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Immunophenotypic portrait of leukemia-associated-phenotype markers in B acute lymphoblastic leukemia

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Abstract

Background: Multiparametric flow cytometry (MFC) is an essential diagnostic tool in B acute lymphoblastic leukemia (B ALL) to determine the B-lineage affiliation of the blast population and to define their complete immunophenotypic profile. Most MFC strategies used in routine laboratories include leukemia-associated phenotype (LAP) markers, whose expression profiles can be difficult to interpret. The aim of our study was to reach a better understanding of 7 LAP markers' landscape in B ALL: CD9, CD21, CD66c, CD58, CD81, CD123, and NG2.

Methods: Using a 10-color MFC approach, we evaluated the level of expression of 7 LAP markers including CD9, CD21, CD66c, CD58, CD81, CD123, and NG2, at the surface of normal peripheral blood leukocytes ($n = 10$ healthy donors), of normal precursor B regenerative cells ($n = 40$ uninvolved bone marrow samples) and of lymphoblasts ($n = 100$ peripheral blood samples or bone marrow samples from B ALL patients at diagnosis). The expression profile of B lymphoblasts was analyzed according the presence or absence of recurrent cytogenetic aberrations. The prognostic value of the 7 LAP markers was examined using Maxstat R algorithm.

Results: In order to help the interpretation of the MFC data in routine laboratories, we first determined internal positive and negative populations among normal leukocytes for each of the seven evaluated LAP markers. Second, their profile of expression was evaluated in normal B cell differentiation in comparison with B lymphoblasts to establish a synopsis of their expression in normal hematogones. We then evaluated the frequency of expression of these LAP markers at the surface of B lymphoblasts at diagnosis of B ALL. CD9 was expressed in 60% of the cases, CD21 in only 3% of the cases, CD58 in 96% of the cases, CD66c in 45% of the cases, CD81 in 97% of the cases, CD123 in 72% of the cases, and NG2 in only 2% of the cases. We confirmed the interest of the CD81/CD58 MFI expression ratio as a way to discriminate hematogones from lymphoblasts. We observed a significant lower expression of CD9 and of CD81 at the surface of B lymphoblasts with a t(9;22)(*BCR-ABL*) in comparison with B lymphoblasts without any recurrent cytogenetic alteration ($p = 0.0317$ and $p = 0.0011$, respectively) and with B lymphoblasts

harboring other cytogenetic recurrent abnormalities ($p = 0.0032$ and $p < 0.0001$, respectively). B lymphoblasts with t(1;19) at diagnosis significantly overexpressed CD81 when compared with B lymphoblasts with other recurrent cytogenetic abnormalities or without any recurrent alteration ($p = 0.0001$). An overexpression of CD58 was also observed in the cases harboring this abnormal cytogenetic event, when compared with B lymphoblasts with other recurrent cytogenetic abnormalities ($p = 0.030$), or without any recurrent alteration ($p = 0.0002$). In addition, a high expression of CD123, of CD58 and of CD81 was associated with a favorable prognosis in our cohort of pediatric and young adult B ALL patients. We finally built a risk score based on the expression of these 3 LAP markers, this scoring approach being able to split these patients into a high-risk group (17%) and a better outcome group (83%, $p < 0.0001$).

Conclusion: The complexity of the phenotypic signature of lymphoblasts at diagnosis of B ALL is illustrated by the variability in the expression of LAP antigens. Knowledge of the expression levels of these markers in normal leukocytes and during normal B differentiation is crucial for an optimal interpretation of diagnostic cytometry results and serves as a basis for the biological follow-up of B ALL.

KEYWORDS

B-cell acute lymphoblastic leukemia, leukemia associated phenotype, multicolor flow cytometry

1 | INTRODUCTION

Multiparametric flow cytometry (MFC) is systematically used as a biological diagnosis tool for B acute lymphoblastic leukemia (B ALL), generating critical data on maturation arrest, lineage assignment, phenotypic aberrations, and expression of therapeutic targets by B lymphoblasts (Bene et al., 1995; De Zen et al., 2000; Hrusak & Porwit-MacDonald, 2002). The phenotypic portrait of B ALL is marked by a great complexity with heterogeneity of profiles, reflected in particular by the expression of leukemia-associated phenotype (LAP) markers. These markers can be associated with recurrent genetic abnormalities. In addition, they can be relevant for measurable residual disease (MRD) assessment, with comparable results to data obtained with molecular methods if an adequate number of markers are used and a sufficient acquired number of events is evaluated (Ryan et al., 2009; van Dongen et al., 2012). Two categories of LAP markers can be found on B ALL blasts populations. The first category of markers can be evaluated by MFC through an approach termed "Difference From Normal" (DFN), with two different sub-kinds of profiles. Tumorous cells can indeed harbor asynchronous LAP, corresponding to antigens physiologically restricted to other stages of B cell differentiation. LAP markers included in this DFN category can also be both expressed by normal B precursors and B ALL blasts, but at a significant different level of expression. Second, B ALL blasts can express the so-called "aberrant LAP markers," defined as antigens physiologically expressed by other lineages than B cells (Cherian & Soma, 2021; Griesinger et al., 1999). During the biological follow-up of B ALL patients, these LAP markers are essential to allow distinction between normal regenerating B-cell precursors and B lymphoblasts. However, this discrimination can be challenging because normal bone marrow B-cell precursors, also known as hematogones, share many immunophenotypic characteristics with B lymphoblasts. A perfect knowledge of normal immunophenotypic patterns associated with normal B-cell maturation, normal marrow regeneration, and of the diversity of the immunophenotypic profiles of B lymphoblast populations is thus required.

In 2012, the Euroflow consortium published guidelines with standardized MFC panels for the diagnosis of hematologic malignancies (van Dongen et al., 2012). The Euroflow strategy dedicated to the phenotypic evaluation of B ALL blasts are first based on a backbone for the identification and the gating of the lymphoblast population. It also relies on characterization markers, sub classification markers, markers allowing differential diagnosis, markers for the distinction of lymphoblasts from normal B cells and LAP markers. Documented at the time of diagnosis, the expression profile of these markers is crucial for accurate assessment of MRD during the follow-up of B ALL patients.

The aim of our study was to reach a better understanding of 7 LAP markers' landscape in B ALL of the Euroflow strategy: CD9, CD21, CD66c, CD58, CD81, CD123, and NG2. MFC analyses were performed on bone marrow and peripheral blood samples of a cohort of 100 de novo B ALL patients diagnosed in our center and of a series of healthy donors, to determine the level of expression of these markers in normal peripheral leukocytes, regenerating hematogones and B lymphoblasts at diagnosis of B ALL.

2 | METHODS

2.1 | Samples

We retrospectively re-analyzed MFC data obtained in our routine from a total of 100 newly-diagnosed patients with B ALL evaluated in our center during a period of 5 years. Immunophenotypic analysis was performed in $n = 90$ fresh bone marrow (90%) and in $n = 10$ fresh peripheral blood samples (10%). Patients included 65 children (median age: 3 years, age range: 1–17 years, $n = 27$ females, $n = 38$ males) and 35 adults (median age: 58 years, age range: 18–82 years, including $n = 5$ young adults with age ≤25 years; $n = 18$ women, $n = 17$ men, see Table S1 for biological characteristics of the patients). The diagnosis of B ALL was established according to World Health Organization (WHO) criteria, including cytologic, immunophenotypic and genetic/cytogenetic analyses.

