A Dissection of the CD45/Side Scatter "Blast Gate"

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Abstract

CD45/side scatter (SS) gating is widely used for isolating blasts by flow cytometry (FC). However, other cells contaminate the "blast gate" (BG); CD45/SS gating is thus imprecise, particularly when there are few blasts. We studied the BG contents in 21 myelodysplastic syndromes (MDSs), 14 myeloproliferative neoplasms (MPNs), 7 chronic myelomonocytic leukemias (CMMLs), and 40 nonneoplastic control samples using 4-color FC with cluster analysis. There were no significant differences across groups in the median percentage of BG events represented by blasts (14.7%-22%), granulocytes (23.3%-33.2%), lymphocytes (2.1%-3.2%), and erythroids (1.0%-9.8%). Monocytes were a larger percentage of BG events in CMML (24.2%). Basophils averaged 35.4% of the BG in MPNs. The percentage of blasts within the BG averaged 94.2% in control samples vs 88.2% in MDSs and 80.7% in CMMLs. Blasts averaged about 20% of events in the BG. About 10% to 20% of blasts fell outside the BG in CMMLs and MDSs. Our data highlight pitfalls in using a traditional BG for blast analysis in nonacute myeloid disorders.

CD45/side scatter (SS) gating is the most commonly used approach to blast isolation. This technique is imprecise, however, as many cell populations other than blasts contaminate the "blast gate" (BG), including granulocytes, monocytes, basophils, hematogones, erythroid precursors, and lymphocytes.[1] Rigidly using a CD45/SS gate to enumerate and/or immunophenotypically characterize blasts is, therefore, problematic, especially in cases with low blast counts. Many recent studies have attempted to characterize immunophenotypic aberrancies in nonacute myeloid disorders (myelodysplastic syndromes [MDSs] and myeloproliferative neoplasms [MPNs]); in these settings, traditional gating approaches can lead to inaccurate enumeration and/or assignment of inappropriate antigen expression on blasts.[2] For example, failure to identify a prominent basophil population (CD11b+) contaminating the BG may cause erroneous assignment of CD11b expression to blasts, a neoplasia-specific blast immunophenotype.[2] In addition, CD34– blasts, which are described in several studies in the early MDS flow cytometry literature, may represent contaminating cell populations, as the authors of these studies used CD45/SS gating as the sole blast isolation method.[3,4] Furthermore, blasts that show decreased CD45 expression (a neoplasia-specific immunophenotypic finding)[2] may not be accurately enumerated using such a gating strategy. As flow cytometry becomes increasingly studied as an ancillary tool in the diagnosis of disorders with low blast counts, recognition of the limitations of traditional gating approaches is imperative.

There are virtually no data in the literature that provide a detailed analysis of the composition of the BG in neoplastic or nonneoplastic bone marrow samples. As the contamination of the BG with other cell populations has practical implications,

we sought to quantitatively evaluate the contents of the CD45/SS BG in cases of nonacute myeloid disorders, including MDSs, MPNs, and chronic myelomonocytic leukemia (CMML), using 4-color flow cytometry and cluster analysis, a method designed to delineate all relevant cell populations.

Materials and Methods

We retrospectively reviewed the flow cytometry laboratory database at Dynacare Laboratories/Medical College of Wisconsin, Milwaukee, for cases of MDSs, MPNs, and myelodysplastic/myeloproliferative disorders (MDS/MPNs) at diagnosis and cases of cytoses and cytopenias that were received to evaluate for possible MDSs and MPNs. Diagnoses were made according to the 2001 and 2008 World Health Organization criteria for MDSs, MPNs, and MDS/MPNs, as appropriate, using a combination of morphologic and cytogenetic features.[5,6] Two control groups were established, including negative lymphoma staging marrow samples and samples of nonneoplastic cytoses or cytopenias. Cases were classified as nonneoplastic cytoses or cytopenias if they had normal cytogenetic studies, including normal karyotype and/or negative fluorescence in situ hybridization studies, and did not meet the World Health Organization morphologic and molecular criteria for MDSs and MPNs. The immunophenotypic, CBC, and cytogenetics data on these cohorts was previously published.[2] This study was approved by the institutional review board at the Medical College of Wisconsin.

