with the *DUX4r* subtype with switch than in patients with *DUX4r* without switch (*Online Supplementary Table S4*).

Genotype also influences immunophenotype associated with subsequent monocytic switch

We next sought to determine whether the diagnostic immunophenotype predicts, similarly to transcriptome, monocytic switch behavior and whether it can be associated with underlying genetic aberrations.

For these purposes we analyzed the diagnostic immunophenotype of the blast population in 745 patients.

The overall picture appeared to be analogous to that of the transcriptome data: diagnostic immunophenotypes were grouped based on the genetic subtype as seen in the UMAP analysis rather than by the monocytic switch status (Figure 2B).

In our previous study all patients with monocytic switch harbored CD2 expression, although belonging to different genetic subtypes. As we have shown recently,[28] CD2 is expressed in approximately 75% of *DUX4r* patients whereas a newly described marker CD371 (CLL-1) is found in all patients with *DUX4r* (*Online Supplementary Figure S8*).

We also observed the expression of CD2 and CD371 in five of seven and two of five patients with *PAX5*-P80R, respectively. Interestingly, all seven patients with *PAX5*-P80R had homogeneous expression of CD66c. Expression of CD4, a rare aberrant marker in BCP-ALL, was present in four of seven *PAX5*-P80R patients, all of which had cells with a phenotype of $CD34^{neg}33^{pos}$ (in contrast to the other three of seven $CD4^{neg}CD34^{pos}$ patients). Interestingly, CD66c expression was retained on switched monocytoid cells on d+15 in all six patients with available data.

Patients with the *ZNF384r* subtype were often classified as having acute leukemia of ambiguous lineage (ALAL) using the EGIL classification (six of 11 patients).

The typical immunophenotypes of the main three subtypes with switch (*DUX4r*: $CD10^{pos}CD20^{neg}CD34^{pos}CD2^{pos}CD371^{pos}$; *PAX5*-P80R: $CD10^{neg/pos}CD66c^{>75\%}CD2^{<50\%}CD4^{neg}CD34^{pos}CD33^{<50\%}$ or $CD10^{neg/pos}CD66c^{>75\%}CD2^{pos}CD4^{pos}CD34^{neg}CD33^{pos}$; ZNF384r: $CD10^{<50\%}CD13^{pos}CD66c^{neg}CD34^{pos}CD135^{pos}CD24^{<60\%}$), are shown in the *Online Supplementary Figure S8*.

Discordance between flow cytometry- and polymerase chain reaction-determined minimal residual disease reflects different switching dynamics in individual genetic subtypes

Loss of B-cell markers during monocytic switch interferes with B-cell-oriented MRD analysis by FC. As we showed previously in our pilot study[1] and now in a significant cohort of patients, switched monocytoid blasts maintain leukemic IG/TR rearrangements despite completely losing the B-cell phenotype. We thus analyzed the influence of switch on MRD detection by comparing FC and PCR MRD quantitation results in the genotype subsets most prone to switch.

We had only limited data for d+8 PB samples from *PAX5*-P80R (n=3) and *ZNF384r* patients (n=2). Spearman R for *DUX4r* (n=31) and patients with monocytic switch outside these three genetic subtypes (n=11) was 0.7 and 0.72, respectively (*Online Supplementary Figures S6* and *S7*).

But, as shown in Figure 3, FC underestimation of MRD led to pronounced discordance at d+15 in the *PAX5*-P80R patients. The concordance of the FC and PCR MRD positivity/negativity at the 10^{-3} level was 91% for the *DUX4r* subtype and 82% for the *ZNF384r* subtype but only 17% for the *PAX5*-P80R subtype. Concordance at the 10^{-1} level, which was determined at this time point as the FC cut-off value for stratifying patients for undergoing high-risk treatment according to the BFM protocols, was 78% for the *DUX4r* subtype and 78% for the *ZNF384r* subtype but only 43% for the *PAX5*-P80R subtype.