Among the 100 patients diagnosed with B ALL, no recurrent cytogenetic abnormality was observed in $n = 25$ patients (25%). A hyperdiploid karyotype (>50 chromosomes) was described in $n = 24$ patients (24%). B lymphoblasts showed a t(12;21)(*ETV6/RUNX1*) in $n = 19$ cases (19%), a t(1;19)(*PBX1/TCF3*) in $n = 5$ cases (5%) or a t(9;22)(*BCR-ABL1*) in $n = 21$ cases (21%). In three patients of the cohort (3%), a t(v;11q23.3) was identified with an unknown *KMT2A* rearrangement partner for one of these three cases, and respectively corresponding to *MLLT3/AF9* and *AFF1/AF4* for the other two cases. In addition, hypodiploidy was described for the remaining three cases (3%).

Cases were also classified in accordance with EGIL (European Group for the Immunological Characterization of Leukemias) classification with the following categories (see Figure S1): I (pro-B: $CD10^-$, intra-cytoplasmic $Ig\mu^-$, $n = 5$ patients, 5%), II (common: $CD10^+$, intra-cytoplasmic $Ig\mu^-$, $n = 67$ patients, 67%), III (pre-B: $CD10^+$, intra-cytoplasmic $Ig\mu^+$, $n = 27$ patients, 27%), IV (mature: surface Ig^+, $n = 1$ patient, 1%).

To evaluate immunophenotypic profiles of the markers of interest in the B normal maturation (normal hematogones of type I and type II, normal mature B lymphocytes), MFC data of 40 bone marrow samples obtained from 36 of the 100 B ALL patients with negative genetic MRD were examined.

In parallel, a total of 10 fresh peripheral blood samples obtained from normal donors (median age: 31 years, age range: 18–66 years) were evaluated in this study, in order to determine normal mean fluorescence intensity (MFI) values for the different evaluated markers in normal circulating B lymphocytes and, if needed, in other normal leukocytes (granulocytes, monocytes, non-B lymphocytes, depending on the selected marker) to use them as normal internal control.

This study was approved by the Institutional Review Board (IRB) of the Montpellier University Hospital (approval number assigned by the IRB: IRB-MTP_2022_09_202201195).

2.2 | MFC analysis

The expression of 25 markers used in our routine procedures for the evaluation of B ALL at diagnosis (CD19, CD20, CD22, CD24, Kappa, Lambda, CD10, CD38, IgM, intra-cytoplasmic Igμ, CD34, nuclear Tdt, CD13, CD33, CD117, CD15, CDw65, CD9, CD21, CD58, CD66c, CD81, CD123, NG2, and CD45) was evaluated using specific antibodies in four MFC tubes, as defined by the Euroflow strategy and as previously published (van Dongen et al., 2012). Among these markers, the expression of Igμ and Tdt was evaluated by intracellular staining, using the Fix and perm© solutions of the kit GAS-002 (Nordic-Mubio©, see Tables S2 and S3 for references and design of the panels). Expression of all remaining markers mentioned above were evaluated at the surface membrane of the cells.

Flow cytometry data were acquired using a 10-color Canto II flow cytometer (Becton Dickinson©) and the FACSDiva© software program (Becton Dickinson©) by collecting 5000–10,000 gated events of interest. The instrument setup and calibration were in accordance with the EuroFlow standard operating procedures, including a daily quality control (van Dongen et al., 2012). To determine the threshold of positivity of each marker, normal leukocytes were considered as internal control populations (each normal sub-population of leukocytes was selected depending on previously described profiles in the literature or determined in this study). A positive expression of each evaluated marker by populations of interest was defined when at least 20% of the population expressed the marker.

After exclusion of doublets on the FSC-H (forward scatter-height) versus FSC-A (forward scatter-area) graph and exclusion of cell debris, B cells (B lymphoblasts, hematogones or normal B lymphocytes, depending on the sample) were selected in the side scatter versus CD45 graph as $CD19^+$ events. Among $CD19^+$ events, B lymphoblasts were selected on the criteria of expression of immature markers (CD34 and/or Tdt). Hematogones were identified as events with a typic B immature profile as previously described (Chantepie et al., 2013; Nagant et al., 2018). The type I of hematogone populations was defined as $CD45^{low}$, $CD19^{low}$, $CD10^{high}$, $CD34^+$, whereas the type II of hematogones was identified with a $CD45^{dim}$, $CD19^{high}$, $CD10^{low}$, $CD34^-$ profile.

2.3 | Prognostic study and risk stratification

Analyses were processed with R.2.10.1 and Bioconductor programs. Univariate and multivariate analysis of markers for patients' survival was performed using the Cox proportional hazard model. Difference in overall survival between groups of patients was assayed with a log-rank test and survival curves plotted using the Kaplan–Meier method (Maxstat R package) (Kassambara et al., 2012). The risk stratification score was built using our previously published methodologies (Alaterre et al., 2022). The risk stratification score is the sum of the Cox beta-coefficients of CD123, CD58, and CD81 expression, weighted by ±1 if the patient expression value for a given marker is above or below the Maxstat reference value of this marker.

2.4 | Statistical analysis

The level of expression of the markers was determined as MFI values. Data were analyzed using Diva Software (Becton Dickinson©) and

Cytobank (Cytobank©, Santa Clara, CA, United States; http://cytobank.org). Statistical comparisons were performed using a Student's *t* test analysis in GraphPad Prism version 9.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com).

3 | RESULTS

3.1 | CD9, a LAP marker with an heterogenous profile of expression during normal B cell maturation and at the surface of B lymphoblasts at diagnosis

CD9 is a member of the tetraspanin family, with a wide cellular and tissue distribution, including cells of the nervous central system (Kaprielian et al., 1995), endothelial cells (Iwasaki et al., 2013), and hematopoietic cells (Reyes et al., 2018). CD9 can contribute to several major cellular functions as motility or proliferation, due to the large diversity of its ligands, allowing a multitude of dynamic interactions, including adhesion molecules or growth factors. In hematopoietic cells, CD9 contributes to the regulation of different functions of the immune system and has been described as displaying a low expression profile in normal peripheral leukocytes (Reyes et al., 2018). In contrast with this observation, Tohami et al. described a significant expression of CD9 at the surface of the monocytes and of the granulocytes (Tohami et al., 2004). In our study, the results were in accordance with this last observation: CD9 was highly expressed by monocytes and, at a lower level, by granulocytes. In mature B lymphocytes the level of expression of CD9 was variable, from a negative to an intermediate level of expression (Table 1, Figure S2A). Normal non-B lymphocytes and granulocytes can thus be used in MFC evaluation as negative internal control and positive internal control, respectively.

During normal B cell maturation, we observed a negative to intermediate expression of CD9 at the stages of hematogones I and II (Figure 1a). Of note, even if no significant difference was observed, our results were partially in accordance with data previously published by Barrena and colleagues, showing that the expression of CD9 was higher in bone marrow B precursors than in peripheral blood B lymphocytes (Barrena et al., 2005).

We then analyzed the level of expression of CD9 in our cohort of 100 cases of B ALL at diagnosis. A heterogeneity was observed, 40% of the cases harboring $CD9^{-}$ blasts and 60% of cases presenting $CD9^{dim}$ or $CD9^{+}$ blasts (see Figure S3A). Of note, the expression of CD9 was globally significantly higher in B lymphoblasts, when compared with type I hematogones (Figure 1a, $p = 0.0248$). Nevertheless, there was a confusing overlap of MFI for a majority of cases (about 80% of the cases).

