The Specificity of Immunophenotypic Alterations in Blasts in Nonacute Myeloid Disorders

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Abstract

Data regarding flow cytometry (FC) in nonacute myeloid disorders is confounded by variable gating strategies and controls limited to normal bone marrow (BM) samples. Blasts in diagnostic BM samples of myelodysplastic syndromes (MDSs), myeloproliferative neoplasms (MPNs), and chronic myelomonocytic leukemias (CMMLs) were compared with 20 nonneoplastic cytopenias/cytoses (CCs) and negative staging BM samples using 4-color FC. Blasts in 10 of 20 CCs showed immunophenotypic differences vs control samples. Immunophenotypic alterations were identified in 18 of 21 MDSs, 11 of 14 MPNs, and 7 of 7 CMMLs vs control samples and 13 (62%) of 21 MDSs, 7 (50%) of 14 MPNs, and 3 (43%) of 7 CMMLs vs CCs. Neoplastic-specific blast immunophenotypic changes included expression of CD7, CD11b, CD15, CD36, and CD56; CD34 overexpression; HLA-DR variability; lack of CD13 and CD33; underexpression of CD13, CD33, CD45, and HLA-DR; and partial loss of CD13, CD33, CD38, and CD117. In all cases, blasts were CD34+. Several blast immunophenotypic alterations are shared in neoplastic and nonneoplastic BM samples. Approximately 40% to 60% of neoplastic BM samples exhibited aberrancies not seen in reactive BM samples.

There is a large and complex literature describing the immunophenotypic features of myelodysplastic syndromes (MDSs) and to a considerably lesser extent myeloproliferative neoplasms (MPNs).[1-13] These studies have focused on aberrant antigen expression and maturation patterns of various cell populations, primarily granulocytes, monocytes, and blasts. For MDSs, scoring systems have been developed based on these descriptions in attempts to aid in diagnosis and guide therapeutic options for patients.[1-8] Studies that have focused on blast populations[2-8] have described various abnormalities, including decreased CD45 expression; underexpression and overexpression of CD13, CD33, CD38, and HLA-DR; lack of CD34; aberrant expression of CD2, CD10, CD11b, and CD15; and expression of the lymphoid-associated antigens CD2, CD5, CD7, CD19, and CD56. However, the specifics of these abnormalities are not always well described. In addition, many of these studies are confounded by variable gating strategies, suboptimal control groups, and skewed and/or poorly characterized patient cohorts.

CD45/side scatter (SS) gating, the most common approach to blast isolation,[14,15] is imprecise because many other cell populations contaminate this gate, including mature and immature monocytes, basophils, granulocytes, hematogones, lymphocytes, and erythroid precursors **Image 1**.[16] In fact, blasts in such a gate frequently represent only a minority of events.[17] Using such a gating strategy may lead to inaccurate reporting of antigen expression patterns on blasts. For example, failure to identify basophils contaminating this gate may result in erroneous identification of CD11b expression on blasts. Flexible, iterative analysis strategies that seek to discriminate distinct population clusters in multidimensional flow cytometry space based on differential patterns of antigen

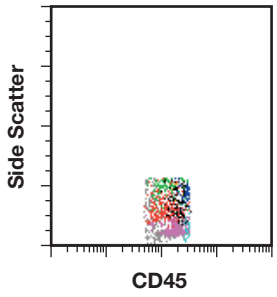

Image 1 CD45/side scatter "blast" gate including blasts (red), granulocytes (green), monocytes (blue), lymphocytes (cyan), hematogones (magenta), basophils (black), and plasma cells (yellow).

expression ("cluster analysis") are more robust techniques for isolating blast populations.[16] Anecdotally, we have seen variability in antigen expression of blasts in nonneoplastic/reactive marrow processes. Therefore, studies using control groups such as "normal" bone marrow (BM) samples from healthy donors or few "reactive" BM samples do not likely fully capture this variability and, thus, overestimate the specificity of certain immunophenotypic changes for myeloid neoplasia. While nonneoplastic immunophenotypic alterations in reactive marrows are sometimes alluded to, such data are uncommonly presented to readers.

Given these confounding factors in much of the flow cytometry literature related to nonacute myeloid disorders, we sought to precisely isolate and immunophenotypically characterize blasts in MDS, MPN, and chronic myelomonocytic leukemia (CMML) cases using an approach designed to delineate all relevant BM populations (cluster analysis) and compare them with normal BM samples and nonneoplastic cytopenias and cytoses. Because we have anecdotally observed myeloblasts in these nonacute myeloid disorders to always be CD34+—a finding not explicitly stated in the literature—we additionally sought to formally confirm this impression.

Materials and Methods

We retrospectively reviewed the flow cytometry laboratory database at Dynacare Laboratories/Medical College of Wisconsin, Milwaukee, for cases of MDS, MPN, and myelodysplastic/myeloproliferative disorders (MDS/MPN) at diagnosis and cases of cytoses and cytopenias that were received to evaluate for possible MDSs and MPNs. All cases underwent morphologic and cytogenetic evaluation for inclusion and classification in the study. This study was approved by the institutional review board at the Medical College of Wisconsin.

Morphologic Studies

Wright-Giemsa–stained peripheral blood smears, bone marrow aspirate smears, and touch imprints and Bouin-fixed, H&E-stained core biopsy specimens and clot sections were examined in each case. When possible, 500-cell aspirate and/or touch imprint differentials were performed.

Cytogenetics and Molecular Diagnostics

Conventional karyotyping was performed from cultured preparations by G banding analysis of 20 metaphases. Fluorescent in situ hybridization (FISH) studies for deletions 5/5q, 7/7q, and 20q; *MLL* gene rearrangements; and trisomy 8 were performed for possible MDS cases, while FISH for t(9;22) was performed for possible chronic myelogenous leukemias (CMLs). Qualitative $JAK2^{V617F}$ mutational analysis was performed using a sequence-specific fluorescent resonance energy transfer hybridization assay.[18]

Classification of Cases

Diagnoses were made by experienced hematopathologists (S.K. and H.O.) according to World Health Organization (WHO) 2001 and WHO 2008 criteria for MDSs, MPNs, and MDS/MPNs, as appropriate, using a combination of morphologic and cytogenetic features.[19,20] Cases were classified as nonneoplastic cytoses or cytopenias if they had normal cytogenetic studies, including normal karyotype and/or negative FISH studies, and did not meet WHO morphologic and molecular criteria for MDSs and MPNs. All cases were reviewed independent of the flow cytometric findings. Negative lymphoma staging BM samples were used as control samples.

Flow Cytometry

EDTA- or heparin-anticoagulated BM aspirate specimens were lysed with an ammonium chloride solution followed by 3 rounds of centrifugation and resuspension with an RPMI/penicillin-streptomycin solution. 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. After incubation for 30 minutes at 1°C to 9°C in the dark, the cells were washed with phosphate-buffered saline containing azide, followed by fixation in 1% formaldehyde. All antibodies were products of Becton Dickinson (Franklin Lakes, NJ). Isotype controls were performed in all cases.

Data Analysis

Flow cytometric data were acquired using a FACSCalibur (February 2006 to February 2009) or FACSCanto (March

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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 **Image 2**.