At d+33, all three subtype groups showed poor correlation between the FC and PCR MRD values (Figure 3) compared to previously published data.[18,29] The concordance of the FC- and PCR-determined MRD at the level of 10^{-3} was 55% for the *DUX4r* subtype, 56% for the *ZNF384r* subtype and 33% for the *PAX5*-P80R subtype.

Monocytic switch rarely caused MRD discrepancies in the other genetic subtypes. When analyzed separately, among 23 patients in the $DUX4r^{neg}$*PAX5*-P80R^{neg}*ZNF384r*^{neg} subset and in which monocytic switch was observed, FC MRD at the appropriate level of sensitivity (defined in the Methods) was measured in 21 and 16 patients on d+15 and d+33, respectively (*Online Supplementary Figure S7*). The concordance at d+15 for the 10^{-1} and 10^{-3} levels was 86% and 90%, respectively. The concordance at d+33 at the 10^{-3} level was 76%.

At week plus 12 majority of samples were PCR (86%) and FC (97%) MRD negative. Concordance for *DUX4r* (n=44), *PAX5*-P80R (n=5), *ZNF384r* (n=8) and cases with switch outside these subtypes (n=21) was 82%, 80%, 100% and 90%, respectively (*Online Supplementary Figures S6* and *S7*).

Prognostic relevance of monocytic switch

From a clinical perspective, it is important to know whether monocytic switch is of prognostic relevance and whether the phenotype changes during relapse. All but one

patient with monocytic switch was treated according to standard BCP-ALL frontline protocols. In the whole study cohort, eight of 80 patients with monocytic switch relapsed. Of the eight relapsed patients, six patients relapsed with an identical BCP-ALL subtype to the subtype at diagnosis (two had the *BCR-ABL1* subtype, one had the *DUX4r* subtype, one had the HHD subtype, one had the *ZNF384r* subtype, and one had the B-other rest subtype). Interestingly, only one of those patients (*ZNF384r*) developed monocytic switch during induction therapy for relapse. Two patients (one with the *DUX4r* subtype and one with the *KMT2Ar* subtype) relapsed with monocytic AML. In all three patients with the presence of monocytoid blasts at relapse, we proved IG/TR rearrangements in the