When comparing the different WHO subgroups of B ALL patients with recurrent cytogenetic abnormalities (see Figure S3B), we observed a significant higher expression of CD9 at the surface of hyperdiploid B lymphoblasts in comparison with other subgroups of recurrent cytogenetic alterations (Figure 1b, $p = 0.0032$), confirming the results obtained by Pierzyna-Switala et al. (2021). We also observed a significant lower expression of CD9 at the surface of B lymphoblasts with a t(9;22)(BCR-ABL) in comparison with hyperdiploid B lymphoblasts and with B lymphoblasts without any recurrent cytogenetic alteration (Figure 1b, $p = 0.0020$ and $p = 0.0317$, respectively). In addition, as previously published (Blunck et al., 2019), a significant lower expression of CD9 was encountered at the surface of B lymphoblasts harboring a t(12;21) when compared with B lymphoblasts with other recurrent cytogenetic abnormalities ($p = 0.0021$), or without any recurrent alteration ($p = 0.0095$, Figure S3C).

3.2 | CD21, a LAP marker not expressed during normal B cell maturation and rarely expressed by B lymphoblasts at B ALL diagnosis

CD21 is also referred as complement receptor type 2 (CR2). It can regulate the activation of the complement, contributes to the BCR signaling by an association with CD19 and CD81 and acts as an EBV receptor. It has been described as expressed by B lymphocytes, T cells following their activation, thymocytes and follicular dendritic cells (Roozendaal & Carroll, 2007; Tsoukas & Lambris, 1988; Zabel et al., 1999). In accordance with these results, we observed that, among the evaluated leukocytes in normal peripheral blood samples, CD21 was only expressed by normal B lymphocytes (Table 1, Figure S2B). These cells can thus be used in MFC evaluation as

TABLE 1 Differential leukemia-associated-phenotype antigen expression in normal peripheral blood leukocytes.

	B lymphocytes	Other lymphocytes (T + NK cells)	Monocytes	Granulocytes	Basophils
CD9	Negative (−) to Dim +	−	+ (Bright)	+	NE
CD21	+	−	−	−	NE
CD58	−	Negative (−) to Dim +	+	+	NE
CD66c	−	−	−	+	NE
CD81	+	+	+ (Dim)	Negative (−) to Dim +	NE
CD123	−	−	−	−	+ (Bright)
NG2	−	−	−	−	NE

Note: + indicates antigen consistently expressed; +/− indicates antigen expressed in several cases; − indicates antigen consistently negative, NE: not evaluated.

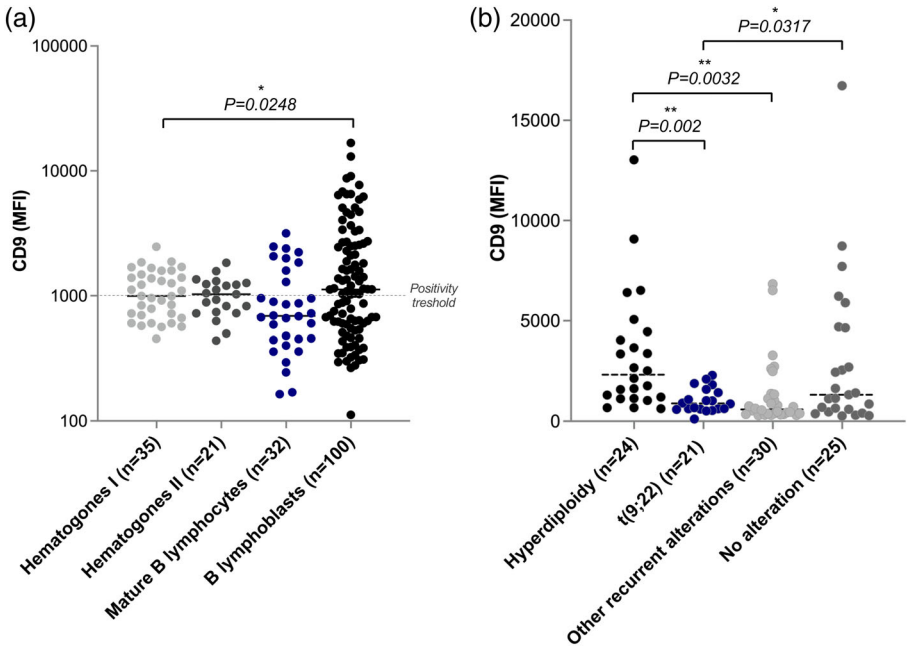

FIGURE 1 A low level of expression of CD9 was observed during normal B cell maturation, whereas it was expressed by half of the cases of B ALL at diagnosis. (a) A low level of expression of CD9 (MFI) was observed in normal hematogones (types I, $n = 35$ and II, $n = 21$) and normal mature B lymphocytes ($n = 32$). No significant difference was observed when these sub-populations were compared. The expression of CD9 by B lymphoblasts at diagnosis ($n = 100$) was heterogeneous, some of the cases overexpressing this marker in comparison with hematogones. (b) The level of expression of CD9 in hyperdiploid B lymphoblasts ($n = 24$) was significantly higher in comparison with B lymphoblasts harboring other recurrent cytogenetic alterations ($n = 30$). The level of expression of CD9 in B lymphoblasts harboring a t(9;22) ($n = 21$) was significantly lower in comparison with B lymphoblasts with no other recurrent cytogenetic alteration ($n = 25$) and in comparison with hyperdiploid B lymphoblasts. [Color figure can be viewed at wileyonlinelibrary.com]

positive internal control, whereas monocytes and granulocytes can be employed as negative internal control populations.

In contrast with normal mature B cells, CD21 was not expressed by the two sub-populations of normal hematogones (Figure 2, $p < 0.0001$), in accordance with previously published data (Barrena et al., 2005; Uckun, 1990).

In B lymphoblasts at diagnosis, we observed that only three cases among the 100 evaluated samples were CD21$^+$ ($n = 3\%$, Figure 2), including one case with a dim expression of CD21 associated to the presence of a t(12;21)(*ETV6/RUNX1*) and two cases with a higher expression of CD21, harboring a t(9;22)(*BCR/ABL*) (see Table S4 for biological characteristics of these three patients at diagnosis).

3.3 | CD58, a marker modestly expressed during normal B cell maturation and frequently found at the surface of B lymphoblasts at diagnosis of B ALL

CD58, also known as lymphocyte-function adhesion antigen 3 (LFA-3), is a heavily glycosylated member of the immunoglobulin super family, contributing to key events in immune activation. Its cellular distribution is very large, including at the surface of the blood leukocytes. Two isoforms are described: a GPI anchored form and a type I transmembrane form (Zhang et al., 2021).

In normal peripheral blood samples, we observed that CD58 was expressed by monocytes and granulocytes, in accordance with previously published results (Krensky et al., 1983). B lymphocytes were CD58$^-$, whereas the expression of this marker at the surface of non-B lymphocytes was negative to dim (Table 1, Figure S2C). Normal B lymphocytes can thus be used in MFC evaluation as negative internal control population, and monocytes or granulocytes as positive internal control populations.

