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Deciphering stage 0 hematogones by flow cytometry in follow-up bone marrow samples of pediatric B—Acute lymphoblastic leukemia cases: A potential mimicker of residual disease after anti CD19 therapy

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Abstract

CD19 is frequently targeted for immunotherapy in B cell malignancies, which may result in loss of CD19 expression in leukemic cells as an escape mechanism. Stage 0 hematogones (Hgs) are normal CD19-negative very early B cell precursors that can be potentially mistaken for CD19 negative residual leukemic cells by flow cytometry (FCM) in B cell acute lymphoblastic leukemia (BCP-ALL) cases treated with anti CD19 therapy. Our main objective was to characterize and study the incidence of stage 0 hematogones in follow-up bone marrow samples of pediatric BCP-ALL cases. We analyzed the flow cytometry standard files of 61 pediatric BCP-ALL cases treated with conventional chemotherapy and targeted anti-CD19 therapy, for identifying the residual disease and normal B cell precursors including stage 0 Hgs. A non-CD19 alternate gating strategy was used to isolate the B cells for detecting the residual disease and stage 0 Hgs. The stage 0 Hgs were seen in 95% of marrow samples containing CD19+ Hgs. When compared with controls and posttransplant marrow samples, the fraction of stage 0 Hgs was higher in patients receiving anti CD19 therapy ($p = 0.0048$), but it was not significant when compared with patients receiving chemotherapy ($p = 0.1788$). Isolated stage 0 Hgs are found in samples treated with anti-CD19 therapy simulating CD19 negative residual illness. Our findings aid in understanding the stage 0 Hgs and its association with CD19+ Hgs in anti CD19 therapy and conventional chemotherapy. This is crucial as it can be potentially mistaken for residual disease in patients treated with anti CD19 therapy.

KEYWORDS
anti-CD19 therapy, CD19-negative B cell precursors, flow cytometry, hematogones, measurable residual disease

1 | INTRODUCTION

There is an increased use of immunotherapy for treating relapsed and refractory hematological malignancies. The antigen, which is commonly targeted in immunotherapy for B cell malignancies is CD19 (Hammer, 2012; Viardot et al., 2020). It is the pan B cell marker expressed throughout the B cell maturation from early B cell precursors up to plasma cell differentiation (Wang et al., 2012). It is also used as a gating marker in flow cytometry (FCM) for diagnosis and residual disease detection posttreatment in B cell malignancies for the above

reason. FCM is the most commonly used technique for detecting residual disease in ALL due to its advantage over molecular methods (Brüggemann & Kotrova, 2017; Correia et al., 2021). Currently, there are four groups of drugs that have been developed to target CD19, (i) unconjugated monoclonal antibodies like Inebilizumab and Tafasitamab, (ii) antibody drug conjugates like Denintuzumab mafodotin and Loncastuximab tesirine, (iii) molecules that recruit T cells to kill CD19+ cells by antibody dependent cellular cytotoxicity called bispecific T cell engager (BiTE) like Blinatumomab, and (iv) chimeric antigen receptor (CAR) T cell therapy like tisagenlecleucel, axicabtagene ciloleucel, and lisocabtagene maraleucel (Gambella et al., 2022; Yin et al., 2021).

