Use of Four-Color Flow Cytometric Assay for Discrimination of Hematogone from Lymphoblast: Critical Issue for MRD Assessment in B-ALL Patients

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Abstract
Background: Hematogones are normal B-cell precursor which can be seen in different physiological and pathological conditions. Due to variation in B-cell acute lymphoblastic leukemia (B-ALL) blasts immunophenotyping and interference of hematogones in minimal residual disease (MRD) assessment, precise discrimination of hematogones is very crucial. The purpose of this study was to evaluate the expression pattern of surface markers in hematogones and compare them with lymphoblasts.
Material and Methods: In this applied study, flow cytometric analysis was performed using Coulter FC-500 and MXP software in 4-color combination and 6 different tubes. In this study, 85 patients diagnosed with acute lymphoblastic leukemia were evaluated. Out of these patients, 45 were boys and 40 were girls. Patients aged from 1 to 15 years old. In addition, 27 bone marrow samples from other patients aged 4 to 18 years were included in this investigation. These samples had been obtained for other diagnostic purposes, such as immune thrombocytopenic purpura and juvenile idiopathic arthritis.
Results: During flow cytometric analysis, hematogones showed expressions of CD19, CD20, CD22, CD10, CD45, CD81, CD123, CD9, CD34 (partial expression), and tdt (partial expression). In these patients, hematgones were negative for CD66c expression. Lymphoblastic cells were positive for CD19, CD20 (in some cases), CD22, CD10, CD45, CD81, CD123, CD58, CD9, CD66c, CD34 (in most cases), and TDT. CD81 mean fluorescence intensity (MFI) in hematogones was higher than that in lymphoblasts. (112.5 (30-251) vs. 17.5 (5-30); P<0.0001)
Conclusion: According to findings of this study, it seems that the use of CD81, CD58, CD123, CD66c, CD9, and CD81 MFI in combination with B-Cells associated markers can be very effective in differentiating hematogone from lymphoblast.
Keywords: B-ALL, Hematogone, Lymphoblast, MRD

Introduction

Hematogones (B-lymphocyte precursors), originally recognized by their morphologic features in bone marrow smears, are found in small numbers in most marrow specimens analyzed by flow cytometry. They are reportedly occurring in large numbers in some healthy infants and young children and in a variety of diseases in both children and adults (1 and 2). Hematogones are often identified by 4-color flow cytometry with the co-expression of CD10 and CD19 and low-intensity CD45 expression (3). Early hematogones express CD34, TDT, CD10, CD19, and CD38, while intermediate

hematogones express CD19, CD10, CD20, and CD38 and are usually negative for CD34 and tdt. Late hematogones are negative for CD34, TDT, with dim expression of CD38, CD10, CD19, and CD20. Knowledge about the properties and biologic functions of hematogones is still limited; however, recent data suggest that bone marrow hematogones could be a good prognostic factor in acute myeloid leukemia (AML). Recent flow cytometry studies to quantify hematogones at various intervals revealed that hematogones reflect prognosis not only for regeneration of post-therapy bone marrow but also for future outcome (4).

In pediatric acute lymphoblastic leukemia (ALL), minimal residual disease (MRD) measured during treatment reflect the overall response to chemotherapy (5). MRD assessment can be used to refine the treatment of ALL by identifying patients who need more intensive chemotherapy while eliminating the risk of unnecessary treatment toxicities in other patients. As a result, this response-adapted therapy is increasingly becoming the appropriate approach for clinical management of ALL. However, the number of leukemic cells that persist during chemotherapy retains prognostic significance even in the treatment protocols based on this strategy (6 and 7).

After inducting chemotherapy in pediatric with diagnosis of B-ALL, negative minimal residual disease is significantly associated with increased overall survival (OS) and events free survival (EFS) (8). Evaluation of minimal residual disease after treatment has a higher prognostic value in children than adults (9).

Despite the introduction of a standardized protocol, even experienced laboratories have difficulty with B-cell acute lymphoblastic leukemia (B-ALL) MRD assessment using flow cytometry. Some of these difficulties can be resolved with education; but even with education, identification of hematogones remains a challenge for some laboratories.

Transferring these results to other labs with less experience indicates the need for caution in transferring MRD testing from reference laboratories, and suggests that the introduction of MRD testing as a part of routine clinical management of B-ALL patients may require additional training and educational resources (10).