When evaluating normal B cell maturation, we confirmed a dim to positive expression of CD58 by early hematogones, with a gradual reduction of this level of expression during differentiation to the mature B cell stage, with a significant difference between hematogones I or II and B mature lymphocytes ($p = 0.0040$ and $p = 0.0009$, respectively, Figure S4A), as previously observed (Chen et al., 2001; Don et al., 2020; Lee et al., 2005).

In our cohort of B ALL at diagnosis, B lymphoblasts were in majority CD58$^+$ (96%). Among positive cases, the level of expression of this marker was heterogeneous, forming a continuum, from a dim expression to a high expression (Figure S4B). A significant higher expression was observed in B lymphoblasts in comparison with hematogones I and II ($p < 0.0001$, Figure S4A), as previously observed (Lee et al., 2005; Nagant et al., 2018; Veltroni et al., 2003). However, an overlap of MFI values between 80% of the populations of B lymphoblasts and the populations of hematogones was reported in our cohort, limiting the utility of CD58 as an isolated MRD marker in these cases.

When comparing the different WHO subgroups of B ALL patients with recurrent cytogenetic abnormalities (see Figure S4C), we observed a significant overexpression of CD58 in cases with

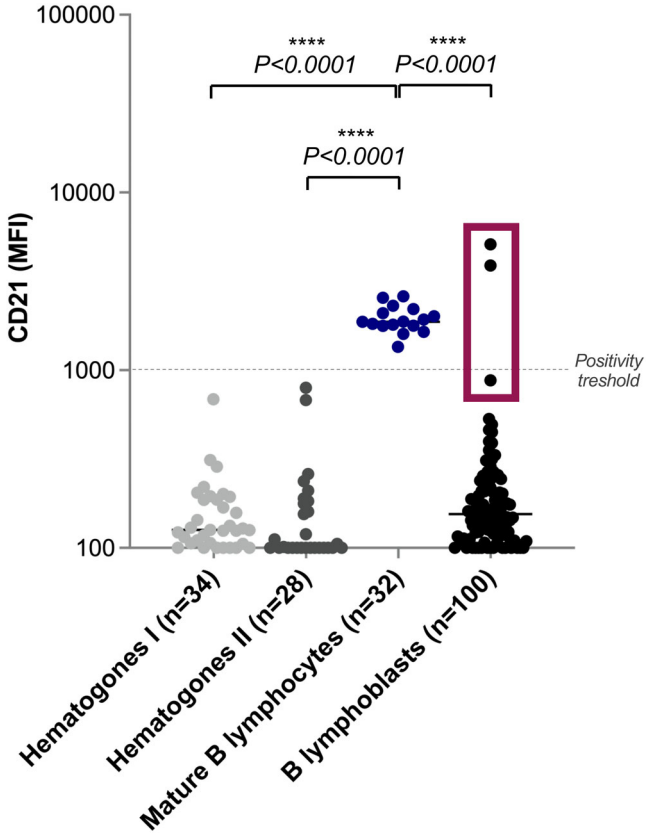

FIGURE 2 CD21 was not expressed at immatures stages during normal B cell maturation and was rarely expressed at the surface of B lymphoblasts at diagnosis. CD21 was not expressed at the surface of normal hematogones (types I, $n = 34$ and II, $n = 28$), in contrast with B mature lymphocytes ($n = 32$). Only 3% of the cases of B ALL diagnosis expressed CD21 at the surface of B lymphoblasts (see the red frame). [Color figure can be viewed at wileyonlinelibrary.com]

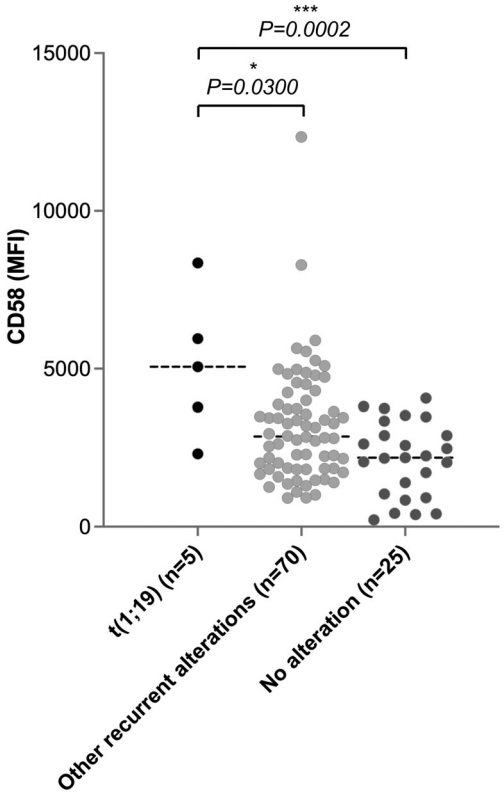

FIGURE 3 CD58 was overexpressed at the surface of B lymphoblasts harboring a t(1;19). CD58 was overexpressed at the surface of B lymphoblasts harboring a t(1;19) ($n = 5$), in comparison with blasts with other recurrent alterations ($n = 70$) or without any recurrent cytogenetic abnormality ($n = 25$).

hyperdiploidy ($p = 0.0007$, Figure S4D), as already known in the literature (Pierzyna-Switala et al., 2021). In addition, we also observed an overexpression of this marker in cases with t(1;19) when compared with B lymphoblasts with other recurrent cytogenetic abnormalities ($p = 0.0300$), or without any recurrent alteration ($p = 0.0002$, Figure 3), an observation that needs to be confirmed in others cohorts with a larger number of patients.

3.4 | CD66c, an aberrant LAP cross-lineage marker

CD66c, also known as Carcino Embryonic Antigen related Cell Adhesion Molecule 6 (CEACAM6), is a well described multi-functional glycoprotein expressed at the surface of epithelia and in normal hematopoiesis, by granulocytes and their precursors (Boccuni et al., 1998; Stocks et al., 1996). As a consequence, during MFC evaluation at B ALL diagnosis, granulocytes can be used as positive internal control, whereas monocytes and normal lymphocytes can be employed as negative internal control populations (Table 1, Figure S2D). In addition, CD66c was not expressed by normal hematogones (Figure S5A). At diagnosis of B ALL, CD66c has been considered as being the most frequently aberrant myeloid antigen present at the surface of B lymphoblasts (Kalina et al., 2005). In our cohort, CD66c was expressed by 45% of the cases at diagnosis, with a heterogeneous profile, ranging from a low expression to a very high expression of this surface marker (Figure S5B).

In addition, when exploring the level of expression of this marker within the different subgroups of patients with recurrent cytogenetic abnormality (Figure S5C), our results confirmed previous observations in favor of an absence of CD66c expression in B lymphoblasts with t(12;21)(*ETV6/RUNX1*), in comparison with B lymphoblasts harboring other recurrent cytogenetic abnormalities ($p = 0.0016$) or with B lymphoblasts without any recurrent cytogenetic alteration ($p = 0.0439$, Figure S5D). We also confirmed an overexpression of this marker at the surface of B lymphoblasts at B ALL diagnosis with t(9;22)(*BCR/ABL*) ($p = 0.0070$, Figure S5E), as largely observed previously (Guillaume et al., 2011; Kiyokawa et al., 2014; Owaidah et al., 2008).