Normal cell populations were identified as follows: hematogones, low forward and side scatter, CD10+, CD22(moderate+), CD38(moderately bright+), HLA-DR+, CD34(subset+), and CD45(moderate to dim+); monocytes, intermediate forward and side scatter, CD11b+, CD14(bright+), CD33(bright+), CD36(bright+), CD38+, CD45(bright+), CD64(bright+), CD15(variable+), and CD34–; lymphocytes, low forward and side scatter and CD45(bright+); basophils, tight cluster with intermediate forward scatter, low side scatter, CD38+, CD11b+, CD13(moderately+), CD33(moderately+), CD45(moderate to bright), and CD34–; maturing granulocytes, moderate to high forward and side scatter, CD15(bright+), CD33+, CD45(moderately+), CD64(moderately+), and HLA-DR–; and erythroids, variable but predominantly low forward and side scatter, CD36+, CD34(predominately–), CD45(negative to dim+), and CD64–.[21,22] Nonviable cells and debris were removed based on very low forward and side scatter. Dysplastic monocytes or promonocytes were recognized by bright CD45 expression and variable patterns of CD13, CD14, and HLA-DR.[23] 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. Blasts were enumerated by averaging the results of 2 tubes, CD34/CD14/CD45/CD38 and CD36/CD64/CD45/CD34.

Positive antigen expression was defined as at least 20% of the blast population showing fluorescence above the background isotype control staining for the same cell population at an isotype cutoff of 2%. The blast immunophenotypes of the negative lymphoma staging BM samples were recorded. Immunophenotypic alterations were defined as at least a quarter log shift of the blast population compared with the lymphoma staging control samples or the cytoses/cytopenias, as appropriate. Although it is routine laboratory procedure to adjust instrument settings over time to maintain consistent levels of fluorescence intensity, there is the possibility of slight shifts due to instrument or reagent fluctuation. Consequently, when possible, levels of antigen expression were compared with other internal cell populations. In addition, any such shifts would likely affect the control group to a similar extent as the study groups.

Bright CD34 expression was specifically defined as greater than half a log shift of the population compared with the control samples, whereas a quarter log shift was designated as slightly bright CD34 expression. CD45 expression was defined with respect to granulocytes (Image 2). CD15 expression was defined as gross overexpression in the blast population.

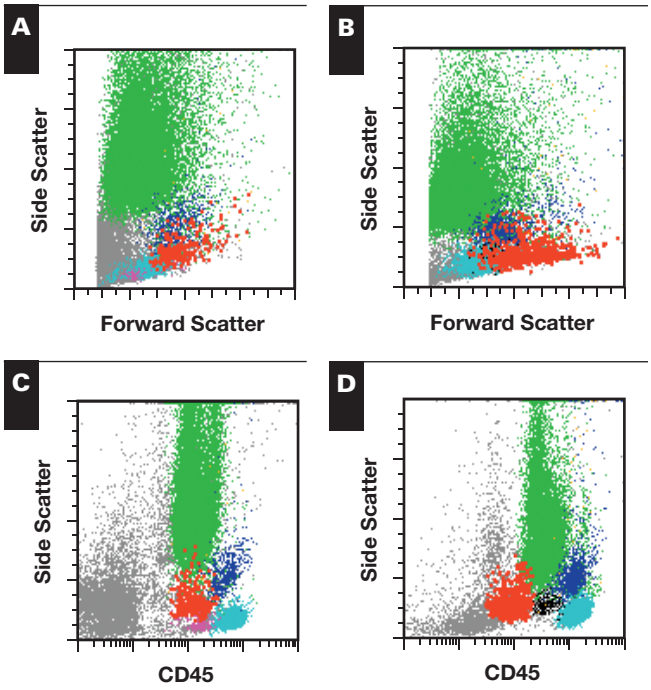

Image 2 Cluster analysis of control samples (**A** and **C**) and neoplastic bone marrow samples (**B** and **D**) illustrating CD45 and scatter properties. Blasts, red; granulocytes, green; monocytes, blue; lymphocytes, cyan; hematogones, magenta; basophils, black; and plasma cells, yellow.

Results

Nonneoplastic Cases

The cytoses/cytopenias group (n = 20) included samples from 13 women and 7 men, ranging in age from 32 to 87 years (median, 58.5 years), and the negative lymphoma staging control group (n = 20) consisted of samples from 13 men and 7 women, ranging in age from 21 to 78 years (median, 54.5 years) **Table 1**. Blast counts by flow cytometry ranged from 0.01% to 3.2% in cytoses/cytopenias (median, 0.48%) and 0.08% to 1.12% in control samples (median, 0.39%). Blast counts by morphologic studies ranged from 0% to 4% in cytoses/cytopenias (median, 1.0%) and 0% to 2% in control samples (median, 1.0%) (Table 1). CBC data are presented in Table 1. BM examinations were performed for cytopenias in 18 of 20 cytoses/cytopenias, including 5 anemias, 2 pancytopenias, 4 anemias and thrombocytopenias, 3 leukopenias/neutropenias, 2 thrombocytopenias, and 2

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Table 1
Patient Demographics and Selected Laboratory Data*

	Control Samples (n = 20)	Cytopenias/Cytoses (n = 20)	MDSs (n = 21)	MPNs (n = 14)	CMMLs (n = 7)
Age (y)	54.5 (21-78)	58.5 (32-87)	60 (44-91)	55.5 (32-73)	77 (58-91)
Sex					
Male	13	7	11	6	7
Female	7	13	10	8	0
CBC					
Hemoglobin concentration (g/dL)	13.6 (9.8-16.5)	10.7 (7.9-14.6)	9.4 (6.2-13.7)	12 (8-15.2)	9.8 (7.9-14.7)
WBC count ($\times 10^9$/L)	7.5 (3.4-23.2)	4.8 (2.5-14.5)	3.3 (1.4-16.8)	20 (7.8-349)	19.2 (7.2-48.5)
Platelet count ($\times 10^3$/μL)	324 (179-469)	172 (22-851)	108 (33-598)	474 (260-2,823)	135 (43-263)
Percentage of blasts					
Morphologic studies	1.0 (0-2)	1.0 (0-4)	3.4 (0-14.8)	1.0 (1-2)	4.6 (0.6-9.8)
Flow cytometry	0.39 (0.08-1.1)	0.48 (0.01-3.2)	1.2 (0.07-7.1)	0.43 (0.09-3.8)	0.67 (0.07-6.7)

CMML, chronic myelomonocytic leukemia; MDSs, myelodysplastic syndromes; MPNs, myeloproliferative neoplasms.
* Data are given as median (range), except for sex, which is the number of patients. Hemoglobin and platelet values are given in conventional units; conversions to Système International (SI) values are as follows: hemoglobin (g/L), multiply by 10.0; platelet count ($\times 10^9$/L), multiply by 1.0. WBC values are given in SI units; for conversion to conventional values (/μL), divide by 0.001.

anemias plus leukopenia and in 2 of 20 cytoses/cytopenias for cytoses, including 1 thrombocytosis (with anemia) and 1 thrombocytosis with leukocytosis **Table 2**. Follow-up clinical information was available in 16 of 20 cytoses/cytopenias cases. In these 16 cases, after negative BM examination findings, the cytopenias and cytoses were clinically attributed to the following: 7 of 16 drug-related cytopenias, 3 of 16 chronic renal insufficiency–related anemias, 2 of 16 iron deficiency anemias, 2 of 16 immune thrombocytopenias, and 2 of 16 aplastic anemias (Table 2). Only 1 patient with aplastic anemia had a repeated BM biopsy, which was without evidence of hematolymphoid neoplasia 2 months later.

