Cutting Edge: Flow Cytometry in Myelodysplastic Syndromes

Arjan A. van de Loosdrecht, MD, PhD, and Theresia M. Westers, PhD

Abstract
Flow cytometry (FC) is recognized as one of the important tools in the diagnosis of myelodysplastic syndromes (MDS), especially when cytomorphology and cytogenetics are inconclusive. Minimal requirements to analyze dysplasia using FC have been proposed by the International Workshops from the European LeukemiaNet on Standardization of FC in MDS. These should enable a categorization of FC results in cytopenic patients as “normal,” “suggestive of,” or “indicative of” MDS. Notably, FC should be considered as part of an integrated diagnosis, rather than as a separate technique. This article highlights tools to implement FC in the diagnostic workup of MDS. Current advances in FC for diagnosis and prognosis of MDS are also introduced. (JNCCN 2013;11:892–902)

Morphology is not always clear-cut in the diagnosis of myelodysplastic syndromes (MDS).1 Hence, in clinical practice, cytogenetics is currently considered the most important parameter because the karyotype has diagnostic, prognostic, and therapeutic implications. However, a need exists for additional diagnostic markers, especially in cytopenic patients with normal or inconclusive cytogenetics. Flow cytometry (FC) can play a key role in this process. It has been introduced as an important co-criterion in the diagnosis of MDS and was recently identified as a recommended tool in the diagnostic workup of MDS if performed according to a standardized method as defined by the European LeukemiaNet (ELNet)2–4 (L. Malcovati, MD, unpublished data, June 2013). Analysis of bone marrow cells with FC can identify specific aberrations in both immature and maturing compartments among different hematopoietic lineages. Current WHO 2008 recommendations recognize the presence of 3 or more FC aberrancies as indicative of MDS.3 Moreover, recent guidelines recommend FC analysis in the diagnostic workup of MDS (L. Malcovati, MD, unpublished data, June 2013).

Reports from the ELNet workshops on the standardization of FC in MDS provide recommendations for implementing FC and propose minimal criteria for the diagnosis and prognostic evaluation of MDS and other cytopenias.3,4 This article summarizes current concepts and guidelines, and introduces recent advances.

Initial Screening for MDS With FC
Because MDS comprises a heterogeneous group of myeloid neoplasms, it is unlikely that a single specific marker can discriminate MDS from other cytopenias. Therefore, the presence of multiple aberrancies has a higher predictive value for MDS than single aberrancies.6–9 As shown in a multicenter study on behalf of the ELNet, application of only 4 parameters enables low-risk MDS without other specific markers (ie, cytogenetics or ring sideroblasts) to be distinguished from nonclonal cytopenias.10 The cardinal parameters within this score are

1. An increased percentage of CD34+ progenitor cells of nucleated cells in a bone marrow sample (≥2%)
2. A decreased number of progenitor B cells within the CD34+ compartment (≤5%)
3. A decreased or increased CD45 expression on myeloid progenitor cells compared with that of reference lymphocytes (≤4.0 or ≥7.5)
4. A decreased sideward light scatter (SSC) of neutrophils compared with that of reference lymphocytes (≤6.0)

From the Department of Hematology, Cancer Center Amsterdam, VU University Medical Center, Amsterdam, the Netherlands. Submitted January 23, 2013; accepted for publication April 29, 2013.

The authors have disclosed that they have no financial interests, arrangements, affiliations, or commercial interests with the manufacturers of any products discussed in this article or their competitors.

Correspondence: Arjan A. van de Loosdrecht, MD, PhD, Department of Hematology, Cancer Center Amsterdam, VU University Medical Center, De Boelelaan 1117, 1081 HV, Amsterdam, the Netherlands. E-mail: a.vandeloosdrecht@vumc.nl
Displayed reference ranges were determined from a large cohort of patients with nonclonal cytopenia. In assessing whether data of a particular patient were beyond the reference range, the presence of 2 or more of these specific aberrancies identified 70% of patients with low-risk MDS with a specificity of 93%. This simple diagnostic score is a start; however, for reliable clinical application in a single patient, its sensitivity may need improvement.