Despite all the recent advances in the evaluation of MRD with flow cytometry, hematogones is still considered as a confounding factor. As a result, further recognition of the immuno-phenotype characteristics of hematogones could be helpful in standardizing and reporting MRD more accurately. On the other hand, in some pathological processes, increased hematogones can imitate a disease similar to ALL; in such a situation, a complete understanding of the immunophenotype of the hematogones can prevent false diagnosis.

The purpose of this study was to evaluate the expression pattern of surface markers in hematogones and compare them with lymphoblasts, which is very important for the isolation and differentiation of hematogon from the lymphoblast, especially in evaluating MRD in B-ALL patients.

Materials and Methods

Given that the evaluation of the expression pattern of lymphoid markers and other markers helps in separation of hematogones from lymphoblasts, in this applied study we investigated 85 patients diagnosed with ALL. Out of these patients, 45 were boys and 40 were girls. Patients aged from 1 to 15 years old. In addition, 27 bone marrow samples from other patients aged 4 to 18 years were included in this investigation. These samples had been obtained for other diagnostic purposes such as ruling out leukemia in patient with juvenile arthritis and patient with isolated thrombocytopenia or infectious disease. This study was

approved by ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.REC.1398.95). For all patients, all markers needed for diagnosis of B-ALL were used. These markers included HLA-DR, CD3, CD5, CD13, CD33, CD19, CD20, CD22, CD10, CD38, CD34, and tdt; however, additional markers were also used for more specific separation of lymphoblasts from hematogones and correct diagnosis. These new markers were CD81, CD58, CD123, CD66c, and CD9. BM sample was received in EDTA as anticoagulant and prepared following the manufacturer's instructions. For the assessment of hematogones and lymphoblasts, 4-color combination was used for flow cytometric evaluation. Flow cytometric evaluation was performed with coulter FC-500 instrument and MXP software with 4-color combination in Takhte Tavous Pathobiology Lab. All markers were analyzed according to dim, mod, or bright expression and MFI for better distinction of hematogones from lymphoblasts. For better assessment of hematogones from lymphoblasts, 4-color combination in 6 different tubes was used. Antibody combination included CD19 (ECD), CD10 (PE), CD45 (Percp), CD81 (FITC)-CD19(ECD), CD10 (PE), CD81 (Percp), CD58 (FITC)- CD19 (ECD), CD123 (PE), CD81 (Percp), CD10 (FITC) -CD10 (Percp), CD19 (ECD), CD34 (PE), CD81 (FITC)-CD19 (ECD), CD10 (Percp), CD81 (FITC), CD66c (PE)- CD10 (PE), CD19 (ECD), CD34 (Percp), and CD9 (FITC). To evaluating expression pattern of CD38, CD20, CD22, and tdt, two separate tubes was used. For the assessment of expression pattern in hematogones and lymphoblasts, multiple quadrants such as CD10 vs. CD19, CD10 vs. CD38, CD10 vs. CD81, CD10 vs. CD58, and CD10 vs. CD9 were analyzed and specific pattern of these markers expression and difference of expression pattern were evaluated in both hematogones and lymphoblasts. Gating was performed by CD19 vs. side scatter and then CD10 vs. CD19. After the assessment of the selected population (lymphoblasts or hematogones), clinical data were obtained, and close follow up for each patient was performed to achieve definite diagnosis.

Statistical analysis
Statistical analysis of data was performed using SPSS version16 (P<0.05). The Independent t-test was used for the comparison of difference in MFI between two groups.

Results
Expression profile of CD10, CD19, CD20, CD22, CD34, CD45, and tdt in hematogon and lymphoblast
In the first step, the expression of B-lineage specific markers in hematogones and lymphoblasts was evaluated. In most patients, lymphoblastic cells expressed CD19, CD10, CD34, CD38, tdt, CD22 (dim expression), and in 31 cases, lymphoblasts were negative for CD20 expression and in other investigated patients, blastic cells showed dim to moderate expression of CD20. In a few B-ALL cases, leukemic cells were negative for the expression of CD10 (8 cases). The expression of CD19 in lymphoblast did not differ significantly from that of mature B-cells. The expression of CD10 varied in lymphoblasts, but in most cases, lymphoblasts showed a brighter expression of CD10. In most B-ALL cases, CD34 was positive with different intensity. In B-ALL patients, lymphoblastic cells had dim expression of CD38 and CD20 was negative or showed dim to moderate expression. CD22 expression was seen