Table 2
Clinical Data for Cytopenias/Cytoses

Case No./ Sex/Age (y)	Indication for Bone Marrow Biopsy	Cause of Cytopenia or Cytosis
1/M/87	Anemia, thrombocytopenia	Drug related
2/F/76	Anemia, thrombocytopenia	Unknown
3/F/32	Leukopenia	Drug related
4/M/49	Pancytopenia	Renal disease
5/M/50	Anemia, thrombocytopenia	Aplastic anemia
6/M/32	Pancytopenia	Drug related
7/F/74	Anemia, thrombocytopenia	Aplastic anemia
8/F/42	Leukopenia, anemia	Unknown
9/F/65	Leukopenia, anemia	Drug related, ACD
10/F/45	Leukocytosis, thrombocytosis	Drug related
11/F/58	Thrombocytopenia	ITP
12/F/59	Thrombocytopenia	ITP
13/F/68	Anemia	Unknown
14/F/83	Anemia	Renal disease
15/F/59	Anemia	IDA
16/F/54	Anemia, thrombocytosis	IDA
17/M/71	Anemia	Renal disease
18/M/45	Leukopenia	Drug related
19/F/78	Anemia	Drug related
20/M/52	Leukopenia	Unknown

ACD, anemia of chronic disease; IDA, iron deficiency anemia; ITP, immune-mediated thrombocytopenic purpura.

No cases had definite morphologic evidence of dysplasia. Conventional cytogenetics was performed in 18 of 20 cytoses/cytopenias, with normal karyotypes reported in all analyzed cases. In the cytoses/cytopenias group, FISH for MDS was performed and reported as negative in 11 of 18 cytopenias, and *JAK2*^{V617F} mutational analysis was negative in both of the cytoses cases (2/2).

Neoplastic Cases

The MDS group (n = 21) consisted of samples from 11 men and 10 women, ranging in age from 44 to 91 years (median, 60 years); the MPN group (n = 14) included samples from 8 women and 6 men, ranging in age from 32 to 73 years (median, 55.5 years), and the CMML group (n = 7) included samples from all men, ranging in age from 58 to 91 years (median, 77 years) (Table 1). CBC data are additionally presented in Table 1. Cases of MDS included 1 refractory anemia with ringed sideroblasts; 6 refractory cytopenias with multilineage dysplasia with or without ringed sideroblasts; 4 cases of refractory anemia with excess blasts (RAEB)-1; 2 cases of RAEB-2; 6 cases of MDS, unclassifiable; and 2 cases of therapy-related MDS. Cases of MPNs included 7 chronic myelogenous leukemias (CMLs), 3 cases of primary myelofibrosis, and 4 MPNs, unclassifiable **Table 3**, **Table 4**, and **Table 5**. Blast counts by flow cytometry ranged from 0.07% to 7.1% in MDSs (median 1.2%), 0.09% to 3.8% in MPNs (median 0.43%), and 0.07% to 6.7% in CMMLs (median 0.67%). Blast counts by morphologic studies ranged from 0% to 14.8% in MDSs (median, 3.4%), 0% to 4% in MPNs (median, 1%), and 0.6% to 9.8% in CMMLs (median, 4.6%).

Conventional cytogenetics were available in 20 of 21 MDS cases, with 11 having normal karyotypes and 9 with abnormal karyotypes, including the following abnormalities: isochromosome X; t(3;5); t(18;20); deletions 1p, 3p, 5q, 7, 18, and 20q; and trisomy 8. FISH results for MDS were

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Table 3
Diagnosis, Cytogenetic Data, and Percentage of Blasts in Cases of MDS

Case No./Sex/Age (y)	Diagnosis	Karyotype	FISH	Percentage of Blasts	
				Morphology	Flow Cytometry
1/F/55	RAEB-1	Complex	Complex	5	6.3
2/F/57	RAEB-2	N	–	18	7.1
3/M/76	RCMD	N	–	1	0.2
4/F/89	RCMD	Isochromosome X	–	4	1.74
5/F/76	MDS-U	N	Trisomy 11	3.4	1.97
6/F/87	MDS-U	t(3;5)	del5q	0	2.82
7/M/75	RAEB-1	N	–	5.2	0.24
8/F/59	t-MDS	del7	del7	0	0.22
9/M/78	RCMD	del20q	del20q	2	0.70
10/M/71	MDS-U	N	–	1	0.49
11/F/91	RARS	ND	–	0	0.50
12/M/75	RCMD	N	–	4.8	2.41
13/F/62	MDS-U	N	del7q	2	0.07
14/F/85	RCMD	N	–	4	4.49
15/F/62	RCMD	del7	del7	1	0.70
16/M/67	MDS-U	del1p/del5q; trisomy 8	del5q; trisomy 8	3	1.23
17/M/60	RAEB-2	N	–	14.8	1.21
18/M/79	RAEB-1	N	–	5.6	5.28
19/M/44	MDS-U	del18/del20q; t(18;20)	del20q	2	1.4
20/M/48	t-MDS	del3p/del5q	del5q	3.4	0.47
21/M/75	RAEB-1	N	–	6	2.9

del, deletion; FISH, fluorescence in situ hybridization; MDS, myelodysplastic syndrome; N, normal; ND, not done; RAEB, refractory anemia with excess blasts; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; t, therapy-related; U, unclassifiable; –, negative.

Table 4
Diagnosis, Cytogenetic Data, and Percentage of Blasts in MPNs

Case No./Sex/Age (y)	Diagnosis	Karyotype	FISH t(9;22)	Percentage of Blasts		JAK2
				Morphology	Flow Cytometry	
1/F/32	MPN, U	N	–	1	0.73	–
2/M/54	PMF	N	ND	0	0.12	+
3/F/63	MPN, U	N	–	0	0.32	ND
4/F/73	CML	t(9;22)	+	2	1.14	ND
5/F/50	MPN, U	N	–	1	0.19	+
6/M/73	MPN, U	N	–	1	0.09	+
7/M/32	CML	ND	+	2	1.12	ND
8/F/43	PMF	N	ND	2	0.38	–
9/M/57	CML	ND	+	1	0.42	ND
10/F/44	CML	t(9;22)	+	0	0.28	ND
11/M/36	CML	t(9;22)	+	0	0.44	ND
12/F/74	CML	t(9;22)	+	4	2.08	ND
13/M/77	PMF	N	ND	4	3.83	+
14/F/69	CML	t(9;22)	+	2	1.6	ND

CML, chronic myelogenous leukemia; FISH, fluorescence in situ hybridization; MPNs, myeloproliferative neoplasms; N, normal; ND, not done; PMF, primary myelofibrosis; U, unclassifiable; +, positive; –, negative.

available in all MDS cases with 11 negative results and 10 positive results, including trisomies 8 and 11 and deletions of 5q, 7, 7q, and 20q (Table 3). FISH for t(9;22) was performed in 11 of 14 MPNs and was positive in the 7 CMLs (Table 4). Conventional cytogenetics findings were normal in the remaining 3 MPNs. *JAK2* mutational analysis was available in 6 of 14 MPNs (6/7 non-CML MPNs) and was positive in 4 of 6 (Table 4). Conventional cytogenetics was available for all CMMLs (7/7), with 5 demonstrating normal karyotypes and 2 showing abnormal karyotypes, including the presence of deletion of 12p or trisomy 13 (Table 5).

