Short Communication

Class II–Associated Invariant Chain Peptide Expression Represents a Novel Parameter for Flow Cytometric Detection of Acute Promyelocytic Leukemia

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Because of severe bleeding complications, patients with acute promyelocytic leukemia (APL) have to be treated with all-*trans* retinoic acid immediately following diagnosis. In addition to morphology, flow cytometry contributes to a rapid detection of APL according to phenotypic characteristics of leukemic cells. In some patients, these analyses are inconclusive or even contradictory to diagnosis. Previously, we showed the clinical and functional impact of class II–associated invariant chain peptide (CLIP) in acute myeloid leukemia (AML). This study focuses on the analysis of CLIP expression on leukemic cells to characterize HLA-DR–negative AML, including APL. We demonstrate exclusive and significant CLIP expression in all cases of typical and variant APL, as compared to other HLA-DR–negative non-APL-type AML. CLIP appears to be a highly sensitive and specific flow cytometric marker, resolving discrepant identification of both genetic subgroups. Our findings show the additive value of CLIP analysis for a fast and unequivocal recognition of APL by flow cytometry in conjunction with morphology. (*Am J Pathol* 2011, 179:2157–2161; DOI: 10.1016/j.ajpath.2011.07.027)

Acute promyelocytic leukemia (APL) is characterized by excessive proliferation of abnormal promyelocytes bearing t(15;17)(q22;q12), which results in the expression of the PML-RAR α fusion transcript.¹ Immediate recognition

of this acute myeloid leukemia (AML) subtype is of major importance, as it is associated with coagulation disturbances leading to early death²; rapid treatment with all-*trans* retinoic acid is required to control or prevent these severe complications and improve the prognosis of APL patients.^{3,4} At present, classical morphology showing bundles of Auer rods (ie, elongated aggregates of intracellular material found only in acute myeloid leukemia blasts) is the most commonly used, fast approach for detection before the confirmation of PML-RAR α gene rearrangement for diagnosis of APL. It can also assist in the classification of hypergranular (typical) and hypogranular (variant) subforms of APL, according to the World Health Organization criteria.⁵ In variant APL cases, the leukocyte count is usually increased and is associated with a worse clinical outcome, as compared to typical APL cases.^{6,7} In some patients and in less experienced hands, however, morphological examination is inconclusive and could lead to a delayed or even a false-negative or false-positive recognition of APL.

Another sensitive technique to identify APL is multi-color flow cytometry, which provides distinctive immunophenotypes of leukemic promyelocytes. For both typical and variant APL, bright and homogeneous expression of MPO, CD33, and CD117, heterogeneous expression of CD13, and low expression or absence of CD34, HLA-DR, CD11b, and CD15 are classically found.^{5,8} Still, there are examples of HLA-DR–negative non-APL-type patients that display a similar immunophenotype. CD56 expression has been described for APL, but only for ~20% of cases, with low specificity.^{9,10} Increased side scatter (SSC) is only a feature of typical APL,⁵ whereas the expression of CD2 is mostly restricted to variant APL.^{11,12}

HLA-DR is a molecule that plays a major role in HLA class II antigen presentation; in our previous studies regard-

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ing this issue in AML, APL patients were excluded because of their low or absent HLA-DR expression.^{13,14} One of the key mediators of HLA class II antigen presentation is the invariant chain. A small remnant of this protein, the class II-associated invariant chain peptide (CLIP), can be presented by HLA-DR complexes on the surface of leukemic blasts, resulting in decreased immunogenicity.¹⁵ Interestingly, in APL cases, we observed that CLIP was abundantly expressed at the surface of HLA-DR-negative leukemic promyelocytes. Here, we studied the application of the analysis of CLIP expression in the discrimination of HLA-DR-negative AML subgroups, including APL, by flow cytometry.