In previous published studies, hyperdiploidy was associated with expression of CD66c by B lymphoblasts (Kulis et al., 2022; Pierzyna-Switala et al., 2021). Among the 24 cases of our cohort with hyperdiploid B lymphoblasts, $n = 15$ cases were CD66c$^+$, whereas $n = 9$ cases were CD66c$^-$ (see Figure S5C). The level of expression of CD66c at the surface of hyperdiploid B lymphoblasts was not significantly different when these blasts were compared with B

lymphoblasts without any cytogenetic alteration or with B lymphoblasts grouped in the category "other cytogenetic alterations."

In addition, an absence of expression of CD66c was observed in B lymphoblasts with *KMT2A* rearrangement. However, our cohort comprised only three patients with this profile, and this small number of cases did not allow a statistical study.

3.5 | CD81, a marker highly expressed by hematogones

CD81, is a member of the tetraspanin family, also known as CVID6, S5.7, TAPA1, or TSPAN28. When combined with other tetraspanins in membrane microdomains, CD81 allows constitution of dynamic entities that serve as signaling platforms and contribute to specific immune functions (Hemler, 2005). Its expression profile is characterized by a large tissular distribution in hematopoietic and non-hematopoietic tissues, as already published (Jones et al., 2011; Levy, 2014; Muzzafar et al., 2009). In normal peripheral blood samples, CD81 is expressed by B and T lymphocytes and at a lower level by monocytes. In addition, this surface marker is heterogeneously expressed by granulocytes, ranging from a negative expression to a dim expression (Table 1, Figure S2E). T lymphocytes can thus be used in MFC evaluation as a positive internal control population. Granulocytes can serve as a negative internal control population. However, it must be taking into account that a reliable fraction may weakly express this marker.

During normal B cell differentiation (Figure S6A), we confirmed that CD81 was highly expressed by hematogones and that its expression was significantly down-regulated in B mature lymphocytes, in accordance with previous published data (Barrena et al., 2005; Muzzafar et al., 2009; Nagant et al., 2018). In our cohort of B ALL at diagnosis, B lymphoblasts were in majority CD81^{+} (97%). Among positive cases, a heterogeneous level of expression of this marker was observed, forming a continuum from a dim expression to a high expression (Figure S6B). A significant higher expression was observed in hematogones I ($p < 0.0001$) and II ($p < 0.0001$) in comparison with B lymphoblasts (Figure S6A), as previously observed (Nagant et al., 2018). However, an overlap of MFI values between 31% of the populations of B lymphoblasts and of hematogones was reported in our cohort, limiting the interest of CD81 as an isolated MRD marker in these cases.

Considering that the level of expression of CD58 was higher in B lymphoblasts than in normal hematogones and that, on the contrary, the level of expression of CD81 was higher in normal hematogones, Nagant et al. proposed the CD81/CD58 MFI expression ratio as a way to discriminate these two categories of cells (Nagant et al., 2018). We applied this ratio on the MFI data obtained from our cohort and confirmed the interest of this approach, the ratio significantly discriminating B lymphoblasts from hematogones I ($p < 0.0001$) and from hematogones II ($p < 0.0001$, Figure 4, Table S5).

When comparing the different WHO subgroups of B ALL patients with recurrent cytogenetic abnormalities (see Figure S6C), we observed a significant lower overexpression of CD81 in cases with t(9;22)(BCR-ABL) in comparison with B lymphoblasts harboring other cytogenetic recurrent abnormalities ($p < 0.0001$) or without any recurrent cytogenetic alteration ($p = 0.0011$, Figure 5a). Furthermore, we observed an overexpression of this marker in cases with t(1;19) when compared with B lymphoblasts with other recurrent cytogenetic abnormalities ($p < 0.0001$), or without any recurrent alteration ($p = 0.0001$, Figure 5b), an observation that needs to be confirmed with a larger patient population.

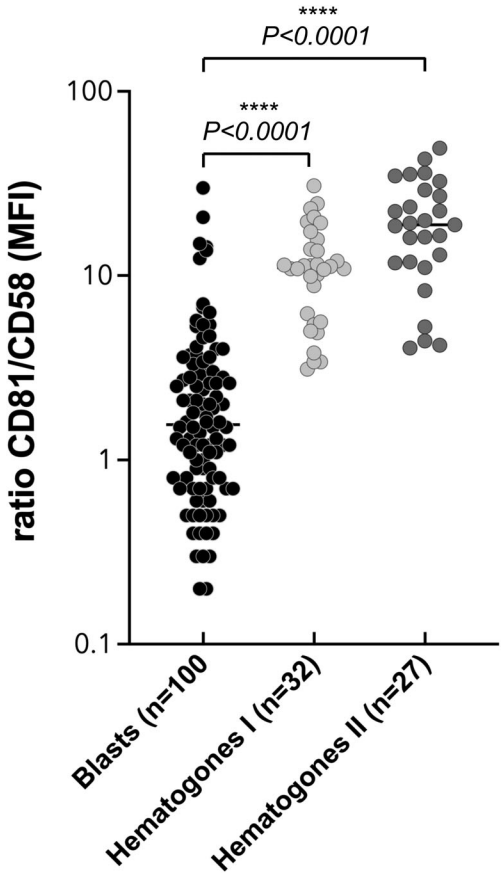

FIGURE 4 Discrimination of B lymphoblasts from hematogones through the use of the ratio CD81/CD58 (MFI).

3.6 | CD123, a LAP marker not expressed during normal B cell differentiation

CD123 corresponds to the alpha chain of IL-3 receptor (IL3R) and is essential for the formation of the high affinity heterodimeric IL3R in association with the beta subunit. In physiological conditions, it is only expressed by plasmacytoid dendritic cells, basophils, and eosinophils (Bras et al., 2019; Han et al., 2008). During MFC evaluation of hematopoietic samples at B ALL diagnosis, basophils can thus be used as positive internal control cells, whereas normal lymphocytes, or granulocytes can be employed as negative internal control populations (Table 1, Figure S2F). During normal B cell maturation, we confirmed an absence of expression of CD123 at the stages of hematogones I and II (Figure S7A) in accordance with data previously published (Das et al., 2020; Djokic et al., 2009).