Flow Cytometry

EDTA- or heparin-anticoagulated bone marrow aspirate specimens were processed as previously described.[2] Cell suspensions (approximately 2×10^6 cells/mL) were incubated with antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein, or allophycocyanin, using the following 4-color combinations: CD10/CD22/CD20/CD34, CD34/CD14/CD45/CD38, CD15/CD33/CD45/CD34, CD16/CD56/CD45/CD11b, CD16/CD13/HLA-DR/CD45, CD7/CD117/CD45/CD34, and CD36/CD64/CD45/CD34. All antibodies were products of Becton Dickinson (Franklin Lakes, NJ).

Data Analysis

Flow cytometric data were acquired using a FACSCalibur (February 2006 to February 2009) or FACSCanto (March 2009 to May 2009) instrument (Becton Dickinson) and analyzed using Becton Dickinson Paint-A-Gate software. Cluster analysis was performed to identify all relevant populations, including hematogones, monocytes, lymphocytes, basophils, granulocytes, and erythroid precursors in each 4-color tube when possible, as previously described **Image 1A**.[2] Blasts were recognized after exclusion of all other populations as cohesive, well-delineated clusters, with consistent light scatter and CD45 expression patterns across multiple tubes. Nonviable cells and debris were removed based on very low forward scatter and SS.

Following cluster analysis, BGs were uniformly applied across all tubes, according to conventions from the literature **Image 1B**.[7-10] The width of the gate was defined as the width of the granulocytes on the CD45 axis; the height was defined as extending up to the main granulocyte population on the SS axis. The percentages of events within the BG were collected. To ensure consistent uniform gating across tubes, these percentages needed to be within 10% of each other.

The cell populations in each BG of the CD34/CD14/CD45/CD38 and CD36/CD64/CD45/CD34 tubes were collected, as illustrated in **Image 1C**. Percentages of blasts,

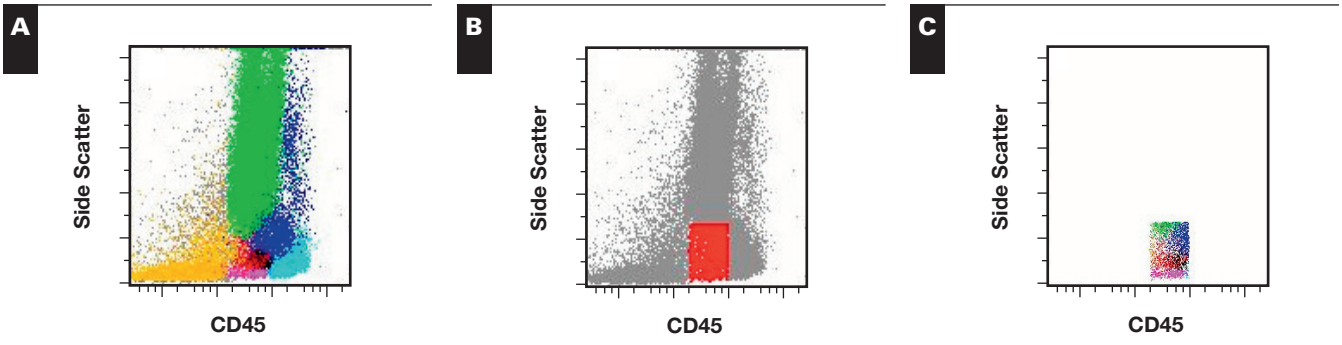

Image 1 **A**, CD45/side scatter histogram of a control specimen analyzed by cluster analysis, showing granulocytes (green), monocytes (blue), lymphocytes (cyan), erythroid precursors (yellow), basophils (black), blasts (red), and hematogones (magenta). **B**, The CD45/side scatter "blast gate" (red) adopted from conventions in the flow cytometry literature and uniformly applied to all cases. **C**, A CD45/side scatter histogram detailing the cellular composition of the blast gate, with granulocytes (green), monocytes (blue), lymphocytes (cyan), erythroids (yellow), basophils (black), blasts (red), and hematogones (magenta).