Materials and Methods

Patients

From a cohort of 297 patients who subsequently presented with AML between 2004 and 2010, 16 cases were diagnosed with APL (5.4%) and 23 with HLA-DR-negative non-

APL-type AML (7.7%). HLA-DR-negative non-APL-type AML patients were defined as cases with less than 20% of HLA-DR-expressing myeloid blasts. Blood and bone marrow samples were collected after obtaining informed consent as part of routine diagnostic procedures at our department and by the rules of our institute and the Helsinki Declaration. All patients were classified according to the World Health Organization 2001 criteria,⁵ and routinely screened for cytogenetics and molecular aberrancies, including t(15;17), PML-RAR α transcripts (bcr1/bcr3), and internal tandem duplication of the *FLT3* gene (*FLT3-ITD*). Cytogenetic risk groups were defined as favorable [t(8;21), t(15;17), or inv(16)], standard (neither favorable nor adverse), or adverse [complex karyotype, -5 or -7, del(5q), abnormality 3q or 11q23], as previously described.¹⁶

Flow Cytometry

The following monoclonal antibodies were used for immunophenotyping of AML cases: PE-conjugated c-MPO

Table 1. HLA-DR-Negative AML

Clinical parameters	APL*	Non-APL†	<i>P</i> value
Number of patients	16	23	
Male/female	6/10	13/10	
Age in years at diagnosis, mean	45 (21–68)	57 (22–75)	0.0079
WHO 2001 classification			
AML with recurrent genetic abnormalities			
AML with t(15;17)(q22;q12)	16 (100)	0	
AML with multilineage dysplasia	0 (0)	4 (17)	
AML not otherwise categorized			
AML without maturation	0 (0)	5 (22)	
AML with maturation	0 (0)	7 (30)	
Acute monoblastic and monocytic leukemia	0 (0)	1 (4)	
Acute megakaryoblastic leukemia	0 (0)	2 (9)	
AML of ambiguous lineage			
Biphenotypic acute leukemia	0 (0)	1 (3)	
AML (not classifiable)	0 (0)	3 (13)	
<i>FLT3-ITD</i>			
Normal	8 (50)	11 (48)	
Heterozygous	8 (50)	9 (39)	
Not done	0 (0)	3 (13)	
Cytogenetic risk group			
Favorable	16 (100)	0 (0)	
Standard	0 (0)	15 (65)	
Adverse	0 (0)	0 (0)	
Not applicable	0 (0)	8 (35)	
Immunophenotype, mean‡			
SSC (ratio)§	3.2 (1.6–4.5)	1.9 (1–3.5)	0.0003
CD34 (%)	27 (0–90)	26 (0–99)	0.3301
c-MPO (%)	94 (84–99)	76 (0–99)	0.6473
HLA-DR (%)	10 (0–44)	4 (0–17)	0.5191
CD13 (%)	89 (55–99)	61 (1–99)	0.1228
CD33 (%)	94 (37–99)	91 (33–99)	0.5826
CD117 (%)	74 (21–97)	75 (1–99)	0.5392
CD11b (%)	6 (1–33)	21 (0–86)	0.0024
CD15 (%)	3 (0–11)	10 (0–99)	0.8078
CD2 (%)	25 (0–78)	2 (0–25)	0.0005
CD56 (%)	10 (0–64)	38 (0–99)	0.0079
CXCR4 (%)	22 (1–97)	48 (8–100)	0.0033
CLIP (%)	90 (82–98)	8 (0–73)	<0.0001

Clinical parameters were not significantly different between groups unless otherwise indicated. *P* values in italics are significant.

*Classification according to morphology and presence of *PML-RAR α* (5).

†*PML-RAR α* -negative AML patients with less than 20% myeloid cells expressing HLA-DR.

‡Numbers in parentheses represent the percentage or range of each variable within the patient group.

§Median SSC ratio between immature myeloid cells and CD45^{bright} lymphocytes in the same sample.

AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; *FLT3-ITD*, internal tandem duplication of the *FLT3* gene; SSC, side scatter; WHO, World Health Organization.

Table 2. Typical and Variant APL

Clinical parameters	Typical APL	Variant APL*	<i>P</i> value
Number of patients	8	8	
Male/female	3/5	5/3	
Age in years at diagnosis, mean (range)	43 (21–65)	47 (37–68)	
t(15;17)	8	8	
PML-RAR α transcript			
bcr1	6 (75)	0 (0)	
bcr3	1 (13)	6 (75)	
Not done	1 (13)	2 (25)	
<i>FLT3-ITD</i> , heterozygous	0	8	
Immunophenotype, mean [†]			
SSC (ratio) [‡]	3.7 (2.6–4.5)	2.6 (1.6–3.4)	0.0281
CD34 (%)	5 (0–17)	48 (2–90)	0.0011
c-MPO (%)	94 (81–98)	95 (84–99)	0.3823
HLA-DR (%)	1 (0–5)	19 (1–44)	0.0030
CD13 (%)	88 (55–99)	89 (68–99)	0.9591
CD33 (%)	97 (94–99)	91 (37–99)	0.7984
CD117 (%)	82 (64–97)	74 (51–95)	0.5054
CD11b (%)	3 (1–5)	8 (2–33)	0.1949
CD15 (%)	4 (1–11)	2 (0–4)	0.3823
CD2 (%)	2 (0–5)	48 (10–68)	0.0002
CD56 (%)	2 (0–8)	19 (1–64)	0.0148
CXCR4 (%)	17 (1–60)	27 (4–97)	0.5728
CLIP (%)	89 (83–98)	91 (82–98)	0.3823

Genetic aberrancies were determined according to routine cytogenetics and molecular analyses. *P* values in italics are significant.