As novel targeted therapies are brought into practice for B cell precursor acute lymphoblastic leukemia (BCP-ALL) cases, we find new difficulties and challenges in detecting residual disease by FCM. One of these is downregulation of CD19, which happens following anti-CD19 targeted treatments (Mikhailova et al., 2022). Loss of CD19 in the leukemic cells is one of the escape mechanisms for BCP-ALL when treated with anti-CD19 therapies (Bueno et al., 2022; Chen et al., 2023; Mikhailova, Gluhanyuk, et al., 2021; Mikhailova, Semchenkova, et al., 2021). This results in the emergence of leukemic cells without CD19 antigen. Hence detecting measurable residual disease (MRD) in BCP-ALL cases treated with anti-CD19 therapy impose a challenge, as an alternate gating approach needs to be used independent of CD19. The common alternate markers for B cell gating include CD22, CD24, and cytoplasmic CD79a (Cherian & Stetler-Stevenson, 2018; Mikhailova et al., 2022). Interestingly, the use of these markers has discovered a population of CD19 negative normal very early B cell precursors that are very immature and yet to develop or acquire CD19 expression (Cherian et al., 2018). They are called very early B cell precursors or stage 0 hematogones (Hgs). These very early B cell precursors can be mistaken as CD19 negative residual leukemic cells in B ALL cases treated with anti CD19 therapy. We attempted to characterize and study the incidence of stage 0 Hgs, and also their patterns of maturation to CD19+ (stage 1, 2, and 3) Hgs (Sędek et al., 2014) in pediatric BCP-ALL cases at different phases of conventional chemotherapy and in targeted anti-CD19 (CAR-T and BiTE) therapy. Few nonmalignant control marrow samples and post hematopoietic stem cell transplant (HSCT) regenerating marrows were also studied.

2 | MATERIALS AND METHODS

We analyzed the MRD FCM standard (.fcs) files of pediatric BCP-ALL cases treated with (i) anti CD19 (both BiTE and anti-CD19 CAR-T cell) therapy, (ii) conventional chemotherapy at different time points of end of induction (EOI), end of consolidation (EOC), post FLA-G (fludarabine, high dose ara-C, and G-CSF) and end of treatment (EOT), and (iii) post HSCT. We also included a few chemotherapy naïve marrows in the study group to compare the incidence of stage 0 Hgs. MRD assessment was not done in two cases, which were refractory to blinatumomab with no clearance of blasts in the peripheral blood and bone marrow. We analyzed the .fcs files for residual disease and also studied the normal B cell precursors including stage 0 Hgs.

MRD assessments were done by multiparametric FCM using a 10 color Beckman coulter Navios Exflow cytometer. The quality check for fluidics, optical alignments, and detectors were done daily with flow check pro and flow set pro fluorospheres as per the manufacturer's recommendations. The first pull bone marrow sample was processed by a bulk lyse, stain, and wash protocol and 1.6 million events were minimum acquired. The sensitivity of our BCP-ALL MRD assay is 0.0002%, which was established with LOB, LOD, and LLOQ assays. More details on sample processing and analysis of MRD in BCP—ALL using CD19 as the gating marker are described in our earlier published article (Thulasi Raman et al., 2020). For BCP ALL cases with dim CD19 at diagnosis and those treated with anti-CD19 therapy, we use two tubes as per the laboratory protocol. The panel of antibodies used is outlined in Table 1. One tube with a panel of markers was used in conventional BCP-ALL MRD assessment and another tube with additional markers for alternate B cell gating (CD22 & CD24) and exclusion (HLA-DR & CD123). CD123 is used to exclude CD22 positive plasmacytoid dendritic cells (PDC's) and basophils, whereas HLA-DR is used to exclude CD24 positive neutrophils and also CD22 positive and HLA-DR negative events.

2.1 | Gating strategy

Analysis was done on Kaluza 2.1 software. After ensuring stable acquisition with time plots, the dead cells and debris were eliminated with FSC versus SSC plot and singlets were selected with FSC-H versus FSC-A. From the viable singlets, events positive for CD22 were gated on HLA DR versus CD123 bivariate dot plot to exclude bright CD123 events (basophils & PDC's) and HLA-DR negative events. These events were labeled as "Refined CD22 events." Similarly, viable events positive for CD24 and HLA-DR were selected and labeled as "Refined CD24 events." A Boolean gate named "Total B cell events" was made with "OR" logic, combining the Refined CD22 events and Refined CD24 events (Figure S1). The total B cell events was studied with all combinations of markers. The stage 0 Hgs were identified and quantified by the immunophenotype described in literature (Cherian

TABLE 1 Two tube 10 color antibody panel.