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granulocytes, monocytes, and lymphocytes within the BG were enumerated by averaging the results of these 2 tubes, while hematogones and basophils were enumerated in the CD34/CD14/CD45/CD38 tube, and erythroids were enumerated in the CD36/CD64/CD45/CD34 tube.

Total blast percentages by flow cytometry (% blasts/total events) were recorded, as an average of the CD34/CD14/CD45/CD38 and CD36/CD64/CD45/CD34 tubes and compared with total blast percentages by morphologic assessment (500-cell differential of Wright-Giemsa–stained aspirate smears). Percentages of overall blasts within the BG were then calculated (blasts in BG/total blasts defined by cluster analysis).

Results

For the study, 42 nonacute myeloid disorder cases were obtained and compared with 40 control cases. The nonacute myeloid disorder group included 21 MDSs (11 men, 10 women; 21-78 years), 14 MPNs (6 men, 8 women; 32-73 years), and 7 CMMLs (all men; 58-91 years). The control group included 20 negative lymphoma staging cases and 20 nonneoplastic cytoses/cytopenia cases (20 men, 20 women; 21-87 years). The MDSs consisted of 1 refractory anemia with ringed sideroblasts, 6 refractory cytopenias with multilineage dysplasia, 4 cases of refractory anemia with excess blasts (RAEB)-1, 2 cases of RAEB-2, 6 cases of unclassifiable MDS, and 2 cases of therapy-related MDS. The MPNs consisted of 7 chronic myelogenous leukemias (CMLs), 3 cases of primary myelofibrosis, and 4 MPNs, unclassifiable.

No statistically significant differences were found between the control groups with respect to BG cell populations (with the exception of median percentage of monocytes in the BG, 4.5% in cytoses/cytopenias vs 8.4% in negative lymphoma staging cases; $P = .02$); thus, these groups were combined for comparison purposes. The median percentage of total events in the BG was higher in CMMLs (7.8%; $P < .001$), MDSs (5.9%; $P = .001$), and MPNs (3.3%; $P = .64$) vs control samples (2.8%).

Median blast percentages by flow cytometry were 0.67% in CMMLs ($P = .04$), 1.2% in MDSs ($P = .004$), and 0.43% in MPNs ($P = .28$) compared with 0.41% in control samples (previously published data)[2] **Table 1**. Median blast percentages by morphologic assessment were 4.6% in CMMLs, 3.4% in MDSs, and 1.0% in MPNs vs 1.0% in control samples (previously published data).[2] The percentage of total blasts that fell within the BG averaged 94.2% in control samples vs 86.5% in MPNs ($P = .12$), 88.2% in MDSs ($P = .02$), and 80.7% in CMMLs ($P = .005$) (Table 1). A CMML case demonstrating significant numbers of blasts outside the BG is illustrated in **Image 2**.

The median (range) percentage of BG events represented by blasts was 14.7% (0.32%-36.6%) in control samples vs 21.1% (1.9%-55%; $P = .285$) in MDSs, 21.5% (3.1%-79.4%; $P = .225$) in MPNs, and 22.3% (1.2%-57.1%; $P = .731$) in CMMLs **Table 2**. There were no statistical differences across groups in median percentage of BG events represented by granulocytes (23.3%-33.2%), lymphocytes (2.1%-3.2%), and erythroids (1.0%-9.8%).