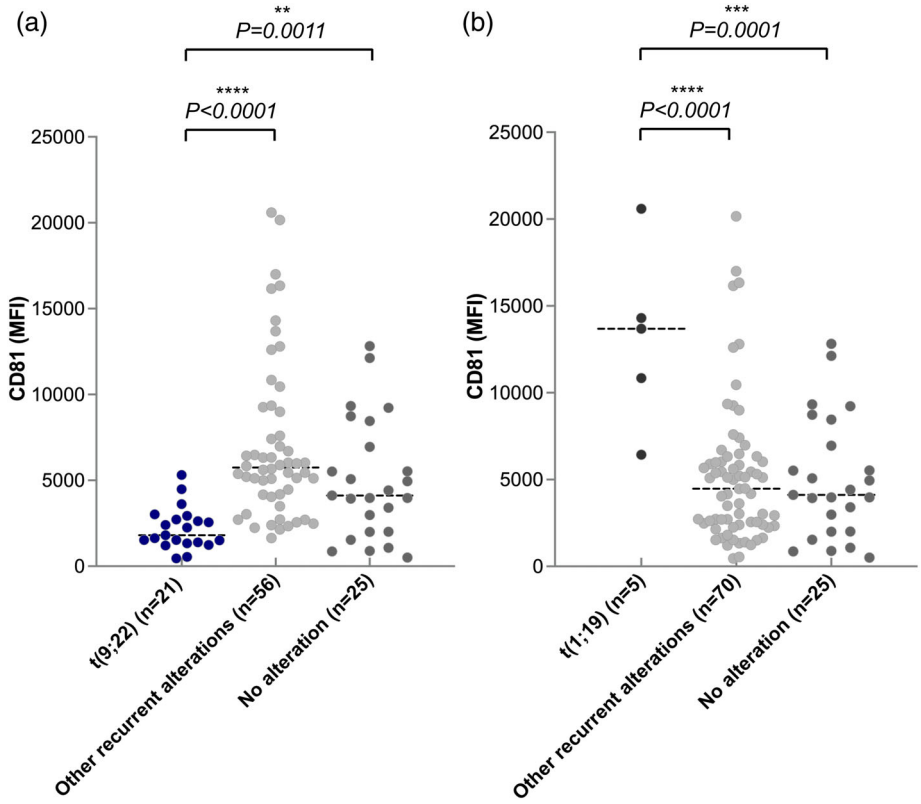

FIGURE 5 The level of expression of CD81 was significantly associated to the translocation t(9;22) and to t(1;19). (a) A low level of expression of CD81 (MFI) was observed in B lymphoblasts harboring a t(9;22) ($n = 21$) in comparison with B lymphoblasts with other recurrent cytogenetic alterations ($n = 56$) or without any recurrent cytogenetic abnormality ($n = 25$). (b) A higher level of expression of CD81 (MFI) was observed in B lymphoblasts harboring a t(1;19) ($n = 5$) in comparison with B lymphoblasts with other recurrent cytogenetic alterations ($n = 70$) or without any recurrent cytogenetic abnormality ($n = 25$). [Color figure can be viewed at wileyonlinelibrary.com]

At diagnosis of B ALL, CD123 was expressed by 72% of the cases, with a heterogeneous profile, ranging from a low expression to a very high expression of this surface marker (Figure S7B).

When exploring the level of expression of this marker within the different subgroups of patients with recurrent cytogenetic abnormality (Figure S7C), a higher expression of CD123 was found at the surface of B lymphoblasts with an hyperdiploidy, in comparison with B lymphoblasts harboring other recurrent cytogenetic abnormalities ($p < 0.0001$) or with B lymphoblasts without any recurrent cytogenetic alteration ($p < 0.0001$, Figure S7D), as already observed by other groups (Bras et al., 2019; Djokic et al., 2009). Of note, the level of expression of CD123 at the surface of B lymphoblasts was significantly higher in samples from children than in samples obtained from adults ($p < 0.0301$, Figure S7E), as described by Bras et al. (2019). Interestingly, B lymphoblasts harboring a t(1;19) at diagnosis did not express CD123 in our cohort. However, this observation needs to be confirmed in a larger patient population.

3.7 | NG2, a LAP rarely expressed by B lymphoblasts at diagnosis

Neuron–Glia protein 2 (NG2), also known as Chondroitin Sulfate Proteoglycan 4 (CSPG4), is a multivalent transmembrane proteoglycan in charge of the sequestration of growth factors, signaling molecules or metalloproteinases and that can facilitate their binding to cell surface receptors and to the extra-cellular matrix. It has been described as expressed by different cells of the central nervous system (Trotter et al., 2010), by muscle and cartilage progenitor cells (Burg et al., 1999), or by cells of the gastrointestinal tract and of endocrine organs (Nicolosi et al., 2015). In our study, and in accordance with a previous published work (Smith et al., 1996), NG2 was not expressed by normal peripheral leukocytes (Table 1, Figure S2G). Therefore, no category of normal leukocytes can serve as a positive internal control during MFC evaluation of hematopoietic samples. In addition, NG2 was not expressed during normal B cell differentiation (Figure S8A).

In our cohort of 100 patients newly diagnosed with B ALL, only two cases were identified with lymphoblasts $NG2^+$ (2%, Figure S8B), with a first case harboring a low level of expression of this marker (MFI = 1128) and, for the second case, a higher level of expression (MFI = 6227). In B ALL, the expression of NG2 is known to be associated with *KMT2A* rearrangement (Schwartz et al., 2003). In our cohort, the 2 $NG2^+$ cases were carrying this category of abnormality (a t(v;11q23.3) with an unknown partner for the first case, and a t(v;11q23.3) with a rearrangement between *KMT2A* and *AFF1* = *AF4* for the second case). Of note, in our cohort a third case harbored a t(v;11q23.3) with a rearrangement between *KMT2A* and *MLLT3* = *AF9*, but the B lymphoblasts corresponding to this case did not express NG2.

3.8 | CD123, CD58, and CD81 expression is associated with a prognostic value in pediatric and young adult B ALL patients and could be used for their risk stratification

We investigated the prognostic value of the 7 LAP markers included in our flow cytometry panel in the cohort of pediatric B ALL patients