*Classification of APL subforms according to morphology.⁵

[†]Numbers in brackets represent the percentage or range of each variable within the patient group.

[‡]Median SSC ratio between immature myeloid cells and CD45^{bright} lymphocytes in the same sample.

APL, acute promyelocytic leukemia; bcr, breakpoint cluster region, an isoform of PML-RAR α ; *FLT3-ITD*, internal tandem duplication of the *FLT3* gene; PML, promyelocytic leukemia; RAR, retinoic acid receptor; SSC, side scatter.

(Dako, Glostrup, Denmark), CD13 (BD Biosciences, San Jose, CA), CD33 (BD Biosciences), CD117 (BD Biosciences), CD11b (BD Biosciences), CD56 (BD), anti-CXCR4 (12G5 clone; BD PharMingen, San Diego, CA), and anti-CLIP (cerCLIP.1 clone; Santa Cruz Biotechnology, Santa Cruz, CA); FITC-conjugated CD15 (BD Biosciences), CD11a (BD Biosciences), CD18 (BD Biosciences), CD2 (Dako), and anti-HLA-DR (L243; BD Biosciences); PerCP-conjugated CD45 (BD Biosciences); and APC-conjugated CD34 (BD Biosciences). Staining of cells was performed by following local standard operating protocols. Leukemic cells were analyzed by gating for the immature myeloid cell compartment, defined as CD45^{dim}/SSC^{low-int}; in this compartment, the percentage of marker-positive cells was assessed as compared to unstained cells. For comparison, we also investigated normal promyelocytes (SSC^{high}/CD34⁺/CD117⁺) from bone marrow aspirates of four different healthy donors. Expression data were acquired on a FACSCalibur flow cytometer and analyzed by using CellQuestPro software (both BD Biosciences).

Statistical Analysis

We compared the differences in marker expression between patient groups by the Mann-Whitney *U*-test. We regarded *P* ≤ 0.05 as significant.

Results and Discussion

The main clinical and immunophenotypic characteristics of APL and HLA-DR-negative non-APL-type AML pa-

tients are demonstrated in Table 1. All of the patients with APL harbored t(15;17)(q22;q12), whereas no rare variants such as t(11;17)(q23;q21) or t(5;17)(q35;q21) were encountered. None of the HLA-DR-negative non-APL cases had cytogenetic abnormalities. There were no significant differences in age, sex, or *FLT3-ITD* (Table 1). Strikingly, *FLT3-ITD* was present in all variant APL cases, and in none of the typical APL cases (Table 2). As expected, standard immunophenotypic analysis of leukemic cells in APL showed significantly increased SSC and CD2 expression, and decreased expression of CD11b and CD56, as compared to HLA-DR-negative non-APL-type AML (Table 1). Also, consistent with previous reports,^{6,11,17} we found a significantly low SSC and high expression of CD34, CD2, and CD56 in variant APL, in comparison with typical APL (Table 2).

In search of new immunophenotypic markers to distinguish APL from HLA-DR-negative non-APL-type AML, researchers recently described reduced CXCR4 expression on leukemic promyelocytes,¹⁸ which we were able to confirm in our patient cohort (*P* = 0.0042, Table 1; 69% sensitivity and 74% specificity). Furthermore, as previously shown,¹⁷ reduced expression of CD11a and CD18 was seen in our cohort of APL cases, as compared to non-APL cases (data not shown). Thus, decreased expression of certain markers is prevalently associated with APL. In contrast, we observed a significantly high expression of CLIP on all leukemic promyelocytes (both typical and variant subforms) when compared to HLA-DR-negative non-APL-type blasts (means of 90% ± 6% and 8% ± 17%, respectively; *P* < 0.0001, Table 1). Only in 2 of 23 HLA-DR-negative non-APL cases was an increased