One pitfall of this score is hemodilution that may cause underestimation of the progenitor compartment. Moreover, a relative decrease in B-cell progenitors is also seen in the elderly population without MDS, and can result from an increase in myeloid progenitor cells, especially in high-risk MDS. Furthermore, myeloid progenitors may be aberrantly CD34−. Reference ranges for CD45 and SSC in a particular center may differ slightly because of differences in fluorochromes and instrument settings.

### Additional Means to Assess Dysplasia With FC

Assessment of an accumulation of FC aberrancies beyond the 4 cardinal parameters might strongly enhance sensitivity, and hence support the diagnosis of MDS. The accumulation of FC aberrancies is reflected by the FC scoring system. An overview of these parameters is given in the following paragraphs and assists in clinical reporting (Tables 1 and 2). For example, discrimination of normal and abnormal myeloid progenitor cells can be assessed through evaluation of several markers such as CD34, CD117, HLA-DR, and lineage infidelity markers. Simultaneous analysis of the maturing myelomonocytic compartment adds valuable information.

Additionally, analysis of erythroid differentiation might provide valuable information, particularly within low-risk MDS. Table 3 summarizes current agreement on the FC parameters that are considered most relevant for diagnosing MDS.

Few applications are available for FC analysis of the megakaryocytic lineage. A recent publication introduces the analysis of platelets in peripheral blood as a reliable substitute. Validation of this procedure is currently ongoing. Standard morphology and immunohistochemistry are therefore still recommended for analyzing dysplasia in this cell lineage.

### Recommendations for Evaluation of Dysplasia in the Immature and Maturing Myelomonocytic Compartment

Recommendations on technical issues, such as sample preparation, instrument setup and quality assessment, acquisition of data (eg, number of events), and gating strategy, are extensively described in the ELNet reports. No standard panels are available within the ELNet working group at the moment.

Evaluation of dysplasia necessitates knowledge of expression levels in appropriate bone marrow controls. Bone marrow aspirates of age-matched healthy volunteers and other nonclonal cytopenic patients should be available. For standardization of analysis and interpretation of results between patients and controls, a standardized gating strategy, color code for subpopulations, and synchronization of dot-plot display (identical x and y axes orientation for same antibodies) are recommended.

### Evaluation of Dysplasia in the Immature Myeloid Progenitor Compartment

The combination of CD45 and SSC provides a first means to identify progenitor cells (diminished CD45 [CD45^dim] and low/intermediate SSC [SSC^low/int]; Figure 1). This immature cell compartment is very heterogeneous; therefore, antibody combinations such as CD45/CD34/CD117/HLA-DR and CD45/CD34/CD123/HLA-DR are instrumental in distinguishing myeloid progenitor cells from other populations (eg, B-cell precursors, plasma cells, monoblasts, basophils, erythroblasts, and plasmacytoid dendritic cell precursors) that might show overlapping CD45 and SSC features. Granular progenitors or degranulated neutrophils may overlap and should be defined accordingly (Figure 1). Antibody combinations, including markers such as CD15, CD24, CD10, CD11b, and HLA-DR, can be useful in this respect.

In low-risk MDS in particular, percentages of progenitor cells are low. However, dysplastic immature myeloid progenitors in MDS may have an aberrant immunophenotype that distinguishes them from normal progenitors. Some of the observed aberrancies in the immature myeloid compartment in MDS are an abnormal intensity or lack of expression of CD45, CD34, CD117, HLA-DR, CD13, and CD33; asynchronous presence of CD11b; and/or the expression of...
lineage infidelity markers such as CD5, CD7, or CD56 (extended list in Table 3). An example of aberrant homogeneously high expression of CD117 is depicted in Figures 2 and 3. These markers should be analyzed not only on CD34\(^+\) progenitor cells but also among CD117\(^+\)/CD34\(^-\) precursors, because CD34 may be aberrantly lost.

Note that several markers, such as CD7 and CD56, can be expressed on a small subset of normal immature myeloid progenitor cells, especially in recovering hematopoiesis. This requires knowledge of expression levels in appropriate bone marrow controls.