Table 5
Cytogenetics Data and Percentage of Blasts in Chronic Myelomonocytic Leukemia Cases

Case No./ Sex/Age (y)	Karyotype	FISH	Percentage of Blasts	
			Morphology	Flow Cytometry
1/M/91	N	–	0.6	0.07
2/M/65	N	–	8	0.67
3/M/82	N	ND	4	0.62
4/M/82	Trisomy 13	–	5.2	3.1
5/M/58	N	–	9.8	6.7
6/M/77	N	ND	4.6	0.43
7/M/74	del12p	ND	3.8	4.46

FISH, fluorescence in situ hybridization; N, normal; ND, not done; –, negative.

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Blast Immunophenotype of Nonneoplastic Cases

All 20 control BM samples had the following blast immunophenotypes: CD7–, CD11b–, CD13(variably+), CD14–, CD15(predominantly–), CD16–, CD33+, CD34+, CD36–, CD38(moderately bright+), CD45(moderately+), CD56–, CD64–, CD117+, and HLA-DR(moderately bright+).

Of 20 cytoses/cytopenias, 10 (50%) had immunophenotypic differences in antigen expression in the blasts compared with the control samples, ranging from 1 to 3 per case (median, 1 per case), for a total of 13 immunophenotypic differences **Figure 1**. Of 10 cases, 8 (80%) had only 1 immunophenotypic difference, while 1 case each (1/10 [10%]) had 2 and 3 immunophenotypic differences. Immunophenotypic differences included overexpression of CD13 (1 case), CD117 (4 cases), and HLA-DR (1 case); slight overexpression of CD34 (1 case); underexpression of CD117 (1 case); and variability in expression of CD33 (2 cases), CD38 (1 case), and CD117 (2 cases) **Table 6** and **Image 3**.

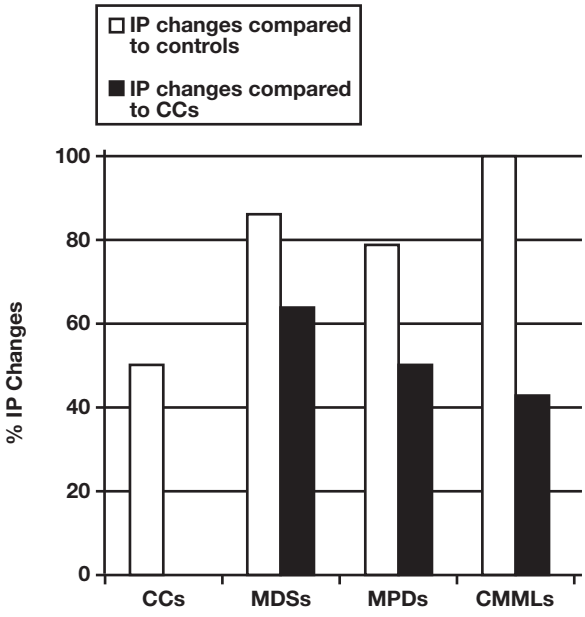

Figure 1 The percentage of immunophenotypic (IP) changes in cytopenias/cytoses (CCs), myelodysplastic syndromes (MDSs), myeloproliferative neoplasms (MPNs), and chronic myelomonocytic leukemias (CMMLs).

Table 6
Immunophenotypic Alterations in Cytopenias/Cytoses

Case No.	Immunophenotypic Alteration
1	CD13(b+)
3	HLA-DR(b+), CD117(dim+)
8	CD117(v+)
10	CD33(v+), CD34(slight b+), CD117(v+)
12	CD117(b+)
15	CD33(v+)
16	CD117(b+)
17	CD117(b+)
18	CD38(v+)
19	CD117(b+)

b, bright; v, variable; +, positive.

Blast Immunophenotype of Neoplastic Cases

Immunophenotypic differences were identified in the blasts of 18 (86%) of 21 MDSs, ranging from 1 to 7 per case (median, 3.0 per case), for a total of 53 immunophenotypic differences compared with control samples (Figure 1), including overexpression of CD13 (2 cases), CD34 (slight, 2 and bright, 4 overexpression), and CD117 (13 cases); expression of CD7 (5 cases), CD11b (1 case), CD15 (1 case), and CD56 (2 cases); underexpression of CD33 (1 case), CD38 (1 case), and CD45 (3 cases); lack of CD33 expression (1 case); and variability in expression of CD33 (6 cases), CD34 (2 cases), CD38 (3 cases), HLA-DR (4 cases), and CD117 (2 cases) **Table 7** (Image 3). The blasts in 13 (62%) of 21 MDSs demonstrated neoplasia-specific immunophenotypic changes not observed in either the control group or cytoses/cytopenias group, ranging from 1 to 4 per case (median, 1.0 per case; total, 24), including underexpression of CD33 (2/21 [9%]), CD38 (1/21 [5%]), and CD45 (3/21 [14%]); expression of CD7 (5/21 [24%]), CD11b (1/21 [5%]), CD15 (1/21 [5%]), and CD56 (2/21 [10%]); lack of CD33 (1/21 [5%]); bright overexpression of CD34 (4/21 [19%]); and variability in expression of HLA-DR (4/21 [19%]). Of these 13 cases, 7 had 1 neoplasia-specific immunophenotypic change, 2 had 2 such changes, 3 had 3, and 1 had 4 changes. An example of an MDS case with several neoplastic-specific immunophenotypic abnormalities in illustrated in **Image 4**.

Immunophenotypic differences compared with control samples were found in the blasts of 11 (79%) of 14 MPNs, ranging from 1 to 5 per case (median, 2 per case; total, 28) (Figure 1), including overexpression of CD34 (slight, 5 cases) and CD117 (2 cases); expression of CD7 (4 cases), CD36 (1 case), and CD56 (1 case); underexpression of CD13 (3 cases), CD45 (1 case), CD117 (1 case), and HLA-DR (2 cases); and variable expression of CD33 (5 cases), CD38 (2 cases), and HLA-DR (1 case) (Image 3 and Table 7). Neoplasia-specific immunophenotypic changes in blasts were identified in 7 (50%) of 14 MPNs, ranging from 1 to 4 per case (median, 1.0 per case; total, 15), including 2 cases with 1 neoplasia-specific change, 3 with 2 such changes, and 1 case each with 3 and 4 changes. Neoplasia-specific immunophenotypic differences included underexpression of CD13 (2/14 [14%]), CD33 (1/14 [7%]), CD45 (1/14 [7%]), CD117 (1/14 [7%]), and HLA-DR (2/14 [14%]); loss of CD13 (1/14 [7%]); expression of CD7 (4/14 [29%]), CD36 (1/14 [7%]), and CD56 (1/14 [7%]); and variable expression of HLA-DR (1/14 [7%]) in the blasts. An example of an MPN case with several neoplasia-specific immunophenotypic abnormalities is illustrated in **Image 5**.