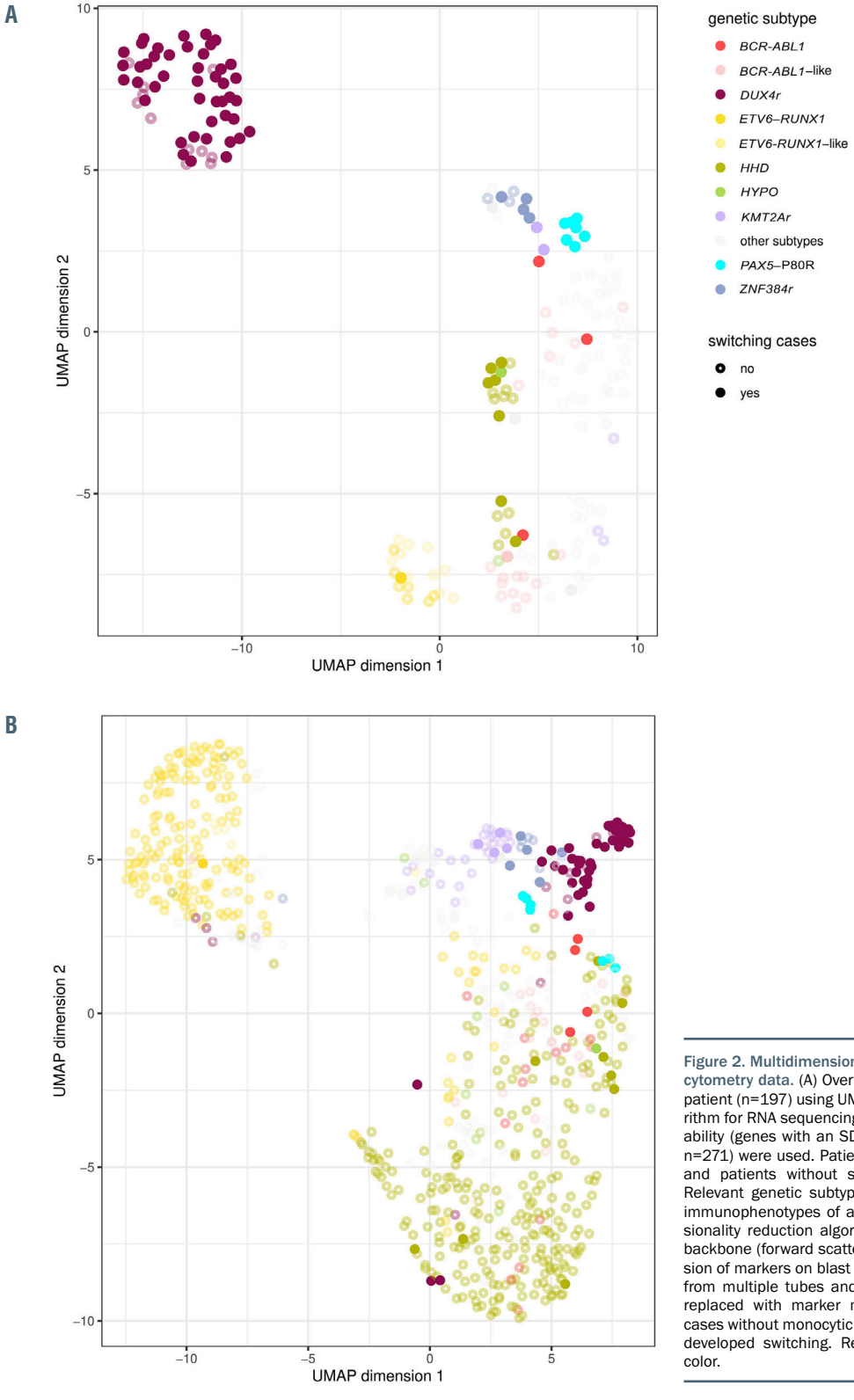

Figure 2. Multidimensional analysis of RNA sequencing and flow cytometry data. (A) Overview of the gene expression found for all patient (n=197) using UMAP as the dimensionality reduction algorithm for RNA sequencing data. Only the genes with the most variability (genes with an SD 0.4-fold higher than the maximum SD, n=271) were used. Patients with switch are shown as full circles, and patients without switching are shown as empty circles. Relevant genetic subtypes are shown in color. (B) Overview of immunophenotypes of all cases (n=745) using UMAP as dimensionality reduction algorithm. Blasts were gated using common backbone (forward scatter, side scatter, CD45). The data (expression of markers on blast population in percentage) were prepared from multiple tubes and merged together. Missing values were replaced with marker median values. Open circles represent cases without monocytic switch, full circles represent cases which developed switching. Relevant genetic subtypes are shown in color.

monocytoid cells identical to those in the original BCP-ALL clone. There was no difference in the event-free survival (EFS) between the prospective patients with or without monocytic switch (5-year EFS, 82±5.5% and 86±1.6% , respectively, Figure 4) also when only patients in the high risk/slow early response group were considered (*Online Supplementary Figure S9*).

Discussion

Monocytic switch provides evidence for the relationship between the monocytic and lymphoid lineages. Rigid models of hematopoiesis assume early separation of the monocytic and lymphoid lineages, but there is increasing evidence of innate immune functions in lymphoid lineages, including phagocytosis.[30] The biological origin of the phenomenon remains to be clarified. Rarely B-cell malignancies of various stages of differentiation under unknown circumstances can undergo transdifferentiation into myeloid/histiocytic malignancies.[31,32] An interesting area for future investigation is the relationship between of monocytic cells to their CD14^{pos} dendritic cells counterparts. CD2, a frequent aberrant marker of BCP ALL blasts prior to monocytic switching, does not clarify their fate as it is known to be expressed in subsets of both dendritic cells[33] and monocytic AML.[34] The constant role of the transcription factor C/EBPα seems to play a role in the process. In our previous work, we found higher expression of *CEBPA* in patients with monocytic switching, which was also true in this extended cohort. C/EBPα directly represses B-cell genes. DiStefano *et al.* reported that C/EBPα can enforce B-cell transcription factor silencing by increasing the expression of the histone demethylase Lsd1 (Kdm1a) and the histone deacetylase Hdac1 at the protein level and that these enzymes are required for the downregulation of B-cell enhancers and the silencing of the B-cell program.[35] *In vitro* models demonstrate that C/EBPα induces the repression of key B-cell regulators such as Foxo1, Ebf1 and Pax5[36].