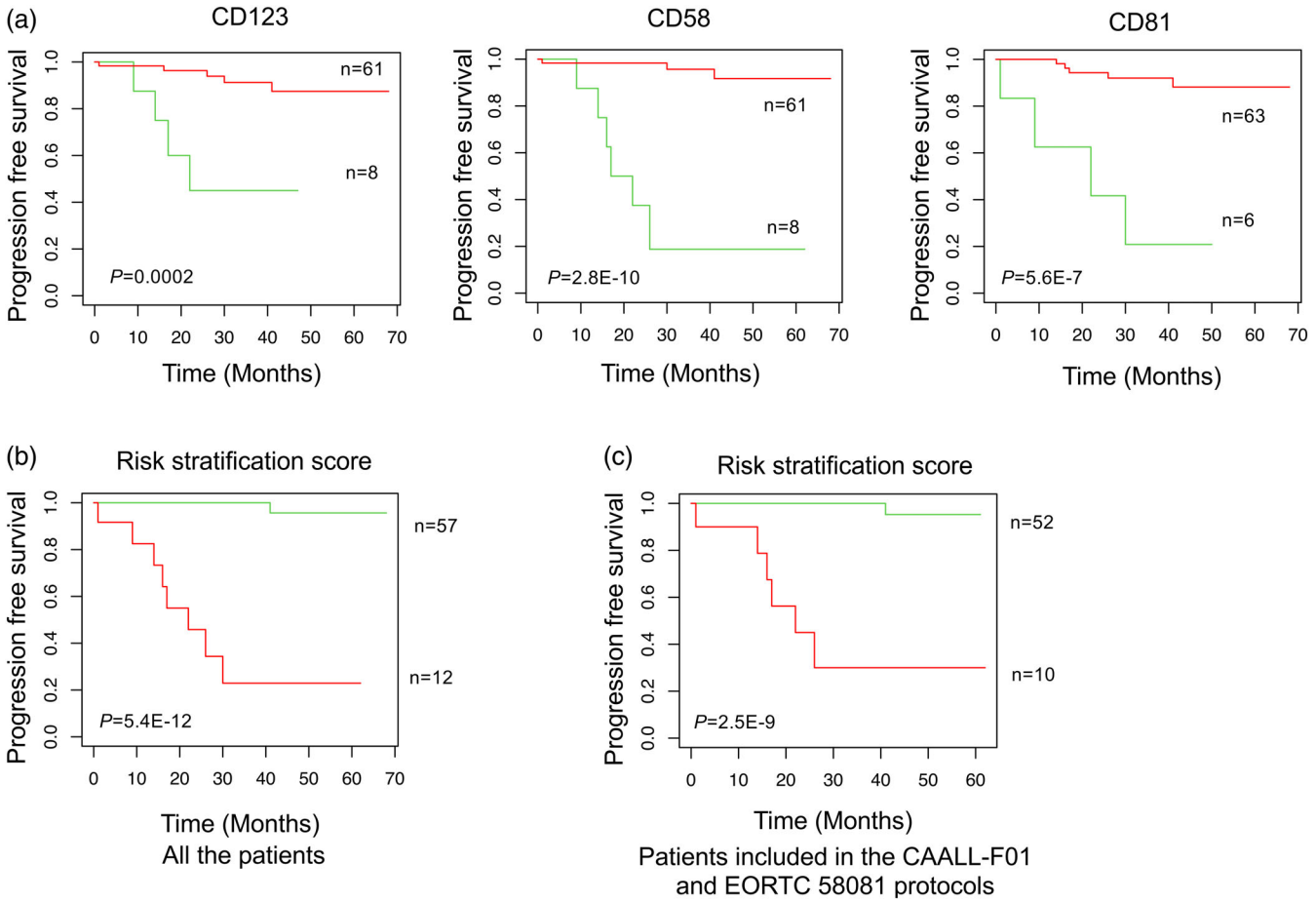

FIGURE 6 Prognostic value of the expression of CD123, CD58, and CD81 in pediatric and young adult B ALL patients. (a) Using Maxstat R algorithm, CD123, CD58, and CD81 expression (MFI obtained by MFC evaluation of B lymphoblasts at diagnosis) was associated with significant prognostic value in pediatric and young adult B ALL patients. High CD123, CD58, and CD81 expression levels were associated with a better outcome. (b) The prognostic value of CD123, CD58, and CD81 expression determined by MFC at diagnosis (MFI) was used to build a risk score splitting all pediatric and young adult patients (all protocols of treatment) into a high-risk group (17%) and a better outcome group (83%) ($P = 5.4 \times 10^{-12}$). (c) The risk score was also validated with data of pediatric patient (1–18 years) without Bcr-Alb rearrangement, to exclude higher-risk patients treated with special protocols. It allowed to split these patients into a high-risk group (16%) and a better outcome group (84%) ($P = 2.5 \times 10^{-9}$). [Color figure can be viewed at wileyonlinelibrary.com]

and young adults with B ALL ($n = 69$, see Table S6 for information about treatment and clinical follow-up). Data from adult patients were not used in this evaluation, as prognosis is generally very different between pediatric and adult patient B ALL populations (Liu et al., 2016). Using Maxstat R algorithm, we identified that CD123, CD58, and CD81 expression was associated with significant prognostic value in pediatric and young adult B ALL patients: a better progression free survival (PFS) was linked with a high CD123, CD58, or CD81 expression (Figure 6a). The prognostic value of CD123, CD58, and CD81 protein expression was then used to build a risk score. This risk stratification score is defined by the sum of the beta-coefficient derived from the Cox model for each marker weighted by −1 or +1 according to the CD123, CD58, and CD81 expression above or below the Maxstat defined cutpoint (Alaterre et al., 2022; Herviou et al., 2021). Pediatric and young adult B ALL patients of our cohort were ranked according to increased risk stratification score value and the Maxstat algorithm was used to define the cutoff associated with the maximum difference in PFS (Figure 6b). The risk stratification score splits these patients into a high-risk group (17%) and a better outcome group (83%, $p < 0.0001$). Comparing the risk stratification score with hyperdiploidy, t(9;22) and t(12;21), only the risk stratification score remained associated with a significant prognostic value (Table S7). We then investigated the prognostic impact of the score in pediatric B ALL patients by removing data of patients younger than 1-year old, older than 18 years old or with a Bcr-Abl rearrangement, to exclude higher-risk patients treated with special protocols and to limit the bias associated with these particular populations. Of interest, the score also presented a significant prognostic value (Figure 6c). Altogether, these data highlight that CD123, CD58, and CD81 expression can be used to identify high-risk pediatric and young adult B ALL patients. When tested in a COX multivariate model, the risk stratification score remained independent from age and hyperdiploidy (Table S7).

4 | DISCUSSION

In this study, using MFC, we analyzed the complexity of the LAP signature of lymphoblasts in a cohort of 100 patients newly diagnosed with B ALL. For the majority of the LAP markers investigated in this study, the expression level in normal leukocytes has been previously described in the literature. Our first goal was to propose a summary view of internal positive and negative populations among normal leukocytes for each of the seven evaluated LAP markers, in order to help the interpretation of the MFC data in routine laboratories (Figure 7).

The MFC technique allows to establish an extensive profile of B lymphoblasts at the time of diagnosis. Some of the expressed markers can be also found at the surface of normal hematogone subpopulations. Indeed, we and others confirmed an overlap in the expression of CD9, CD58, and CD81 between normal regenerative B cells and some B lymphoblasts. As a result, the interpretation of MCF data during the biological follow-up of B ALL patients can be challenging. On the other hand, other markers are not expressed by these normal regenerative B populations, including CD21, CD66c, CD123, and NG2. They allow to distinguish the residual blasts from the hematogones during and after treatment of these patients. A good knowledge of the expression of these markers in normal B populations during their maturation is thus required for an optimal interpretation of the MFC results. In this study, we confirmed the different expression profiles of these LAP markers at the surface of hematogones, in agreement with the literature data. In addition, we compared them to B lymphoblasts profiles. These results have been integrated in the synoptic diagram of the Figure 7, in order to propose a synthetic interpretation tool for routine laboratories. Of note, we confirmed the interest of the CD81/CD58 MFI expression ratio as a way to discriminate hematogones from lymphoblasts, as initially published by Nagant and colleagues in a cohort of 39 B ALL patients (Nagant et al., 2018).

When evaluating the frequency of expression of these LAP markers at the surface of B lymphoblasts at diagnosis of B ALL, we observed a heterogeneity in the expression of these molecules. We could propose three subgroups of markers: markers expressed by the majority of the cases, markers expressed by approximately half of the cases and markers rarely expressed. As already known, CD58 and CD81 were expressed by B lymphoblasts in the vast majority of cases. CD9, CD66c, and CD123 were expressed respectively by 60%, 45%, and 72% of the B ALL cases at diagnosis. CD21 and NG2 were rarely expressed, respectively by 3% and 2% of the cases of our cohort of B ALL patients. Of note, CD21 expression has only very rarely been studied in the context of B ALL diagnosis and to date, the results of only two cohorts of patients are available (Barrena et al., 2005; Ohki et al., 2020). Barrena et al. first observed that 100% of the cases in a group of 12 B ALL patients had an increased expression of CD21. Second, Ohki et al. described an expression of CD21 in 5.9% of the cases in a cohort of 926 patients with B ALL. Our results were more consistent with this second cohort, confirming that CD21 was a rarely expressed antigen at the surface of B lymphoblasts.

Several genetic aberrations have prognostic significance in the context of pediatric B ALL, including translocations and chromosome number abnormalities. A correlation between immunophenotypic profiles of LAP markers and certain recurrent cytogenetic abnormalities has long been established. In this study, we observed new associations between some cytogenetic recurrent aberrations and some profiles of LAP markers. Indeed, we observed a significant lower expression of CD9 and CD81 at the surface of B lymphoblasts harboring a t(9;22). In addition, B lymphoblasts with t(1;19) at diagnosis significantly overexpressed CD81 and or CD58. These results need to be confirmed in larger cohorts of patients.