with variable intensity and tdt was positive in all patients with moderate to bright expression (Figure 1). Evaluation of B-Lineage specific markers expression in hematogones showed fairly similar results to those expressed in lymphoblasts. Hematogones also expressed CD19, CD10, CD38, CD20, and CD22 with partial and dim expression of tdt and CD34. The expression of CD19 in hematogones did not differ significantly from lymphoblasts and it was not helpful in discriminating them. Hematogones showed moderate expression of CD10 and moderate to bright expression of CD38. Early stage hematogones were negative for the expression of CD20, but more mature hematogones expressed CD20 with different intensity and showed dim to bright pattern of expression. CD22 was also positive in hematogones with dim expression. The expression of TDT and CD34 can only be seen in early hematogones and more mature hematogones did not express CD34 and TDT. CD45 is a hematopoietic specific marker and nearly all hematopoietic cells express it. Lymphoblastic cells showed dim expression of CD45 and in some cases, lymphoblastic cells were negative for the expression of CD45, but hematogones showed dim to moderate expression of CD45 with a smear pattern. Based on these finding, the exact differentiation of lymphoblast from homotogon is possible, especially with the expression pattern of CD20 and CD45; however, using these markers did not allow the precise discrimination of early hematogons from lymphoblasts, which could interfere with the evaluation of the MRD in B-ALL. To solve this problem, the difference in the expression of non-specific markers in hematogones and lymphoblasts was evaluated.

Expression profile of CD81, CD58, CD123, CD66c, and CD9 in hematogon and lymphoblast

In the next step, the differences in the expression of CD81, CD58, CD123,

CD66c, and CD9 in hematogones and lymphoblasts were evaluated. Hematogones were positive for the expression of CD81 and showed a moderate to bright expression pattern. The expression of CD58 in hematogones was dim and partial. Hematogones were negative for the expression of CD66c, but with more mature hematogones positive for CD123 expression, they were negative for CD34. CD9 expression was also seen in hematogones with dim to moderate expression (Figure 2). Lymphoblastic cells were also positive for the expression of CD81, but with dim to moderate expression pattern. CD58 was positive in lymphoblastic cells with moderate expression and all lymphoblastic cells expressed CD9 with different intensity and in some cases with brighter expression (Figure 2). In flow cytometry quadrant dot plot, the expression of CD10 vs. CD58 in hematogones and lymphoblastic cells showed different patterns. In lymphoblastic cells, CD10 (FL2) vs. CD58 (FL1) showed a down to up expression pattern but it showed left to right expression pattern in hematogones. Similarly, CD10 (FL2) vs. CD9 (FL1) quadrant dot plot also showed a different pattern in hematogones and lymphoblastic cells. In lymphoblastic cells, CD10 vs. CD9 showed down to up expression pattern but hematogones showed left to right expression pattern (Figure 3). Expression of CD123 was seen in 58% of the patients with B-ALL with different percentage and moderate to bright expression. Hematogones also expressed CD123, especially at stages II and III, which did not show CD34 expression. Expression of CD66c was observed in 39% of the patients. Leukemic cells exhibited dim to moderate expression of CD66c (Figure 2). Lymphoblasts expressing CD9 were also generally positive for CD34 expression, but CD9 positive hematogons were usually CD34 negative.

Difference in CD10, CD38, CD81, CD58, CD123, CD66c, and CD9 MFI in hematogones and lymphoblasts