Figure 1. The use of CLIP for discrimination of APL and HLA-DR-negative non-APL by flow cytometry. **A:** Overview of CD34, HLA-DR, and CLIP expression on leukemic cells from patients diagnosed with typical APL (APL, $n = 8$), variant APL (APLv, $n = 8$), or HLA-DR-negative non-APL-type AML (non-APL, $n = 23$). We assessed CD45^{dim}-gated cells using flow cytometry. Normal promyelocytes (defined as CD34⁺CD117⁺ cells and depicted as normal, $n = 4$) from healthy bone marrow were also analyzed. **B:** Representative examples of patients whose diagnosis is not conclusive according to standard flow cytometric analysis. Patients showed the following immunophenotypes: MPO⁺, CD13⁺, CD33⁺, CD117⁺, CD11b⁺, CD15⁺, CD2⁺, and CD56⁺. Dotplots are shown for the forward scatter (FCS) versus side scatter (SSC), and CD34 versus HLA-DR and CD34 versus CLIP expression. For each patient, the latter two plots are displayed for CD45^{dim}-gated cells. Final diagnosis of these patients is depicted and made according to morphology and analysis of the *PML-RAR α* gene. Note that patient 2 has increased SSC of leukemic cells, a characteristic more frequently observed in patients with typical APL.

CLIP expression of 44% and 73% seen; nevertheless, all APL cases revealed a CLIP expression of at least 82% (Figure 1A). The large mean difference in expression implicates a potential role of CLIP to separate APL from other HLA-DR-negative AML patients. Using CLIP as an additional marker, a correct classification of APL by flow cytometry was obtained for all cases (100% sensitivity and 95% specificity). Despite the significant differences in HLA-DR ($P = 0.0030$), CLIP expression was equally high between typical and variant APL subforms (Figure 1A). In control experiments, normal promyelocytes from

healthy donors revealed only very low surface CLIP expression (mean $3\% \pm 0.3\%$, $P = 0.0029$ as compared to APL, Figure 1A and data not shown). Normally, CLIP associates with HLA class II molecules, but we observed that in APL, the absence of HLA-DR was accompanied by a remarkable lack of all HLA class II isoforms ($n = 6$, data not shown). The underlying mechanism for this HLA class II-independent CLIP presentation is currently not understood and is under investigation.

To illustrate the additive value of CLIP for the flow cytometric recognition of APL, we will discuss three rep-

representative AML cases (Figure 1B). Leukemic cells of these cases revealed a similar expression pattern of routinely analyzed markers; ie, high levels of MPO, CD117, and CD33, heterogeneous levels of CD13, low levels of CD11b, CD15, CD2, and CD56 (data not shown), and absence of CD34 and HLA-DR (Figure 1B). The increased SSC in patients 1 and 2 reflecting granular blasts or granulocytic differentiation suggests typical APL for both cases. Because the lower SSC in patient 3 could also fit the immunophenotype of the variant APL form, this case might be diagnosed with either APL or HLA-DR-negative non-APL-type AML. According to cell morphological scrutiny, patient 1 was classified as typical APL, patient 2 as HLA-DR-negative non-APL, and patient 3 as variant APL, demonstrating a discrepancy between flow cytometric and morphological analysis for patients 2 and 3. After subsequent examination of the *PML-RAR α* gene and conventional cytogenetics, patient 1 was indeed diagnosed with typical APL, but patients 2 and 3 were negative for this translocation and thus regarded as HLA-DR-negative non-APL-type AML. Consistent with this, leukemic cells of patients 2 and 3 (non-APL) did not express CLIP in contrast to the high CLIP expression found for patient 1 (typical APL; Figure 1B). This shows that standard immunophenotyping and morphological examination can be misleading, but that incorporation of CLIP analysis can distinguish APL, including variant APL, from HLA-DR-negative AML.

Multicolor flow cytometry of surface markers on leukemic cells is considered one of the fastest and easiest-to-use techniques to identify genetic subgroups in patients with AML. For the identification of APL, progress has been made in intracellular analysis of *PML-RAR α* fusion proteins by flow cytometry,¹⁹ but it is not yet common practice. In the present study, we introduce cell surface CLIP expression (>80%) as a positive marker in case of HLA-DR-negative AML to characterize APL by the use of flow cytometric analysis alone. Irrespective of the requirement for molecular diagnostic testing, inclusion of this marker in present AML immunophenotyping protocols may attribute to rapid identification of APL, thereby shortening the time to treatment of APL patients.

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