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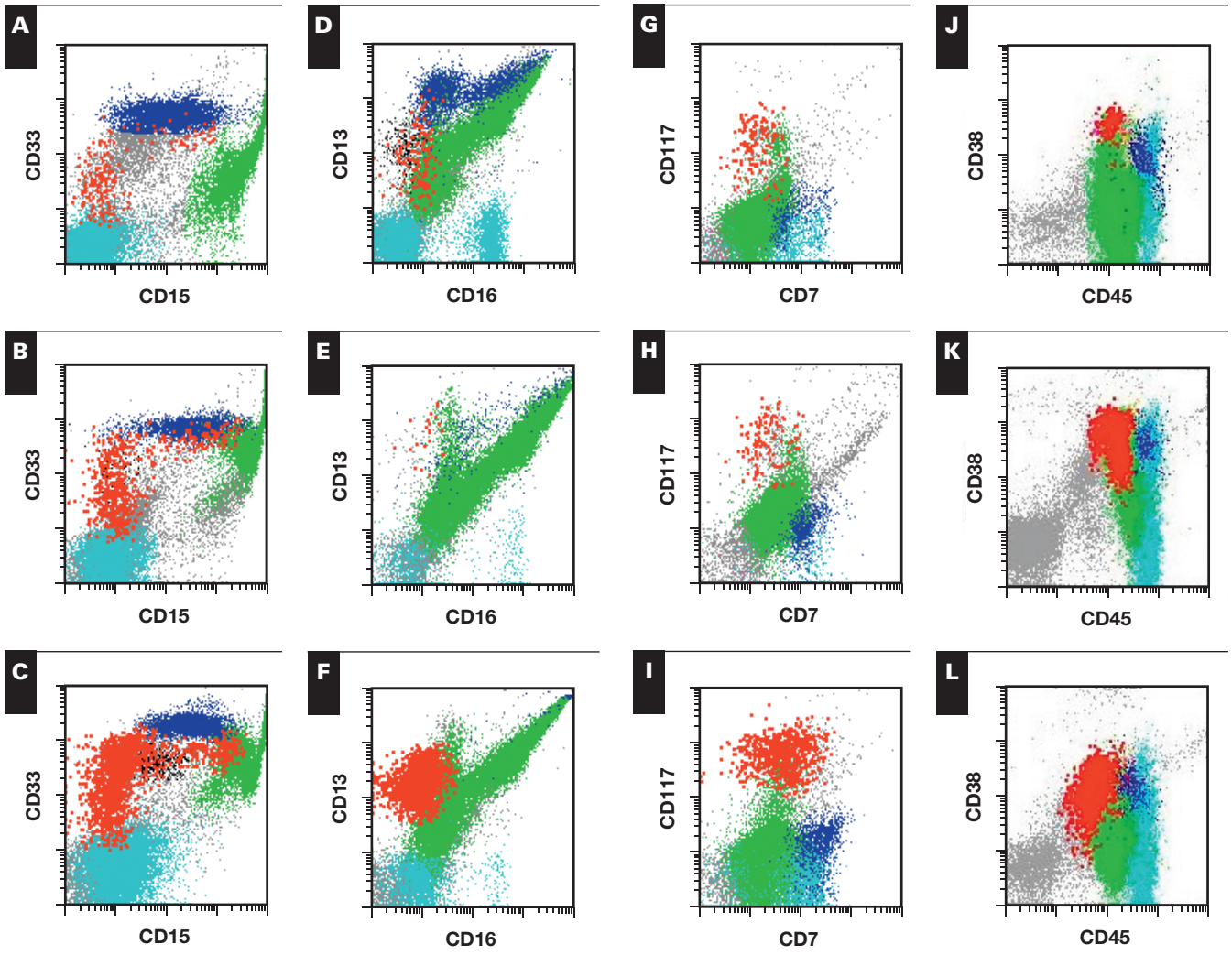

Image 3 Flow cytometric diagrams of control samples (**A**, **D**, **G**, and **J**), cytopenias/cytoses (**B**, **E**, **H**, and **K**), and neoplastic cases (**C**, **F**, **I**, and **L**) illustrating the shared immunophenotypic alterations between cytopenias/cytoses and neoplastic cases. **A**-**C**, CD33 variability in blasts in cytopenias/cytoses and a myelodysplastic syndrome (MDS) case. **D**-**F**, CD13(bright+) blasts in cytopenias/cytoses and a chronic myelomonocytic leukemia case. **G**-**I**, CD117(bright+) in cytopenias/cytoses and a myeloproliferative neoplasm. **J**-**L**, CD38 variability in blasts in cytopenias/cytoses and an MDS case. Of note, in the MDS case (**L**), the CD38(dim+) blasts are dimmer for CD45, which is in contrast with normal blast maturation patterns in which the CD38(dim+) blasts are slightly brighter for CD45. Blasts, red; granulocytes, green; monocytes, blue; lymphocytes, cyan; hematogones, magenta; and basophils, black.

Immunophenotypic differences compared with control samples were found in all CMML cases (7/7 [100%]), ranging from 1 to 3 per case (median, 2 per case; total, 16), with 1 case showing 1 immunophenotypic difference, 3 cases showing 2 differences, and 3 cases showing 3 differences (Figure 1, Image 3, and Table 7). These immunophenotypic differences included overexpression of CD13 (3 cases), CD117 (5 cases), and HLA-DR (1 case); expression of CD7 (1 case), CD15 (1 case), and CD56 (1 case); underexpression of CD45 (1 case); and variable expression of CD33 (1 case) and HLA-DR (2 cases). Of 7 CMMLs, 3 (43%) had neoplasia-specific immunophenotypic changes, including expression of CD7, CD15, and CD56 (each 1/7 [14%]); underexpression of CD45 (1/7 [14%]); and variable expression of HLA-DR (2/7 [29%]).

In summary, immunophenotypic changes exclusive to neoplastic blasts included expression of CD7 (5/21 MDSs, 4/14 MPNs, and 1/7 CMMLs), CD11b (1/21 MDSs), CD15 (1/21 MDSs and 1/7 CMMLs), CD36 (1/14 MPNs), and CD56 (2/21 MDSs, 1/14 MPNs, and 1/7 CMMLs); bright overexpression of CD34 (4/21 MDSs); variability of HLA-DR (4/21) MDSs, 1/14 MPNs, and 2/7 CMMLs); lack of CD13 (1/14 MPNs)

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Table 7
Immunophenotypic Alterations in Neoplastic Cases

General Diagnosis/ Case No.	Specific Diagnosis	Immunophenotypic Alteration
MDS		
1	RAEB-1	CD34(slightly b+), CD117(b+), CD117(v+)
2	RAEB-2	CD15(p+), CD38(p+), CD56(p+), CD117(b+)
4	RCMD	CD33(v+)
5	MDS, U	CD33(v+), HLA-DR(v+)
6	MDS, U	CD117(b+)
8	t-MDS	CD7+, CD45(dim+), CD117(b+)
9	RCMD	CD15(p+), CD33(p+), CD117(b+)
10	MDS, U	CD33(p dim+), CD117(b+)
11	RARS	CD33(v+)
12	RCMD	CD7(p+), CD33(v+), CD38(v+), CD34(b+), CD45(dim+), CD117(b+), HLA-DR(v+)
14	RCMD	CD13(b+), CD33–, CD38(v+), CD45(dim+), HLA-DR(v+)
15	RCMD	CD117(b+)
16	MDS, U	CD7(p+), CD34(slightly b+), CD117(v+)
17	RAEB-2	CD11b(p+), CD13(b+), CD34(b+), CD38(v+), CD56(p+), CD117(b+), CD117(v+)
18	RAEB-1	CD34(b+), CD117(b+), HLA-DR(v+)
19	MDS, U	CD7(p+), CD33(v+), CD117(b+)
20	t-MDS	CD7+, CD117(b+)
21	RAEB-1	CD33(v+), CD34(b+), CD34(v+), CD117(b+)
MPN		
1	MPN, U	CD33(v+), HLA-DR(v+)
2	PM	CD7(p+), CD45(dim+)
3	MPN, U	CD7(p+), CD13(dim+), CD33(v+), CD34(slightly b+)
4	CML	CD7(p+), CD13(p+), CD33(v+), CD117(p+)
6	MPN, U	CD7(p+)
7	CML	CD13(p+), CD33(v+), CD34(slightly b+)
8	PM	CD38(v+)
9	CML	CD33(v+), CD117(b+)
11	CML	CD117(b+)
13	PM	CD13(b+), CD33(p+), CD34(slightly b+), CD38(v+), HLA-DR(dim+)
14	CML	CD34(b+), CD36(p+), CD56(p+), HLA-DR(dim+)
CMML		
1	—	CD13(b+), CD117(b+)
2	—	CD117(b+), HLA-DR(b+)
3	—	CD13(b+), CD117(b+)
4	—	CD13(b+), CD117(b+), HLA-DR(v+)
5	—	CD15(p+), CD33(v+), CD56(p+)
6	—	CD117(b+)
7	—	CD7(p+), CD45(dim+), HLA-DR(v+)

b, bright; CML, chronic myelogenous leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; p, partial; RAEB, refractory anemia with excess blasts; RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; t, therapy-related; U, unclassifiable; v, variable; +, positive; –, negative.