In our study we show that monocytic switch behavior is not limited to a single genotypic subset and that various leukemia genotypes show different propensities to switch to monocytic cells. In agreement with our previous report, in this study, the majority of patients whose blasts switched to monocytoids were categorized in the B-other subset (70% of patients with monocytic switch compared to 21% of patients without switch; Fisher's exact test P<0.00001). New genomic methods – namely, RNA-seq – were recently used to discover new recurring genetic subtypes within the mixture of the genotypes thus far labeled B-other.[2,3,24,25,37–40] Three of these subsets, *DUX4r*, *PAX5*-P80R, and *ZNF384r*, were frequently associated with switch, constituting the majority of the patients with switch in this study. We did not identify a known subtype-defining genetic aberration (B-other rest) in only 3.8% of the patients with monocytic switch (Table 1). Interestingly, all patients with *PAX5*-P80R presented with monocytic switch.

Although it did not reach statistical significance (probably due to the low number of patients), a switching phenomenon was observed in 24% of cases with *BCR-ABL1*. McClellan *et al.* found that primary *BCR-ABL1*-positive BCP-ALL cells could be reprogrammed into macrophage-like cells through exposure to cytokines in vitro or by transient expression of the transcription factor C/EBPα or PU.1.[41] Surprisingly, monocytic switch was not significantly more frequent in the *KMT2Ar* subtype than in other sub-