Monocytes were a larger percentage of the BG events in CMML (24.2%; $P = .03$) vs combined control samples (6.4%), while monocytes averaged 2.8% ($P = .01$) in MPNs and 7.6% in MDSs ($P = .61$) compared with control samples. Basophils averaged 35.4% of the BG in MPNs vs 12.7% in control samples ($P = .001$), 11.5% in MDSs ($P = .968$), and 3.1% in CMMLs ($P = .002$) vs controls. Median hematogone

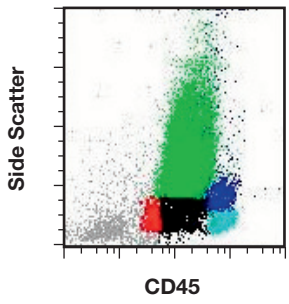

Image 2 A case of chronic myelomonocytic leukemia with increased numbers of blasts (red) showing decreased CD45 expression, which are not represented in the "blast gate" (black) (granulocytes, green; monocytes, blue; lymphocytes, cyan).

Table 1
Total Blasts by Flow Cytometry and Percentage of Blasts in the BG in Control Samples, MDSs, MPNs, and CMMLs*

	Control Samples	MDSs	MPNs	CMML
Total blasts by flow cytometry (%)	0.41 (0.01-3.2)	1.2 (0.07-7.1) $P = .004$	0.43 (0.09-3.8) $P = .28$	0.67 (0.07-6.7) $P = .04$
Blasts in the BG (%)	94.2 (33-100)	88.2 (33-98.5) $P = .02$	86.5 (50-99) $P = .12$	80.7 (57.2-91.3) $P = .005$

BG, blast gate; CMML, chronic myelomonocytic leukemia; MDSs, myelodysplastic syndromes; MPNs, myeloproliferative neoplasms.
* Data are given as median (range). *P* values represent comparisons with control samples.

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Table 2
Composition of the "Blast Gate" in the Various Cohorts*

	Control Samples	MDSs	MPNs	CMML
Blasts (%)	14.7 (0.32-36.6)	21.1 (1.9-55) $P = .285$	21.5 (3.1-79.4) $P = .225$	22.3 (1.2-57.1) $P = .731$
Granulocytes (%)	25.6 (10-60.2)	30.8 (9.1-79.2) $P = .412$	23.3 (7.6-34.9) $P = .961$	33.2 (10.8-65.7) $P = .665$
Monocytes (%)	6.4 (0.14-25.2)	7.6 (0.05-53.6) $P = .611$	2.8 (0.04-9.5) $P = .01$	24.2 (0.27-65.6) $P = .033$
Basophils (%)	12.7 (0-53.4)	11.5 (0-27) $P = .968$	35.4 (0-68.7) $P = .001$	3.1 (0-7.1) $P = .002$
Lymphocytes (%)	2.1 (0-12.3)	3.2 (0-13.3) $P = .265$	2.1 (0.24-4.9) $P = .521$	2.5 (0.19-10.8) $P = .687$
Hematogones (%)	10.5 (0-38.9)	1.3 (0-12.4) $P < .001$	2.2 (0-18.5) $P < .001$	0.48 (0-1.7) $P = .002$
Erythroids (%)	4.7 (0-26.1)	9.8 (0-58.2) $P = .213$	3.7 (0-25.6) $P = .298$	1.0 (0-2.7) $P = .085$

CMML, chronic myelomonocytic leukemia; MDSs, myelodysplastic syndromes; MPNs, myeloproliferative neoplasms.
* Data are given as median (range). *P* values represent comparisons with control samples.

values averaged 0.48% of the BG in CMMLs ($P = .002$), 1.3% in MDSs ($P < .001$), and 2.2% in MPNs ($P < .001$) vs 10.5% in control samples (Table 2).

Discussion

In the early 1990s, the CD45 vs right-angle light scatter gating strategy was proposed as superior to the traditional, forward-angle vs right-angle light scatter gate for the identification of normal cell populations and acute leukemia immunophenotyping.[7,8] This gating strategy, and more specifically the BG, has been used recently to assign blast immunophenotypes in studies of low-blast-count disorders, such as MDSs and MPNs.[3,4,11-13] However, as it is well recognized that different cell populations contaminate this BG, several authors have used more robust methods of analysis for this purpose, including cluster analysis and CD34 back-gating, in nonacute myeloid disorders.[2,11,13-15] Indeed, failure to recognize these contaminating cell populations may lead to inaccurate blast counts and imprecise blast immunophenotypes. Given a lack of detailed data in the literature, we chose to enumerate and compare the BG populations in normal bone marrows and low-blast-count myeloid neoplasms.