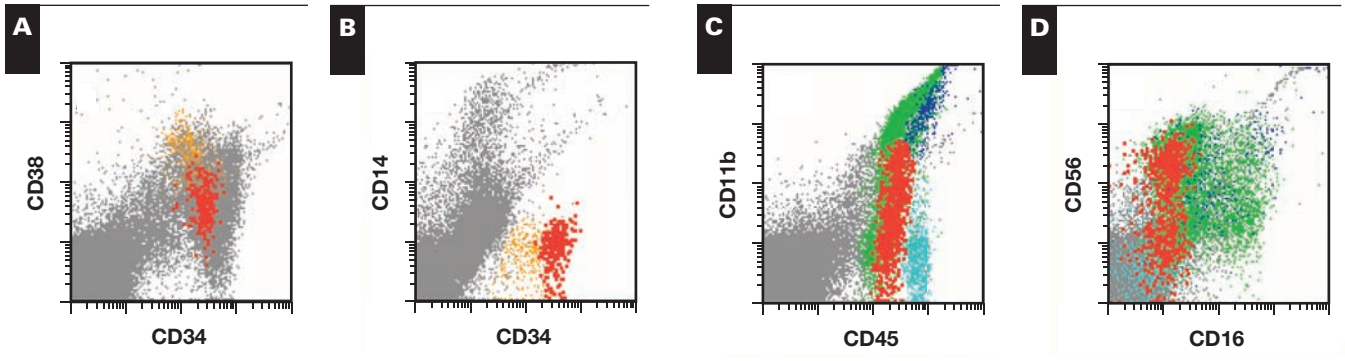

Image 4 Neoplasia-specific immunophenotypic changes in a myelodysplastic syndrome case. **A** and **B**, CD38(variable+) blasts, including a CD34(bright+) subset (red) and a CD34(moderate+) subset (yellow), which may be seen as a regenerative, nonneoplastic change. **C** and **D**, CD11b and CD56 partial expression on blasts. Blasts, red; granulocytes, green; monocytes, blue; lymphocytes, cyan; hematogones, magenta; and basophils, black.

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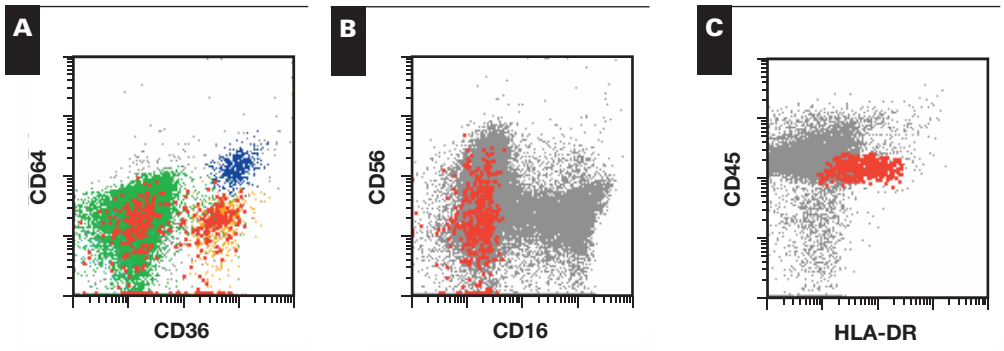

Image 5 Neoplasia-specific immunophenotypic changes in a myeloproliferative neoplasm. **A**, CD36 expression on blasts. **B**, CD56 partial expression on blasts. **C**, HLA-DR(dim+) blasts. Blasts, red; granulocytes, green; monocytes, blue; lymphocytes, cyan; hematogones, magenta; basophils, black; and yellow, erythroids.

and CD33 (1/21 MDSs); underexpression of CD33 (1/21 MDSs), CD45 (3/21 MDSs, 1/14 MPNs, and 1/7 CMMLs), and HLA-DR (2/14 MPNs); and partial loss of CD13 (2/14 MPNs), CD33 (1/14 MPNs), CD38 (1/21 MDSs), and CD117 (1/14 MPNs) **Image 6**. Neoplasia-specific immunophenotypic alterations are summarized in **Table 8** and **Table 9**.

All blasts in all neoplastic and nonneoplastic cases were CD34+.

Discussion

Several studies have described immunophenotypic alterations in myeloblasts in MDSs, but fewer studies characterize blast immunophenotypes in the other nonacute myeloid disorders, MPNs and MDS/MPNs. However, various limitations are evident in many previous studies. Some have used imprecise gating strategies, such as CD45/SS gating, that may result in inaccurate assignment of blast immunophenotypes due to gate contamination by other cell types.[2,5,7,8] In other studies, control groups were limited to normal donor BM samples or healthy volunteer BM samples, which may not capture the potential deviations of blast immunophenotypes in reactive states.[7,8,24] Still others provide little detail of the characteristics of their patient cohorts, clouding conclusions drawn about blast immunophenotypes.[1,3,8,12,13] Finally, the criteria applied to define immunophenotypic aberrancy are often poorly detailed and/or highly subjective.[1,2,5-7] These factors make it difficult to compare results across studies and to apply published findings to routine clinical practice.