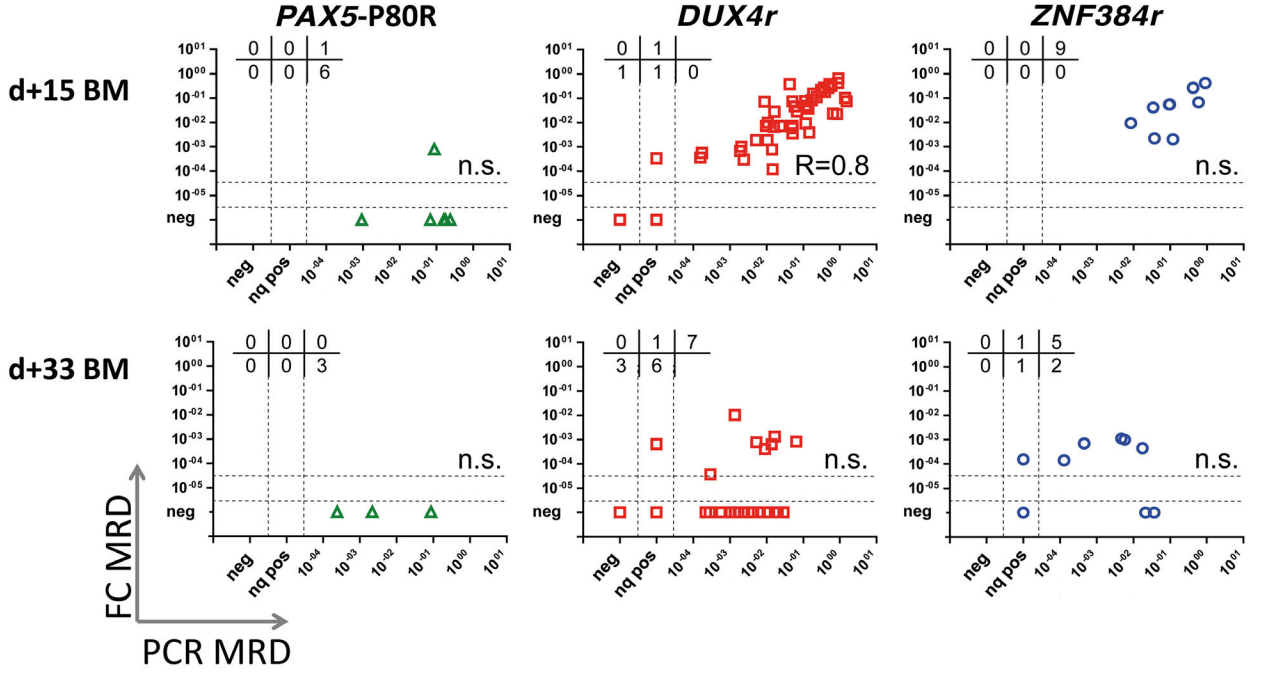

Figure 3. Correlation of the mimal reidual disease results obtained by flow cytometry and polymerase chain reaction in selected patient groups. Only samples with appropriate measured sensitivity are shown (the flow cytometry [FC] sensitivity is 0.0001 if the polymerase chain reaction [PCR]-determined minimal residual disease [MRD] <0.01; for samples with PCR-determined MRD ≥0.01, the FC measurement sensitivity is at least one log value lower than the actual PCR-determined MRD log value). In the upper part of each graph, the number of patients with MRD values FC^{pos}/PCR^{neg}, FC^{pos}/PCR$^{nq\ pos}$, and FC^{pos}/PCR^{pos} (upper lane); and FC^{neg}/PCR^{neg}, FC^{neg}/PCR$^{nq\ pos}$, and FC^{neg}/PCR^{pos} (bottom lane) are indicated. Spearman's rank correlation coefficient indicated if the P-value was <0.05. Nq pos: nonquantifiable positivepositive; BM: bone marrow.

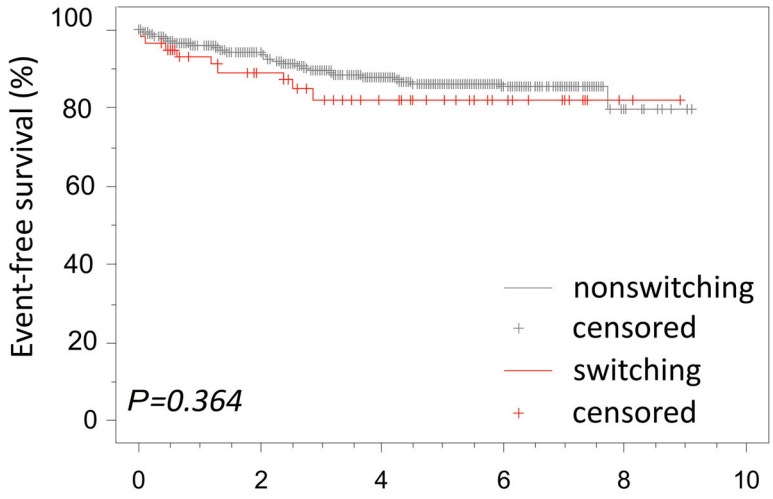

Figure 4. Event-free survival of B-cell precursor acute lymphoblastic leukemia patients with and without switch. The prospective cohort (n=725) is shown. One patient was lost to follow-up.

types, although *KMT2Ar* leukemias can present with a mixed phenotype. However, due to the heterogeneity of *KMT2Ar* leukemias and the limitations of our cohort, the correlation of monocytic switch with specific *KMT2Ar* subtypes should be verified in larger cohorts.

The results from the multidimensional analyses of the gene expression profiles (GEP) and immunophenotypes showed that the patients with monocytic switch coclustered based on genotype. Similarly, Alexander *et al.*[42] recently showed that gene expression-based clustering primarily distinguished genetic subtypes irrespective of MPAL (mixed phenotype acute leukemia) status. Together, these findings show that monocytic switch is a behavior with varying propensity across BCP-ALL genotypes rather than being exclusive to a distinct genetic subtype of leukemia.