The last part of our study was dedicated to the potential prognostic value of these LAP markers in our cohort of pediatric and young adult B ALL patients. A high CD123 expression was associated with a favorable prognosis, in accordance with data published by another group (Das et al., 2020). However, contradictory results have been described in other studies (Liu et al., 2019). CD123 is essential for the formation of the high affinity heterodimeric IL-3 receptor in association with the beta subunit (Miyajima et al., 1993). In physiological conditions, it is mainly expressed by plasmacytoid dendritic cells, basophils, and eosinophils. In hematological malignancies, CD123 is widely expressed by cancer cells in different entities, including hairy cell leukemia, acute myeloid leukemias, mastocytosis, or B lymphomas (Munoz et al., 2001). In B ALL, an overexpression of CD123 is known to be associated with hyperdiploidy and has been described as a reliable MRD marker (Bras et al., 2019). Furthermore, it is a promising therapeutic target and several strategies have been under evaluation, including the development of anti-CD123 monoclonal antibodies, CD3/CD123 bispecific antibodies or chimeric antigen receptor-modified T cells (CAR T) targeting CD123 in several hematological malignancies (Testa et al., 2019).

In addition, we also observed that a high expression of CD58 was associated with a better outcome in accordance with previous results (Archimbaud et al., 1992). Besides, we identified that a high expression of CD81 was also associated with a more favorable outcome. We combined the expression of CD123, CD58, and CD81 to build a risk score for pediatric and young adult patients. This scoring approach allowed us to split these patients into a high-risk group and a better outcome group, an observation that needs to be confirmed and validated in other independent cohorts of B ALL young patients. Of note, since many years, prognosis evaluation of pediatric B ALL patients have been based on the results of the genetic evaluation of the B lymphoblasts, as indicated by the criteria of WHO classification of Haematolymphoid Tumors. Therefore, this MFC scoring system could be a complementary tool, giving a first rapid indicative evaluation of prognosis, until the availability of the genetic results. However, it is again stressed that these results need to be confirmed in larger studies.

In conclusion, we explored the diversity of 7 LAP markers evaluated by a MFC approach in the context of diagnosis of B ALL. Variability of expression of these antigens during normal B cell differentiation, at the surface of normal leukocytes and of B lymphoblasts, as well as their association to several recurrent cytogenetic abnormalities, create a real complexity of the MFC data. A perfect knowledge of these markers is thus crucial for an optimal interpretation of MFC results of B ALL patients.

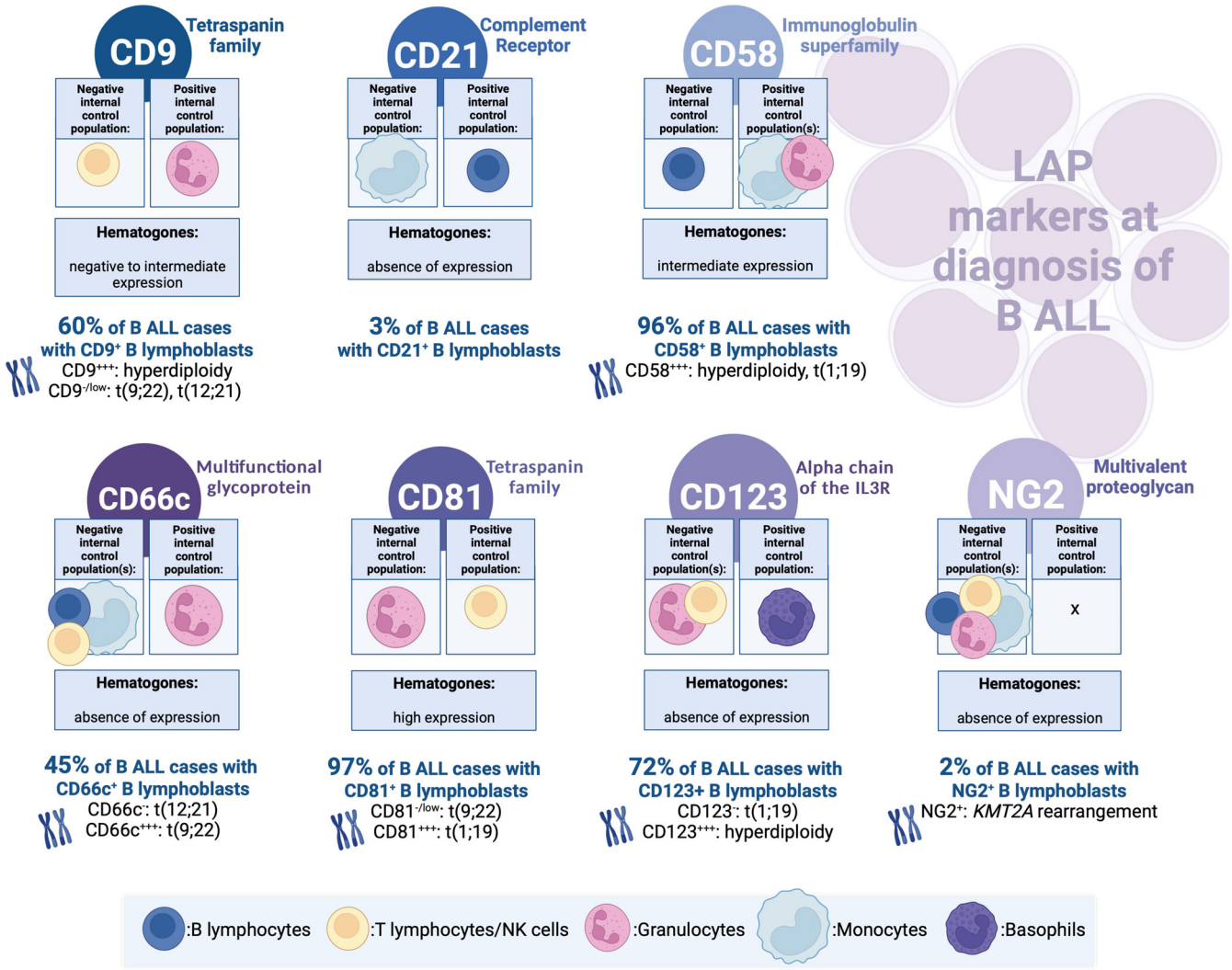

FIGURE 7 An overview of the immunophenotypic portrait of leukemia-associated-phenotype markers in normal leukocytes, during normal B cell differentiation and at diagnosis of B ALL from the results of this study. Created with BioRender.com [Color figure can be viewed at wileyonlinelibrary.com]

AUTHOR CONTRIBUTIONS

Emilia Boris and Valentin Montagnon participated to the collect of the data, to the analysis of the data, to the interpretation of the results. Alexandre Theron participated to the collection and to the analysis of prognosis data. Nicolas Rouquier was in charge of the analysis of the normal samples. Marion Almeras participated to the collect of the data. Jérôme Moreaux participated to the analysis of prognosis data, interpreted the data and the statistical analysis. Caroline Bret designed and performed the study, collected the data, performed the data analysis, interpreted the data and statistical analysis. All authors contributed to the manuscript writing and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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