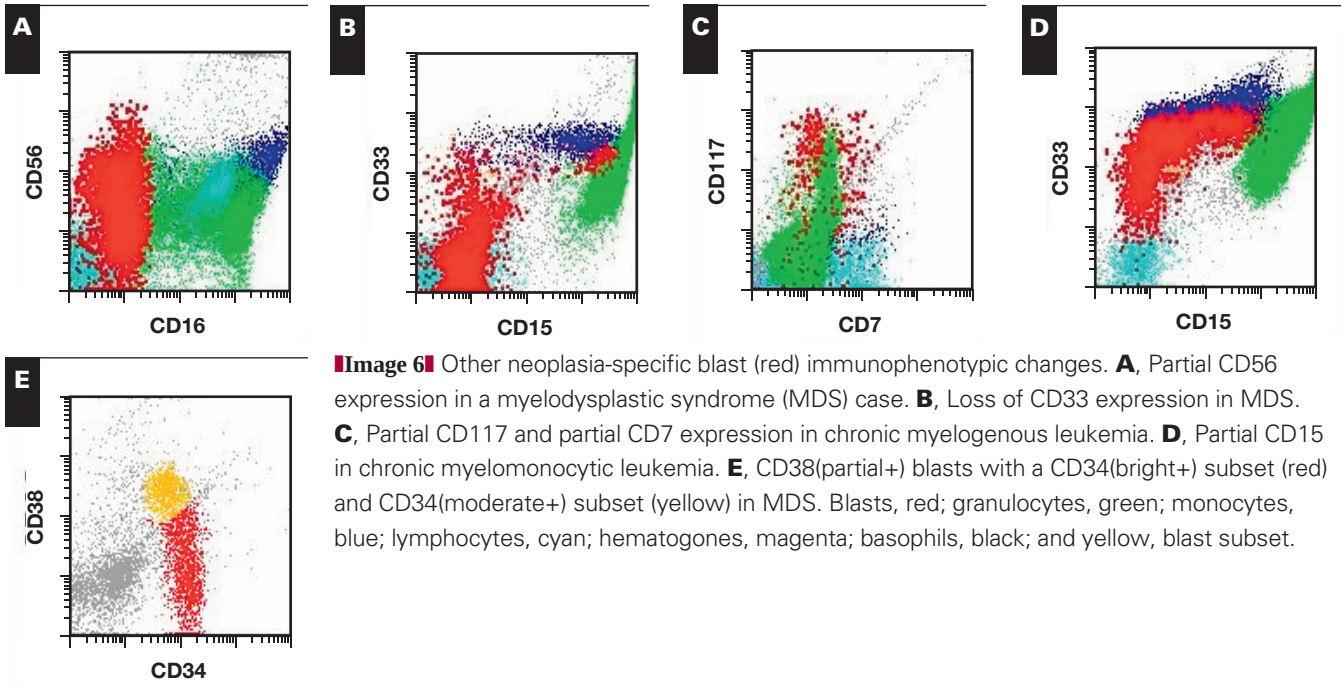

Image 6 Other neoplasia-specific blast (red) immunophenotypic changes. **A**, Partial CD56 expression in a myelodysplastic syndrome (MDS) case. **B**, Loss of CD33 expression in MDS. **C**, Partial CD117 and partial CD7 expression in chronic myelogenous leukemia. **D**, Partial CD15 in chronic myelomonocytic leukemia. **E**, CD38(partial+) blasts with a CD34(bright+) subset (red) and CD34(moderate+) subset (yellow) in MDS. Blasts, red; granulocytes, green; monocytes, blue; lymphocytes, cyan; hematogones, magenta; basophils, black; and yellow, blast subset.

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Table 8
Summary of Shared and Neoplasia-Specific Blast Immunophenotypic Changes

Shared blast immunophenotypic alterations
CD13(b+)
CD33(v+)
CD34(slightly b+)
CD38(v+)
CD117(b+)
CD117(dim+)
CD117(v+)
HLA-DR(b+)
Neoplasia-specific blast immunophenotypic alterations
CD7(p+)
CD11(b+)
CD13(p+)
CD13–
CD15(p+)
CD33(p+)
CD33–
CD34(b+)
CD34(v+)
CD36(p+)
CD38(p+)
CD45(dim+)
CD56(p+)
CD117(p+)
HLA-DR(dim+)
HLA-DR(v+)

b, bright; p, partial; v, variable.

Table 9
Summary of Neoplasia-Specific Immunophenotypic Alterations in MDS, MPN, and CMML

MDS
Expression of CD7, CD11b, and CD56
Overexpression of CD15 and CD34
Variable expression of HLA-DR
Underexpression of CD13, CD33, CD38, and CD45
Loss of CD33
MPN
Expression of CD7, CD36, and CD56
Variable expression of HLA-DR
Loss of CD13
Underexpression of CD45 and HLA-DR
Partial loss of CD13, CD33, and CD117
CMML
Expression of CD7 and CD56
Variable expression of HLA-DR
Overexpression of CD15
Underexpression of CD45

CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm.

Our purposes in this study were to characterize the spectrum of immunophenotypic alterations of blasts in nonacute myeloid disorders and to assess the specificity of these findings for myeloid neoplasia by using 4-color flow cytometry with a broad, uniform panel of antibodies. Our study differs from those in the literature in several respects. First, as already stated, we believe our "cluster analysis" technique is more robust than the often-used CD45/SS gating strategy. Our method uses carefully constructed antibody combinations designed to identify all relevant populations, including blasts, granulocytes, monocytes, hematogones, lymphocytes, erythroid precursors, and basophils. Based on an iterative analysis using various combinations of fluorescence parameters and light scatter properties, distinct clusters are identified in 6-dimensional flow cytometry "space" and assigned arbitrary colors. This method does not make assumptions about the immunophenotypic characteristics of populations, instead relying on the characteristics of populations to drive the analysis, and, thereby, accommodates potential deviations from normal in neoplastic populations. Because blasts account for only approximately 15% to 20% of events in a traditional CD45/SS "blast gate,"[17] studies that used this strategy may have assigned inaccurate blast immunophenotypes. For example, Ogata et al[8] described CD11b expression in approximately 50% of MDS blasts, a finding that has not been reproduced by other authors; most studies, including ours, have described very few MDS cases with CD11b+ blasts.[2,4,7] The discrepancy likely results from contamination of the blast gate with granulocytes, monocytes, and/or basophils or potential immunophenotypic changes resulting from the blast enrichment procedure used in their study.

One outcome of our current analysis was the confirmation that blasts in nonacute myeloid disorders (and in normal BM samples) are uniformly CD34+, in contrast with acute myeloid leukemias, in which the blasts may, in some cases, partially or completely lack CD34. While this has been our anecdotal experience, 2 reports in the literature identify low percentages of MDS cases with CD34– blasts.[2,8] Notably, both of these studies used CD45/SS gating as the sole gating strategy and, therefore, may have erroneously included nonblast populations in their analyses. Many of the more recent studies have used CD45/SS gating along with CD34 back-gating to examine blast immunophenotypes in MDSs, an approach that assumes all blasts in these disorders are CD34+.[1,4,6,7] Our results support this approach. However, given the relatively small number of neoplastic cases analyzed in our series, the finding of CD34 expression on all blasts in nonacute myeloid disorders requires further validation in larger series.

Because of the subjective nature of pattern assessment prevalent in existing literature, we sought to develop more objective means of assessing neoplasia-associated immunophenotypic features. For this reason, we chose to examine only blast immunophenotypes in this study to avoid the inherent difficulties in standardizing assessments of complex maturation patterns. This is a relatively unique approach because antigen expression on several myeloid populations has been more commonly examined. To assess abnormal levels of expression of antigens normally present on myeloblasts, blasts in neoplastic myeloid disorders were compared with blasts in a large set of nonneoplastic BM samples that were processed and analyzed in an identical manner. When applicable, antigen

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expression was also compared with other internal populations. Aberrancy was defined as at least a quarter log shift in antigen expression, providing an objective means of assessing such changes. To assess expression of antigens not normally present on myeloblasts (maturation-associated or cross-lineage), we rigorously compared with background fluorescence in blasts in an isotypic control tube in each case.

An important feature of our study design that differentiates it from much of the existing literature is that we have compared our findings in the neoplastic cases with 2 separate control groups: negative lymphoma staging BM samples (normal samples) and nonnormal, but nonneoplastic marrow states. This has allowed us to characterize alterations in blast immunophenotypes that appear to be neoplasia-specific, although these findings are certainly limited by our relatively small cohort. While much of the literature presents findings in relation to proposed scoring systems, we instead describe blast immunophenotypes that appear to be specific to neoplastic myeloid disorders. We believe that this approach is simpler to apply in practice. Notably, in this respect, we found that immunophenotypic alterations were present in blasts of half of the nonneoplastic cytosis/cytopenia BM samples, compared with the negative lymphoma staging BM samples. The most common abnormality, overexpression of CD117, was seen in 4 of 20 cytoses/cytopenias cases. Less common blast immunophenotypic alterations included variability in CD117 and CD33; underexpression of CD117; overexpression of CD13, CD34 (slight), and HLA-DR; and variability in CD38 expression, in descending order.