Similarly, switching cannot be predicted using a diagnostic immunophenotype according to our data. Nevertheless, immunophenotype can help to predict genotype, as was shown previously and extended in this study.[39,40,43,44]

Monocytic switch may lead to uncertainty about the continuation of ALL-type therapy. In some patients the phenomenon was very discreet and might be overlooked using routine examination. On the contrary, we observed monocytosis at d+8 of as high as 20,804/μL, 3,969/μL and 15,544/μL for the *DUX4r*, *PAX5*-P80R and *ZNF384r* subtypes, respectively. Although such findings may trigger thoughts of changing to the AML type of treatment, all but one patient with detected monocytic switch achieved complete remission on an ALL type of treatment. One patient died during induction therapy. The EFS for an ALL type of therapy was identical among patients with and without monocytic switch. However, we did observe two patients (one with the *DUX4r* subtype and one with the *KMT2Ar* subtype) who relapsed with monocytic AML, showing that the optimal treatment of such rare patients has yet to be determined. Of note, the majority of the published cases of monocytic relapse after primary BCP-ALL had the *KMT2Ar* genotype.[45] In addition to these AML relapses, we discovered that six patients with detectable monocytic switch relapsed later with BCP-ALL. According to published data, the prognosis of patients with the *DUX4r* or the *PAX5*-P80R subtype does not seem to be unfavorable.[3,38]

The clinical significance of switched monocytoids, *per se*, remains unknown. To date, we have limited evidence about their potential to initiate relapse. Functional tests of those switched cells so far have not been performed. Interestingly, we observed a rapidly enlarging spleen in one patient and progressive liver failure in another patient, most likely caused by infiltrating macrophages, which had identical IG/TR rearrangements as the original malignant B precursors.

Despite several observed features of switched monocytoids in *DUX4r*- and *PAX5*-P80R-mutated patients (*e.g.*, CD45RA and CD2 positivity in *DUX4r*, CD13 negativity and CD66c positivity in *PAX5*-P80R), their interpretation (especially at time points with myeloid regeneration, including time point d+33) is challenging.

Monocytic switch not only creates discordance between MRD levels determined by FC and MRD levels determined by PCR but also affects the availability of CD19 as a therapeutic target. In some current pediatric treatment protocols, these patients can be stratified for anti-CD19 treatment regardless of their CD19 expression levels. Thus far, data are limited regarding the efficacy of such treatment in patients with monocytic switch. Recently, the first case report of myeloid relapse in BCP-ALL patients with a *ZNF384r* subtype after CAR-T therapy was published.[46] However, myeloid relapse in *ZNF384r* patients without targeted therapy has also been described.[47]

In conclusion, we report the frequency of monocytic switch in novel genetic subtypes of BCP-ALL and highlight the discordance between MRD levels determined by FC and PCR during the switch. New markers for discriminating switched monocytoid blasts from nonmalignant monocytes are needed to overcome FC underestimation of MRD levels, which is becoming more relevant with the use of targeted anti-CD19 therapy.

Disclosures

No conflicts of interest to disclose

Contributions

MN analyzed immunophenotypic data, evaluated FC MRD and wrote the manuscript; MZ was responsible for whole-exome and RNA-seq data and wrote the manuscript; KF analyzed RNA-seq data and wrote the manuscript; BV analyzed immunophenotypic data and evaluated FC MRD; LS performed PCR MRD on sorted samples, evaluated PCR MRD levels and analyzed immunophenotypic data; AM analyzed RNA-seq and immunophenotypic data; MB and MR performed the investigation in German patients; EF was responsible for PCR MRD

analysis; TK analyzed the flow cytometry data and designed the sorting; JStu managed the patients and contributed to data collection; LW analyzed whole-exome and RNA-seq data; PS and AK performed the investigation in Slovak patients; JSta performed statistical analyses; JZ and TK supervised the molecular genetic data analysis; OH wrote and reviewed the manuscript; EM designed the research, analyzed the data and wrote the manuscript. All authors have read and approved the final submission of the manuscript.

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