	KO	BV421	FITC	PE	ECD	PC5.5	PC7	APC	APC700	APC750
TUBE 1	CD45	CD73	CD38	CD58	CD22	CD10	CD34	CD19	CD123	CD20
TUBE 2	CD45	HLADR	CD38	CyCD79a	CD22	CD10	CD34	CD19	CD123	CD24

et al., 2018; Mikhailova, Gluhanyuk, et al., 2021; Mikhailova, Semchenkova, et al., 2021). The CD19+ Hgs were studied and quantified from both tubes.

2.2 | Statistics

Unpaired *t*-test was used to compare the frequencies of stage 0 and CD19+ Hgs in bone marrow samples treated with anti-CD19 therapy, conventional chemotherapy, and treatment naïve control. The relationship between stage 0 and CD19+ Hgs was studied using the Pearson correlation coefficient.

3 | RESULTS

We studied the frequency of stage 0 Hgs and its relation to other CD19+ Hgs in targeted anti-CD19 (CAR-T and BiTE) therapy and in follow-up marrows of BCP-ALL children, at different phases of conventional chemotherapy. MRD was analyzed with a two tube—10 color panel with alternate gating markers as described above. A total of 61 cases were enrolled in this study. The stage 0 Hgs were reliably identified with the panel of markers used. The stage 0 Hgs and CD19+ Hgs were found in 45/61 (mean 0.1% [0.0025–0.59]) and 43/61 (mean 4.3% [0.01–31.5]) bone marrow samples analyzed by MFC, respectively (Table 2). The phenotype of stage 0 Hgs was consistent and identical across all the samples and it fell in the respective positions in the predesigned template. They showed expression of CD22 (moderate to bright), CD34 (moderate), CD38 (moderate), CD10 (dim), and CD45 (dim, but more than stage 1 Hgs). They were negative for CD19, CD20, and CD24 and also showed a relatively higher side scatter.

The very early stage 0 Hgs were consistently detected in marrow samples with CD19+ Hgs, irrespective of MRD status (Table 2). Only two samples had no stage 0 Hgs in the presence of CD19+ Hgs. One was a post induction marrow that showed a very few CD19+ Hgs quantified at 0.01% and the other was an EOT marrow with 0.06% CD19+ Hgs. In BCP-ALL cases treated with chemotherapy, stage 0 Hgs were absent in all marrow samples that lacked CD19 positive Hgs. However, in the setting of anti CD19 CAR-T cell therapy, isolated stage 0 Hgs were noted in the absence of other CD19 positive Hgs in all the three samples analyzed. Similarly, one case that received two cycles of blinatumomab showed an isolated stage 0 Hgs population. Although this isolated population raised the suspicion of residual disease in these four cases, they lacked a strong leukemia associated immunophenotype (LAIP) and hence reported as MRD negative. Though stage 0 Hgs are seen with other CD19 positive Hgs in all scenarios except anti-CD19 CAR-T therapy, there was no positive correlation between them in terms of frequency/quantity ($r = -0.0297$; $p = 0.88$). Some cases, which had florid CD19+ positive Hgs, had very few stage 0 Hgs.

Stage 0 Hgs were less frequently found in marrows with positive residual disease. Four out of five MRD positive marrows lacked stage 0 Hgs. Three cases had residual disease of greater than 1% (post blinatumomab and FLAG) and had no stage 0 Hgs. One post consolidation marrow had residual disease of 0.16% but showed stage 0 Hgs of 0.31% and CD19+ Hgs of 2.63%. The frequency of stage 0 Hgs was much lower in marrows assessed at the EOI as compared with other

TABLE 2 Frequency of stage 0 and CD19+ Hgs with MRD status in follow up marrow samples of BCP-ALL children treated with different therapies and time points.