When compared with the negative lymphoma staging control samples, high percentages of MDSs (86%), MPNs (79%), and CMMLs (100%) demonstrated immunophenotypic alterations in blasts. However, when these cases were compared with the cytoses/cytopenias control group, approximately two thirds of the MDSs (62%) and roughly half of the MPNs (50%) and CMMLs (43%) demonstrated neoplasia-specific alterations in blast antigen expression. This finding emphasizes the need to use appropriate control groups when evaluating immunophenotypic alterations in cell populations.

While some studies in the flow cytometry and MDS literature have evaluated blasts in reactive/cytopenic control groups, the specificity of immunophenotypic alterations in the neoplastic myeloid processes is sometimes difficult to ascertain. In our study, neoplasia-specific immunophenotypic alterations in blasts included expression of CD11b, CD36, and the lymphoid-associated antigens CD7 and CD56; underexpression of CD33, CD45, and HLA-DR; lack of CD13 or CD33 expression; partial expression of CD38 and CD117; overexpression of CD15 and CD34(bright); and variable expression of CD34 and HLA-DR. The most common abnormalities observed in neoplastic blasts were CD7 expression, variable HLA-DR expression, bright CD34 expression, decreased CD45 expression, and CD56 expression, in descending order. Several studies have identified similar findings, including the expression of CD7, CD11b, and CD56; underexpression of CD33, CD45, and HLA-DR; partial expression of CD117; and overexpression of CD15 and CD34 on neoplastic blast populations mainly in MDSs.[2-8]

In our study, we describe several previously unreported or previously incompletely characterized neoplasia-specific blast findings, including the partial expression of CD36 in a CML case; partial expression of CD38 in an MDS (RAEB-2) case; and variable expression of HLA-DR in 9 cases. Caution must be used in ascribing diminished CD38 expression in blasts to neoplasia because the most immature myeloblasts are CD34(bright+) and CD38(dim), with increasing CD38 and decreasing CD34 expression seen with maturation.[16,21,25] However, the partial pattern of CD38 expression (a continuous spectrum from entirely negative to positive) in the RAEB-2 case was distinctly different from the variable positivity seen in normal and reactive blast populations (dominant bright positive with a "tail"). Kussick et al[3] described abnormalities in CD38 as a neoplasia-specific blast immunophenotype but did not provide additional details. Similarly, van de Loosdrecht et al[7] described "abnormal expression of HLA-DR" in blasts of patients with MDS and not in control subjects, but no further detail was provided on the specific expression patterns. In addition, this group compared its study cases with a set of healthy volunteers and, therefore, did not establish the specificity of the finding.[7] Finally, the description of CD36+ blasts in neoplastic cases may be unique to our study because this antigen has not often been examined in the literature. Interpretation of CD36 expression on blasts needs to be interpreted cautiously because platelets can adhere to or be coincident with leukocytes and cause misleading immunophenotypes. In our experience, platelet adherence/coincidence results in a pattern of partial or subset expression, with a very similar pattern seen in granulocytes (unpublished data). Thus, cases designated as CD36+ in our study were controlled by demonstrating lack of significant CD36 expression on granulocytes.

As previously discussed, we believe our approach of identifying neoplastic-specific blast immunophenotypes is easier to implement in daily practice, compared with proposed flow cytometric scoring systems. Identification of neoplasia-specific blast immunophenotypes (100% specificity) was 62%, 50%, and 43% sensitive for the diagnosis of MDSs, MPNs, and CMMLs, respectively, in our study. Similarly, Ogata et al[4] described 100% specificity and 59% sensitivity, for 3 or more blast immunophenotypic abnormalities in their MDS cohort, although their objective analysis was dependent on calculation of relative mean fluorescence intensities. In a study that examined a fairly comprehensive profile of antigen expression on blasts in

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addition to granulocytic and monocytic abnormalities, Wells et al[2] described similar findings, including 100% specificity and 55% sensitivity for a flow cytometric score of 3 or more abnormalities in MDSs. In contrast, Truong et al[5] found specificity and sensitivity values of 85% and 75%, respectively, for positive flow cytometric results in their recent study that examined abnormalities in blasts, granulocytes, and monocytes in 12 MDS/CMMLs and 61 nonneoplastic cytopenias. Positive flow cytometric results in that study were defined as discretely increased myeloblasts or aberrant lymphoid antigen expression on blasts or more than 5 maturing myelomonocytic marker abnormalities. Extrapolating from the data presented, the specificity and sensitivity change to 100% and 50%, respectively, in this study, when analysis is limited to blast immunophenotypic abnormalities. In a similar study by the same group, Stachurski et al[6] described specificity and sensitivity of 78% and 84%, respectively, for positive flow cytometric results in MDSs, examining blasts and maturing granulocytes and monocytes. Thus, overall, our approach offers a slightly better sensitivity (62%) than the proposed flow cytometric scoring systems with apparent 100% specificity for an MDS diagnosis, although this conclusion is based on a relatively small series of MDS cases.

Several of our results, specifically detection of expression of antigens not normally present on blasts (eg, CD7, CD11b, CD36, and CD56) should be easily reproducible in other laboratories, provided criteria for reactivity relative to appropriate negative control samples are rigorously applied. However, assessment of variations of expression of antigens normally present on blasts will require that individual laboratories establish their own baseline assessments of blast immunophenotype in nonneoplastic BM samples with their own panels and instrument settings and apply a quality assurance program to ensure consistent results over time. With that caveat, given the objective nature of the blast immunophenotypic changes we have described, it should be feasible to reproduce our results and apply them to routine practice.

In a consensus report published in 2007 on the diagnosis and treatment of MDSs, an international working group recognized abnormal erythroid and/or myeloid phenotypes, as determined by flow cytometry, as a criterion to support the diagnosis of myelodysplasia in the absence of definitive morphologic features (>10% dysplasia in ≥1 lineage), myelodysplasia-defining cytogenetic abnormalities, or elevated blast counts (≥5% blasts), given the appropriate clinical scenario of persistent, unexplained cytopenia(s).[26,27] The working group further recognized the lack of uniform technical and analysis protocols as currently limiting the usefulness of flow cytometry in this diagnosis. Our study was not designed to assess the added value of flow cytometry in the diagnosis of MDS because all cases were defined as such based on the "gold standard" of morphologic studies and/or cytogenetics. However, once certain changes have been established as neoplasia-specific, they would be expected to contribute to the diagnosis of cases that are diagnostically equivocal based on conventional criteria.[5,27,28]

We have presented a detailed analysis of the immunophenotypic alterations observed in blasts in a spectrum of nonacute myeloid disorders. We have described the wide overlap of immunophenotypic alterations that can exist between neoplastic and nonneoplastic cohorts and, more important, have identified neoplastic-specific immunophenotypic findings in blasts of these disorders. Finally, by using a thorough analysis approach unlike others reported in the MDS flow cytometry literature, we have confirmed in our cohorts that blasts in MDSs, MPNs, and CMMLs are uniformly CD34+, allowing a simplified diagnostic approach to blast characterization in the future.

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