Despite the difference in the expression of B-lineage specific markers and other antibodies used in this study, we aimed to achieve a precise method for discrimination between homatogon from lymphoblast. To achieve this goal, in addition to evaluating the difference in the expression of these markers, the difference in mean fluorescence intensity (MFI) in the markers studied was also evaluated between two groups. In this study, the mean fluorescence intensity of CD38, CD10, CD81, CD58, CD9, CD123, and CD66c in hematogones and lymphoblasts was evaluated. According to our experience, hematogones showed brighter expression of CD81 with higher MFI. In this evaluation, hematogones in stage II and III showed higher CD81 MFI (MFI 50 to more than 200) in all samples; whereas in stage I, CD81 expression was moderate with MFI between 30 and 50. Stage I hematogones, in nearly all cases were negative for CD58, CD66c, CD123, and CD9. In some cases, it showed very dim expression of CD58 with low MFI (MFI:0.95-1.3), and expressed CD38 with higher MFI (MFI: 12.4-21.7) and CD10 with MFI between 9.7-14.5. We concluded that MFI equal or higher than 50 was consistent with hematogones in stages II and III and CD81 MFI between 30 and 50, in the absence of aberrant expression of other markers such as CD123, CD66c, and CD58, was consistent with stage I hematogones. However, B-Cell precursors with MFI between 20 and 30, and the absence of CD123, CD66c, and CD58 expression could be considered as normal precursors. Expression of CD81 in lymphoblasic cells showed different results. Expression of CD81 in lymphoblastic cells was dim and MFI in most cases was lower than 20. This experience showed that in B-ALL cases with more mature immunophenotyping such as CD20 expression and absent or dim expression of CD34, lymphoblastic cells showed brighter expression of CD81 with higher MFI to 100 (MFI: 5-100), but in these cases, lymphoblastic cells usually had aberrant expression such as expression of CD58,CD123, CD66c, or brighter expression of CD9. CD58 MFI could also help to distinguish hematogon from lymphoblast. Lymphoblastic cells had higher CD58 MFI (MFI: 6.4-18.2) than hematogones (MFI: 0.9-1.5). It can be concluded that