Time points of MRD assessment	MRD status	Stage 0 Hgs	% stage 0 Hgs of BMNC mean (range)	CD19+ Hgs	% CD19+ Hgs of BMNC mean (range)	% stage 0 among the total Hgs
After anti CD19 CAR-T therapy ($n = 3$)	Undetected in 3/3	3/3	0.15 (0.06–0.3)	0/3	NA	100
After BiTE therapy ($n = 15$)	Undetected in 13/15 Detected in 2/15	11/15	0.03 (0.002–0.07)	10/15	0.38 (0.02–1.5)	16.8 (1.4–38.5)
Post induction (n = 10)	Undetected in 9/10 Detected in 1/10	1/10	0.06	2/10	0.03–0.07	33.3 (0–66.6)
Post consolidation ($n = 8$)	Undetected in 7/8 Detected in 1/8	8/8	0.26 (0.018–0.59)	8/8	2.39 (0.14–4.6)	14.4 (6.7–33.3)
Post FLAG ($n = 2$)	Undetected in 1/2 Detected in 1/2	1/2	0.1	1/2	0.02	83.3
End of treatment ($n = 10$)	Undetected in 10/10	9/10	0.1 (0.06–0.17)	10/10	3.35 (0.01–18.9)	21.4 (0–38.1)
Post HSCT ($n = 9$)	Undetected in 9/9	8/9	0.025 (0.005–0.06)	8/9	12.9 (0.96–19.2)	0.3 (0.1–0.7)
Control ($n = 4$)	NA	4/4	0.08 (0.01–0.14)	4/4	14.6 (1.6–25.16)	0.6 (0.4–0.8)
Total ($n = 61$)	Undetected in 52/57 Detected in 5/57	45/61	0.1 (0.003–0.59)	43/61	4.3 (0.01–31.5)	15.2 (0–100)

Abbreviations: BiTE, bispecific T cell engager; BMNC, bone marrow nucleated cells; CAR, chimeric antigen receptor; Hgs, hematogones; MRD, measurable residual disease.

time points like post consolidation, maintenance, or EOT. The EOI marrow also showed a lower frequency of CD19 positive Hgs. Only one out of 10 post induction marrows showed stage 0 and CD19+ Hgs. All post consolidation marrows showed stage 0 along with other CD19 positive Hgs. Likewise, stage 0 Hgs were consistently seen with CD19 positive Hgs in all nonmalignant control and post HSCT regenerating marrows (Figure 1). The percentage of stage 0 and CD19+ Hgs are shown in Table 2.

The presence of stage 0 Hgs in BiTE therapy was found to be variable. It was found in 11 out of 15 cases (0.026% [0.025–0.0737]) that received blinatumomab. It was absent in four patients who also had no CD19 positive Hgs. In BiTE therapy, stage 0 Hgs are seen as an isolated population in one case, with CD19+ early Hgs in six patients, and with early and late Hgs in four patients.

We compared the frequency of stage 0 Hgs (% of all marrow nucleated cells) in marrows treated with anti CD19 therapy ($n = 18$) against controls ($n = 04$) and marrows treated with conventional chemotherapy ($n = 30$) (Table 3). We did not find significant difference as compared with controls ($p = 0.5850$) and marrows treated with conventional chemotherapy ($p = 0.0914$). But when we compared the proportion of stage 0 Hgs among the total marrow Hgs in anti CD19 treated marrows and controls, we found increased stage 0 Hgs in marrows treated with anti CD19 therapy, which was statistically significant ($p = 0.0048$). However, it was not significant on comparing the proportion of stage 0 Hgs among the total Hgs in marrows treated with anti CD19 therapy against PI, PC, and EOI marrows treated with chemotherapy ($p = 0.1788$).

4 | DISCUSSION

The non-CD19 gating strategy for BCP-ALL cases treated with anti CD19 therapy disclosed two progenitor populations. One is a multipotent lymphoid progenitor with no lineage assignment while the other progenitor shows B cell commitment with the expression of CD22 and is referred to as CD19- early B cell precursors or stage 0 Hgs. Though CD22 precedes CD19 expression in normal B cell maturation, it is not specific and additional evidence for B cell lineage is desired. We confirmed the expression of cytoplasmic CD79a (CyCD79a) in these very early precursors as evidence for B cell lineage (Figure S1). CyCD79a expression precedes CD19 in B cell ontogeny (Dworzak et al., 1998) and many others have also demonstrated the presence of cyCD79a in this stage 0 Hgs, which confirms their B cell lineage commitment (Mikhailova, Gluhanyuk, et al., 2021; Mikhailova, Semchenkova, et al., 2021). This stage 0 population with the expression of CD22 (bright), CD34 (bright), and CD10 (variable) can mimic residual disease in CD19 downregulated BCP-ALL cases and in anti-CD19 treated patients. Interestingly circulating CD22+/CD19-/CD24- progenitors have been reported (Zhou et al., 2023). Literatures are limited on the incidence of this population in different types and phases of BCP—ALL treatment.