To accomplish this, all relevant cell populations were identified initially using cluster analysis, as previously described. A traditional BG was adopted from the literature using reproducible CD45 and SS properties and applied to the analyzed cases. While we recognize that such a method may deviate from more sophisticated analysis techniques, wherein a BG is defined by the population data rather than uniformly applied criteria, this rigorous application allowed for comparison across study groups.

Given expected myeloid expansions in the diseased study groups, it was not surprising that more events were present in the BG of the nonacute myeloid disorders compared with the control groups, although only MDS and CMML comparisons were statistically significant. Blast percentages by flow cytometry and morphologic assessment were higher in each of these groups compared with the control group, which may in part account for the increased numbers of events observed in the BG.

In all groups, blasts averaged approximately 20% of events in the traditional CD45/SS BG. Despite the increased blast percentages identified by flow cytometry and morphologic assessment in CMMLs and MDSs, the median relative percentages of blasts in the BG were the same across all study groups and the control group. Thus, the majority of events in the BG do not represent blasts; instead, granulocytes represent the largest population in the BG (averaging approximately 30% of events) in all groups except MPNs, in which basophils are the largest contributor to the gate (averaging 35% of events). Failure to identify these contaminating populations may overestimate blast percentages, as previously stated. Furthermore, if granulocytes and basophils are not appropriately identified during analysis, blasts may be inaccurately perceived to be asynchronously positive for mature granulocyte antigens (eg, CD11b, CD15, and CD16) or to be underexpressing normally expressed blast antigens (eg, HLA-DR, CD34, and CD117). Since the demonstration of aberrant blast immunophenotypes may support the diagnosis of a neoplastic myeloid process in cases with low blast counts, CD45/SS gating in these clinical scenarios is not ideal, and, therefore, more robust analysis strategies for blast enumeration and immunophenotyping are recommended.[2]

Several of our findings were predictable. For example, monocytes constituted a larger percentage of the BG in CMMLs compared with control samples and the other nonacute myeloid disorders. Similarly, basophils heavily contaminated the BG of MPNs compared with the other studied groups, with both cell types averaging approximately 25% to 35% of the BG events in their respective diseases. Finally, compared with control samples, fewer BG events in MDSs, CMMLs, and MPNs represented hematogones; previous reports have documented diminished hematogones in MDS and CML.[16,17]

Decreased CD45 expression is a well-recognized aberrancy on myeloid and lymphoid blasts.[2,4,11,15,18,19] In our

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study, after identification of the cell populations by cluster analysis, we applied the uniformly defined BG to determine the percentage of blasts that were within and outside the gate, hypothesizing that neoplastic blasts with variable CD45 expression would not be entirely captured by the uniform gating strategy. In fact, our data support this presumption, with the nonacute myeloid disorders having a smaller proportion of the total blast population falling within the BG compared with the control samples. This finding was most pronounced in the CMML cohort, in which a median 81% of the blasts were within the BG vs 94% in control samples. Blasts were observed outside the BG because of decreased CD45 expression (observed only in the neoplastic cases) and/or adherence to granulocytes. Our observations in neoplastic cases support the use of analysis techniques that are driven by the properties of the populations, rather than applying rigid gating strategies that presuppose the characteristics of populations.

We present data on the content of the traditional CD45/SS BG and therein highlight the fraction of events that are, in fact, blasts. The data presented support the use of more robust analysis techniques for enumerating and immunophenotyping blast populations, particularly in disorders with low blast counts. This is especially important as flow cytometry attains a more established role in the diagnosis of low-blast-count disorders such as MDS. The data also have important implications for the approach to minimal residual disease analysis in acute myeloid leukemia.

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