CD58 MFI less than 2 was consistent with hematogones. CD9 showed similar expression in hematogones and lymphoblasts, but with different expression pattern and wide range MFI in lymphoblastic cells. CD9 MFI in lymphoblasts was between 6.8 and 51 and hematogones had MFI between 18 and 25. In this study, hematogones were negative for CD66c expression, as a result, MFI for CD66c was not calculated, but the calculated CD66c MFI in lymphoblasts was 13.2 (9.4-17.7). Expression of CD123 was observed in both hematogones and lymphoblasts. CD123 MFI in lymphoblastic cells was 9.1 (6.2-14.0) and it was 5.5 (3.1-7.2) in hematogones. Significant differences were observed between hematogones and lymphoblasts in terms of CD81, CD58, and CD123 MFI ($p<0.01$). Subsequently, MFI of CD10 and CD38 was also evaluated in lymphoblasts and hematogone. Lymphoblasts had higher CD10 MFI than hematogones, but CD38 MFI was higher in hematogones than lymphoblasts (Table I and Figure 4).
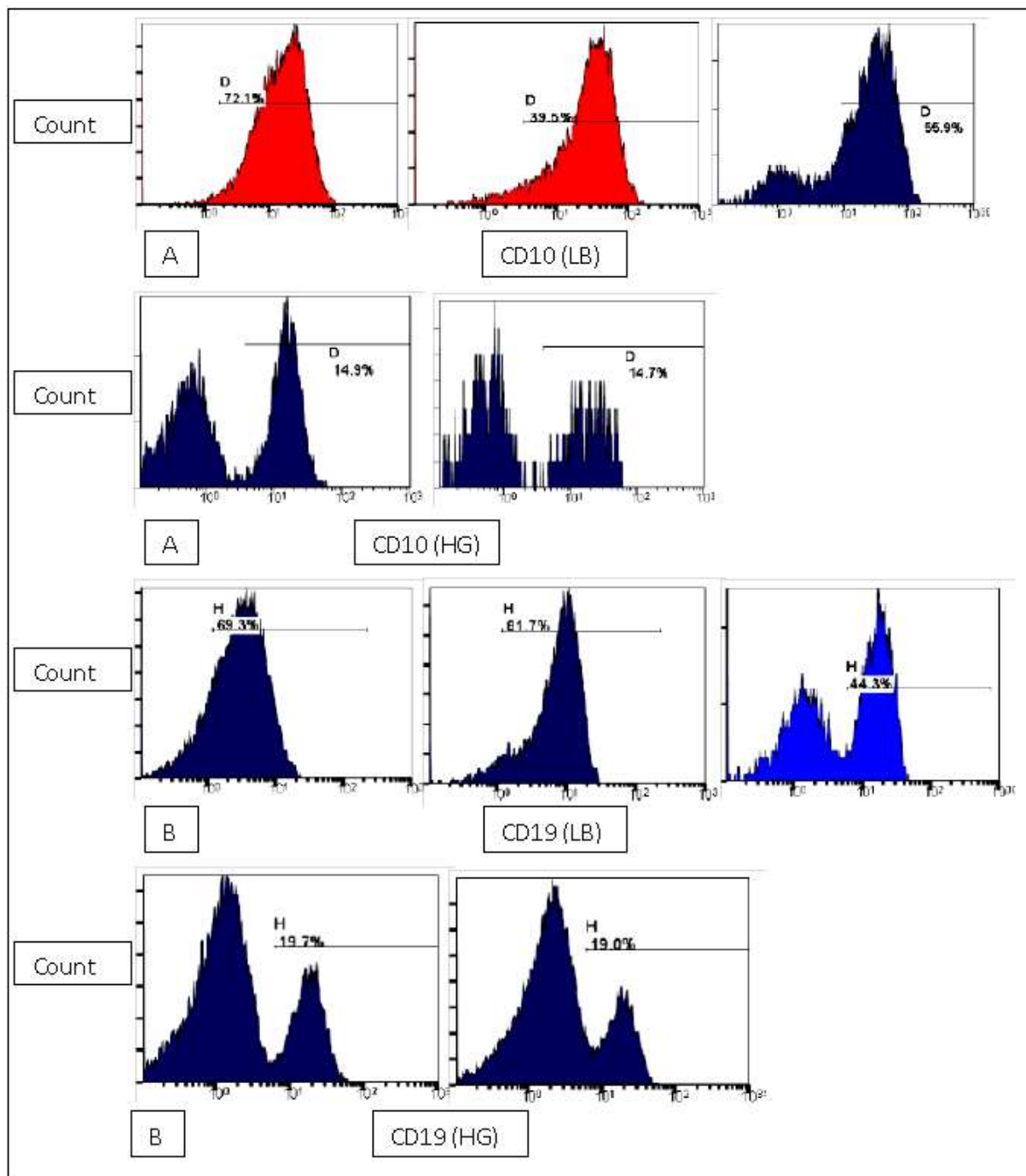
Figure 1. CD19 and CD10 expressions in hematogones and lymphoblastic cells. A. Difference in CD10 expression between HG and LB, B. Difference in CD19 expression between HG and LB