MRD assessment with two tubes is routinely done for BCP-ALL cases treated with anti CD19 therapy, since most labs have 8 or 10 color flow cytometers. One tube for routine standard panel of markers and another with alternate B cell gating markers, such as CD22, CD24, and cyCD79a may be used. Recently a single tube 14 color, 16 parameter panel comprising 15 antibodies has been reported effective for detecting CD19-negative abnormal immature B cells (Gao et al., 2023). However, single tube with above alternate markers at the compromise of aberrant MRD markers like CD73, CD123, CD86, or CD304 is not prudent (Sędek et al., 2019; Tembhare et al., 2018). Isolated usage of CD24 as a B-cell gating marker can miss the stage 0 Hgs and CD19-negative leukemic cells of *KMT2A* rearranged BCP ALL cases that were negative for CD24 at diagnosis (Correia et al., 2021). Hence, a Boolean gate including all events gated with two or three different B cell markers (CD22, CD24, & cyCD79a) is ideal. Cherian et al. (2018) suggested the use of CD66b along with CD24 and CD22 in the second MRD tube of BCP-ALL cases treated with anti-C19 therapy, to exclude CD24 positive neutrophils. However, we recommend the addition of HLA-DR instead of CD66b, as the former can help in excluding both the CD24 positive neutrophils and CD22 positive basophils and PDCs.

The Stage 0 Hgs are phenotypically stable and can be easily identified when high event acquisition of greater than one million is done. CD34 is expressed in both stage 0 and Stage 1 Hgs, but the CD19 negative stage 0 Hgs show increased expression of CD22, CD38, and CD45 and decreased expression of CD10 and CD24 as compared with the CD19+ stage 1 Hgs (Figure 2). They also show relatively higher side scatter compared with stage 1 Hgs. The antigens expressed in the leukemic cells at diagnosis may get modulated with anti-CD19 therapy. The antigen that gets frequently modulated is CD34 (up modulation in 20% and down modulation in 18% of cases) (Mikhailova, Gluhanyuk, et al., 2021; Mikhailova, Semchenkova, et al., 2021). Hence, too much dependency on diagnostic phenotype may lead to erroneous reporting of MRD. As described in CD19+ Hgs, treatment induced immunomodulation in stage 0 Hgs should also be studied (Chatterjee et al., 2021).

Similar to the CD19+ Hgs, the frequency of stage 0 Hgs is low in post induction marrows. This finding agrees with Mikhailova, Gluhanyuk, et al. (2021) and Mikhailova, Semchenkova, et al. (2021). The absence of Hgs in most of the post induction marrows is attributed to the intense chemotherapeutic regimen given during this phase of treatment. Mikhailova, Gluhanyuk, et al. (2021) and Mikhailova, Semchenkova, et al. (2021) reported the absence of stage 0 Hgs in post consolidation marrows too. However, we found stage 0 Hgs consistently in post consolidation marrows along with CD19+ Hgs. They also detected significantly fewer stage 0 Hgs in controls and post HSCT marrows compared with patients after CD19 therapy (Mikhailova, Gluhanyuk, et al., 2021; Mikhailova, Semchenkova, et al., 2021). However, we found no significant difference between these two groups (Table 3).

The stage 0 Hgs was consistently seen in marrow samples after anti-CD19 CAR-T therapy. They were also found in patients treated with blinatumomab. The expansion of stage 0 Hgs without maturation in some cases treated with anti CD19 therapy is not well understood. Whether the anti-CD19 surveillance activity of circulating CAR T cells

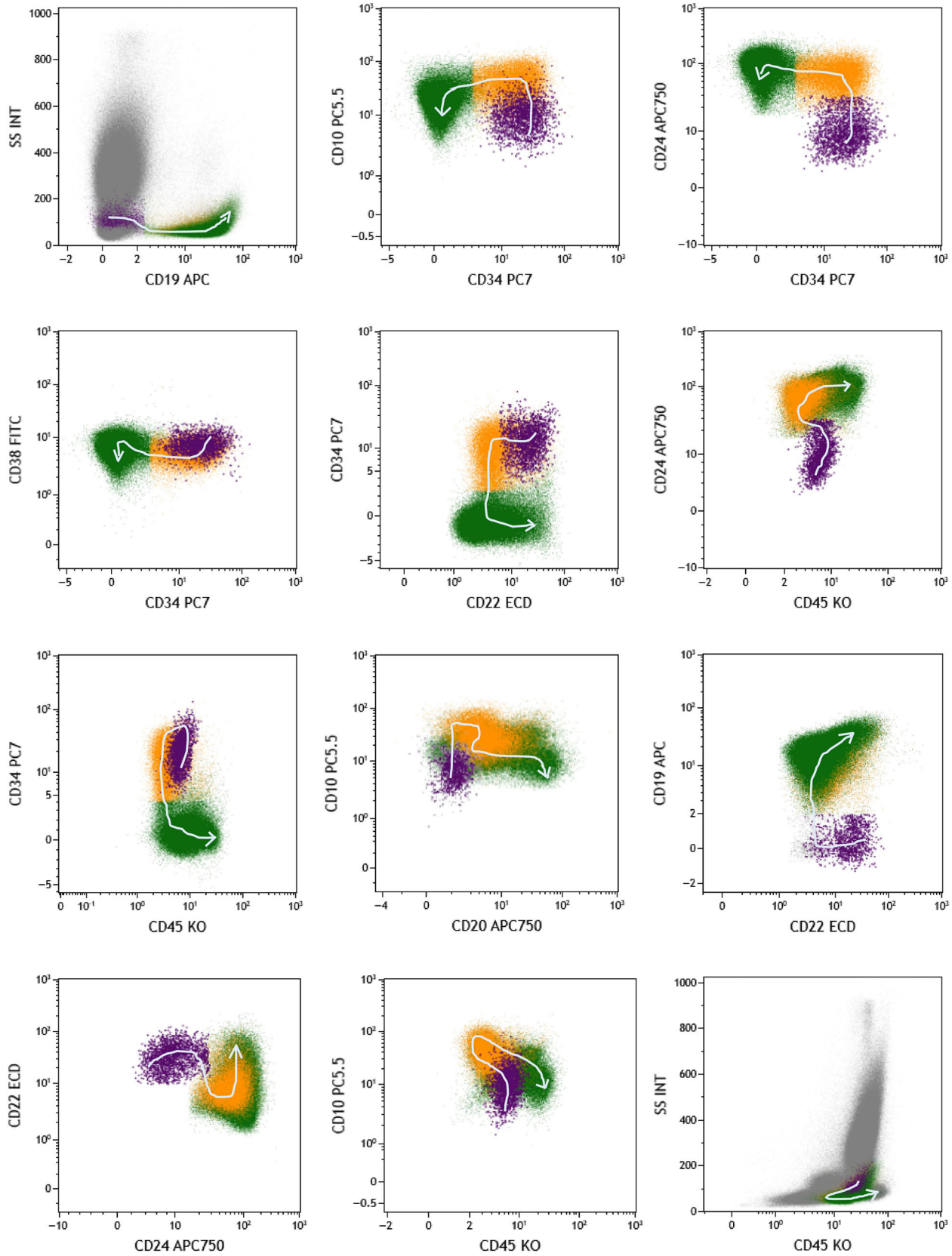

FIGURE 1 Normal maturation patterns of B cell precursors including the CD19 negative (stage 0 Hgs) precursors, shown on two dimensional dot plots with different antigen combinations. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Comparison of stage 0 Hgs and CD19+ Hgs frequencies in anti CD19 treated marrows with control and chemotherapy treated marrows.