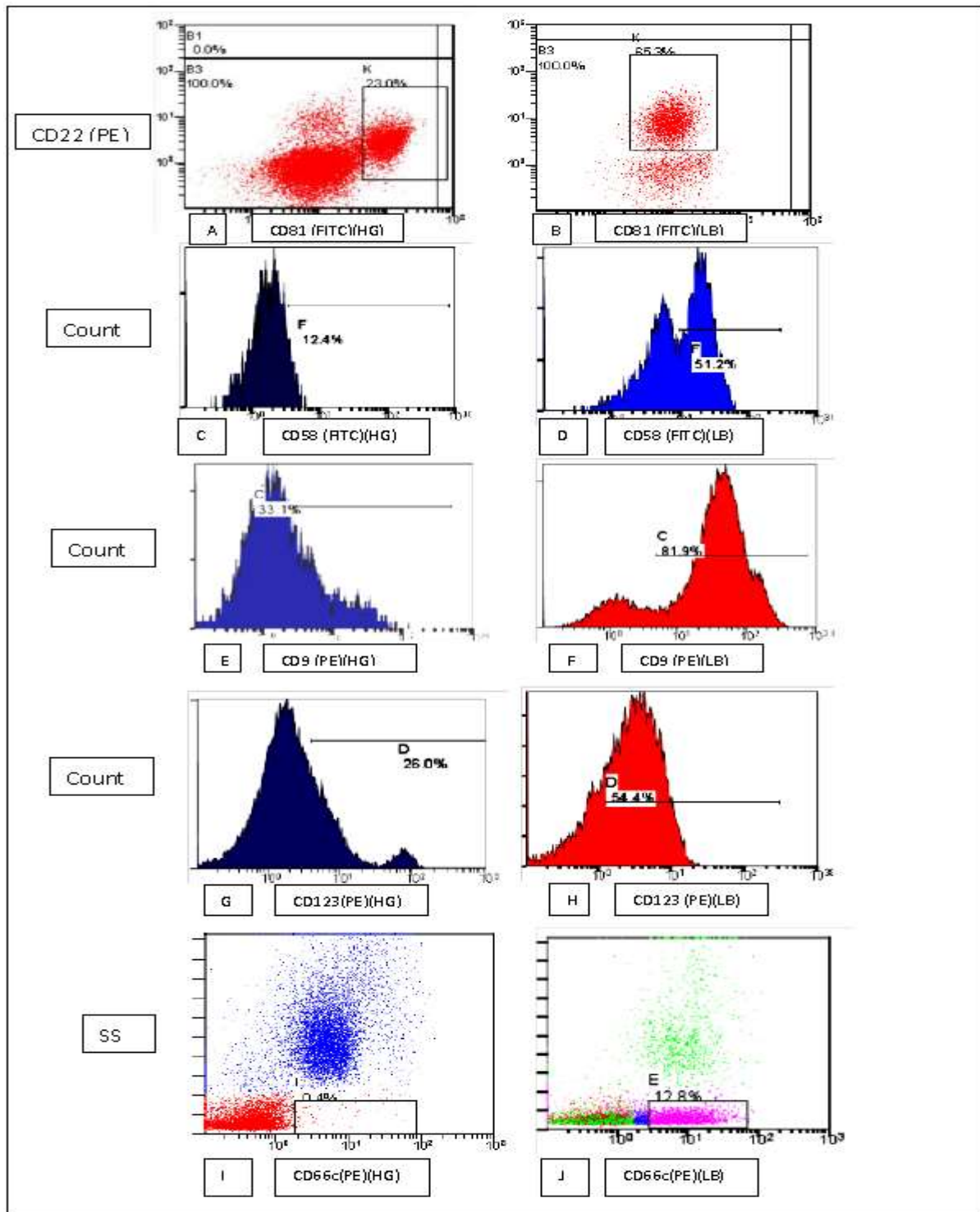
Figure 2. Difference in CD81, CD9, CD123, CD58, and CD66c expressions between hematogones (HG) and lymphoblasts (LB). A. bright expression of CD81 in hematogones, B. Moderate expression of CD81 in lymphoblasts, C. Negative expression of CD58 in HG, D. Moderate expression of CD58 in LB, E. Dim to mod expression of CD9 in HG, F. Brighter expression of CD9 in LB, G. Expression pattern of CD123 in HG and LB (H), I. Negative expression of CD66c in HG,J. Positive expression of CD66c in LB.
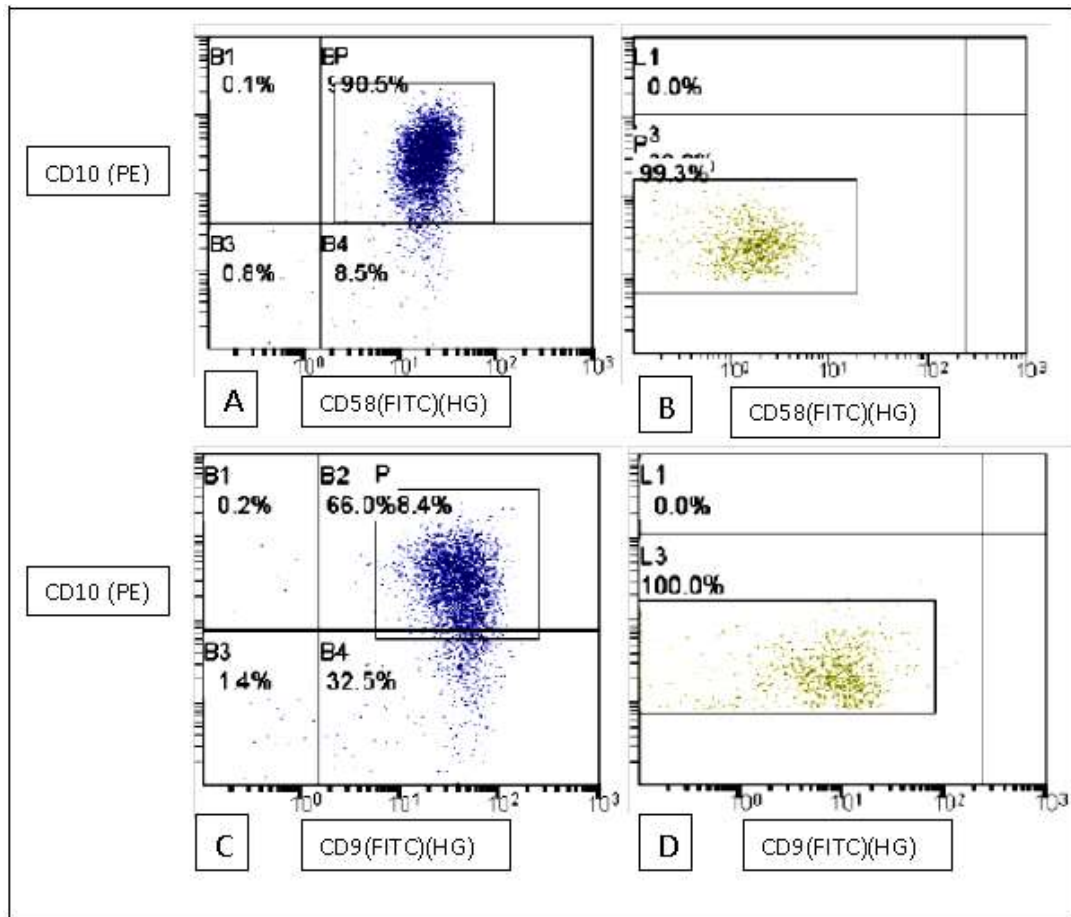
Figure 3. Different expression patterns of CD9 vs. CD10 and CD58 vs. CD10 in hematogones and lymphoblastic cells. In lymphoblastic cells, (A) CD10 (FL2) vs. CD58 (FL1) showed a down to up expression pattern; however, (B) it showed left to right expression pattern in hematogones. In lymphoblastic cells, (C) CD10 vs. CD9 showed down to up expression pattern but (D) showed left to right expression pattern in hematogones.
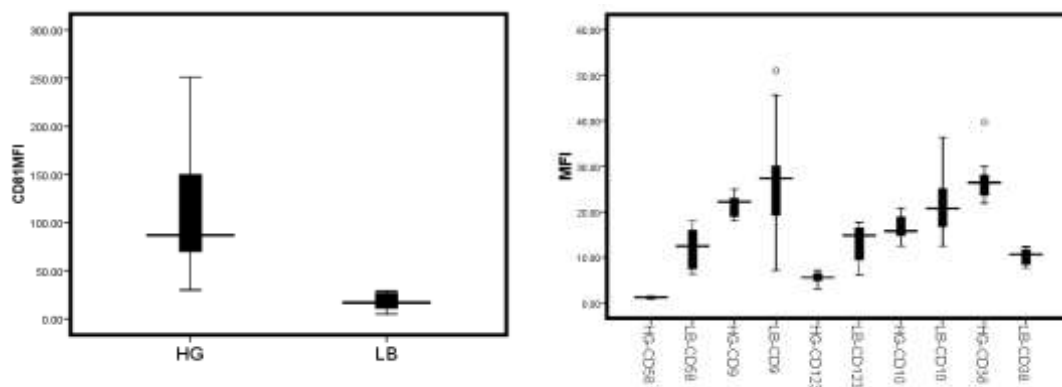