Group comparison	% stage 0 Hgs of BMNC mean (range)	*p* value	% stage 0 among the total Hgs mean (range)	*p* value
After anti CD19 therapy ($n = 18$) versus control ($n = 04$)	0.07 (0.002–0.3)	0.5850	44.5 (1.4–100)	0.0048
	0.08 (0.01–0.14)		0.6 (0.4–0.8)	
After anti CD19 therapy ($n = 18$) versus conventional chemotherapy ($n = 30$)	0.07 (0.002–0.3)	0.0914	44.5 (1.4–100)	0.1788
	0.17 (0.018–0.59)		29.2 (0.9–83.3)	

Abbreviations: BMNC, bone marrow nucleated cells; Hgs, hematogones.

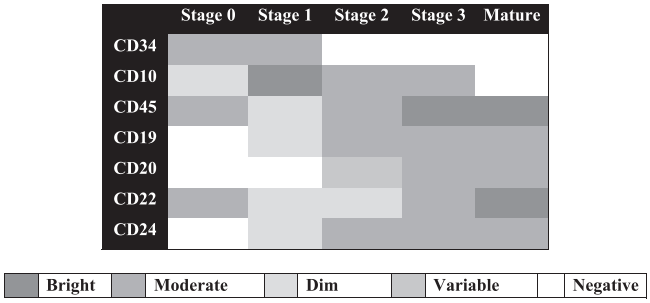

FIGURE 2 Heat map showing intensity of different antigens in hematogone maturation stages.

causes a hindrance for maturing to CD19+ stage 1 Hgs is a question that needs answering. Similarly, the chance of encountering stage 0 Hgs with only stage 1 or with both stage 1 and stage 2 may be related to the time of MRD assessment after stopping the blinatumomab therapy. Cases that are assessed during the course of blinatumomab may show stage 0 Hgs, in the absence of CD19+ Hgs as seen in anti CD19 CAR-T patients. Regenerated CD19+ Hgs may be seen with stage 0 if the marrow is assessed after the completion of treatment cycle and followed by a gap.

The knowledge and identification of these very early Hgs becomes critical in MRD analysis of B ALL cases treated with anti-CD19 therapy, to differentiate them from CD19 negative leukemic events. This population can be misinterpreted as MRD, as it stands out in the different-from-normal approach used for MRD detection. These stage 0 Hgs can present as an expanded and restricted cluster without CD19+ Hgs, especially in patients treated with anti CD19 CAR T therapy. This becomes more complex when the diagnostic blast phenotype matches that of stage 0 Hgs. More MRD markers that may help in differentiation should be added to the panel along with CD45, CD19, CD34, CD38, CD22, CD24, CD10, and CD20. Molecular methods may be an alternative for MRD detection in these cases if immunoglobulin rearrangement at diagnosis is known.

To summarize, we characterized the stage 0 Hgs and compared their incidence in bone marrow samples treated with anti-CD19 therapy, conventional chemotherapy, post HSCT, and control samples. Regardless of MRD status, the very early stage 0 Hgs were consistently seen in marrow samples containing CD19+ Hgs. When compared with controls and posttransplant marrow samples, the fraction of stage 0 Hgs in all B cell precursors was considerably higher in patients receiving anti CD19 therapy, but it was not significant when compared with marrows from patients receiving chemotherapy. In the context of anti CD19 therapy, isolated stage 0 Hgs may be discovered, simulating CD19 negative residual illness. To correctly distinguish them, many MRD markers need to be used with alternate gating markers. In complex situations, molecular techniques may be a reliable substitute. The limitation of this study is the smaller sample size, as many relapsed/refractory BCP-ALL patients could not afford anti-CD19 therapy due to financial constraints. MRD assessment by molecular techniques was not done in our study. Similar to the CD19+ Hgs, the prognostic significance of stage 0 Hgs in MRD negative BCP-ALL cases shall be studied in future (Arabi et al., 2023; Liao et al., 2019).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts 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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