Figure 5. Difference between hematogone and Lymphoblast in terms of MFI

Table I: Difference between Hematogone and Lymphoblast in terms of MFI

CD Markers	Hematogones	Lymphoblasts	P-Value
CD81	112.5 (30-251)	17.5 (5-30)	<0.0001
CD58	1.2 (0.9-1.5)	12.3 (6.4-18.2)	<0.0001
CD9	21.5 (18-25)	26.9 (6.8-51)	0.131
CD66c	Negative	13.2 (9.4-17.7)	----
CD123	5.5 (3.1-7.2)	9.1 (6.2-14.0)	<0.0001
CD10	16.5 (14.2-20.8)	21.7 (12.5-36.4)	0.038
CD38	27.1 (21.9-39.7)	10.2 (8.5-12.4)	0.022

Discussion

In this study, the expression profile of B-Cell associated markers and expression of CD81, CD58, CD9, CD123, and CD66c were examined in hematogones and lymphoblasts. The aim of this study was to prevent misdiagnosis in patients with increased hematogones and exact discrimination of hematogones from lymphoblasts in MRD assessment in B-ALL patients. Increasing hematogones in patients with autoimmune disorders and increasing their number in the patients with ALL after reducing chemotherapy doses could pose many diagnostic challenges. CD81 was expressed both in lymphoblasts and in hematogones with different intensities and MFIs. Hematogones expressed CD81 with higher MFI (more than 30) with moderate to bright expression pattern. Nagant et al., also reported similar results. They showed that hematogones had CD81 MFI between 40.4 and 69.2 and lymphoblastic cells had CD81 MFI between 4.3 and 11.7 (11). In another study by Muzzafar et al., under-expression of CD81 was reported in lymphoblastic cells (12). In our recent study, high CD81 MFIs were observed in hematogones ranging between 30 and more than 200 and most lymphoblastic cells showed CD81 MFI between 5 and 30. According to this study, CD81 can be the best marker for the discrimination of hematogones in different stages of maturation and their separation from lymphoblastic cells. In the present study, CD58 showed a brighter expression in lymphoblastic cells with higher MFI (6.4-18.2), but hematogones expressed CD58 with low intensity and in all cases with low MFI (0.9-1.5). Nagant et al., reported similar results (11). In this study, CD58 MFI in lymphoblastic cells was reported between 1.7 and 5 and MFI between 0.2 and 0.5 in hematogones. In other study, over-expression of CD58 in lymphoblastic cells was also noted, which can be helpful in evaluating MRD (13). In a previous study by Veltroni et al., CD58 expression was significantly higher in lymphoblastic cells (14). The expression of CD9 was seen in both hematogones and lymphoblastic cells with different intensities. Lymphoblastic cells showed CD9 expression with dim to moderate pattern and MFI between 6.8 and 51. In some B-ALL cases, the use of quadrant dot plot (CD10 vs. CD9 or CD38 versus CD9) can help distinguish hematogones from lymphoblastic cells. In such B-ALL cases,

lymphoblastic cells showed brighter expression of CD9. In another study by shaver et al., the importance of CD9 was noted for MRD assessment in B-ALL patients (15). In lymphoblastic cells, CD9 expression can be accompanied by the expression of CD34, but CD9 expressing hematogones do not express CD34, which is critical in MRD assessment. CD123 and CD66c are very critical markers for the assessment of MRD in B-ALL(16). Expression of CD123 was seen in about 58 percent of B-ALL cases with moderate to bright expression. In a recent study by Nagant et al., CD123 expression was noted in 34% of B-ALL cases, but they noted that hematogones were negative for CD123 expression (11). In this study, lymphoblastic cells in 58% of B-ALL cases expressed CD123, but hematogones also expressed CD123, and this expression most commonly was seen in stages II and III hematogones, which were negative for CD34. In a study by Esh SS et al., the expression of CD123 was reported in hematogones and it was shown that CD123 expressing hematogones were negative for CD34 expression (17). In the current study, CD66c expression was observed in 39% of B-ALL cases, but all hematogones were negative for the expression of CD66c. For this reason, CD66c could be the best aberrant marker for the discrimination of lymphoblastic cells from hematogones and critical marker for MRD assessment in B-ALL cases with expression of CD66c. In this study, lymphoblastic cells showed heterogeneous expression profile, but hematogones consistently showed unique expression pattern. All hematogones had a dim to bright expression of CD81, expression of CD123 without co-expression of CD34, very dim expression of CD58, and negative expression of CD66c. Hematogones showed bright expression of CD38, dim to moderate expression of CD10, moderate expression of CD19, dim to bright expression of CD20 and CD45, and mostly negative

expressions of CD34 and tdt. Lymphoblasts had a different expression profile with different intensity. It was found that 31 cases were negative for CD20 expression and 8 cases were negative for CD10 expression. CD81 was consistently expressed in lymphoblastic cells with dim to moderate expression and low MFI (less than 30). However, in some cases, CD81 MFI was more than 30. In such cases, moderate to bright expression of CD58 with higher MFI and or bright expression of CD9 with higher MFI and in the absence of these properties, co-expression of CD123 with CD34 or expression of CD66c could be critical for identifying lymphoblastic cells.

Conclusion
It can be concluded that CD81 and CD58 could be the best markers for the determination of hematogones and its distinction from lymphoblastic cells, and other markers such as CD9, CD123 and CD66c, as complementary markers, are very helpful for more specific and sensitive distinction of hematogones from lymphoblasts.

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Conflict of Interest
The authors have no conflict of interest to declare.

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