### Evaluation of Dysplasia in the Neutrophil Compartment

The combination of CD45 and SSC is regularly applied to identify maturing neutrophils by FC (CD45\(^\text{int}\)/SSC\(^\text{int-bright}\)). CD33, CD64, HLA-DR, and CD11b are useful in distinguishing monocytes and hypogranular neutrophils. Neutrophils display diminished CD33 and CD64 expression, are heterogeneous to bright CD11b, and mostly lack HLA-DR compared with CD33\(^{\text{bright}}\), HLA-DR\(^-\), and CD11b\(^+\) monocytes (Figure 2).

The number of maturing neutrophils partly reflects the ability of the progenitor compartment to proliferate and differentiate, thereby supporting successful hematopoiesis. A decreased amount of neutrophils might indicate disturbed granulopoiesis. One of the most frequently reported FC aberrancies in the maturing neutrophil compartment is an abnormally decreased SSC reflecting hypogranularity. The SSC of maturing neutrophils should be expressed as a ratio relative to that obtained for lymphocytes as an internal reference.

Next to decreased SSC, dysplastic neutrophils can display increased or decreased expression of antigens or an aberrant relationship among 2 or more antigens, such as aberrant relationships between CD13 and CD11b and/or CD13 and CD16 and between CD15 and CD10 (Table 3, Figure 2). Additional dysplastic features of neutrophils are summarized in Table 3.

### Abbreviations
- FC, flow cytometry
- SSC, sideward light scatter
- MDS, myelodysplastic syndromes

### Table 1 Translation of Flow Cytometric Results for Clinic Reports

<table>
<thead>
<tr>
<th>Diagnostic flow score</th>
<th>&lt;2</th>
<th>&lt;2</th>
<th>&lt;2</th>
<th>≥2</th>
<th>≥2</th>
<th>≥2</th>
<th>≥2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysplasia by FC myeloid progenitors</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Dysplasia by FC</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>• Neutrophils (SSC or ≥2 other aberrancies)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Monocytes (CD56 or ≥2 other aberrancies)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Conclusion

**A**

FC results show no signs of MDS. On sustained suspicion of MDS, analysis is to be repeated in 3–6 months, especially when clinical features, such as cytopenias, worsen. Note: FC results do not yet include analysis of erythropoiesis and dysmegakaryopoiesis, and therefore MDS cannot be excluded. If FC results are contradictory with other results, the authors recommend repeating the analysis in due time.

**B**

FC results show signs of myelodysplasia. If FC results are contradictory with other diagnostic parameters, the authors recommend repeating the analysis in due time. Note: If contradictory with other results, the authors recommend repeating the analysis in 6 months or at any moment if cytopenia worsen or suspicion of MDS sustains.

**C**

FC results may fit MDS. These results should always be interpreted in the context of other diagnostic parameters; if contradictory, the authors recommend repeating the analysis in 6 months.

### Abbreviations
- FC, flow cytometry
- MDS, myelodysplastic syndromes
Note that because of genetic polymorphisms, some markers such as CD16 or CD33 may show no-naberrantly low expression levels. Furthermore, decreased expression of CD16 and CD11b has been reported to coincide with apoptosis. Assessment of the CD11b versus CD16 pattern next to that of CD16 versus CD13 can be helpful in this respect. Moreover, loss of glycosyl-phosphatidyl-inositol (GPI)–associated cell surface proteins, such as paroxysmal nocturnal hemoglobinuria (PNH), can cause loss of CD16 expression.4,25,29–33 A neutrophil-associated marker recently introduced as potentially indicative of MDS is the intracellular myeloid nuclear–derived antigen (MNDA), which displays a marked down regulation in MDS. Interestingly, the combined use of standard FC and MNDA improved sensitivity of correct identification of MDS by FC from 78% to 91% (specificity decreased from 91%–81%).34,35

### Evaluation of Dysplasia in the Monocytic Compartment

Morphologic assessment of dysmonopoiesis in MDS is difficult unless marked monocytosis is present. In FC, monocytes can be defined based on their CD45 expression (intermediate-bright22), SSC (intermediate), and useful additional markers such as HLA-DR, CD11b, CD64, CD36, CD33, and CD14. The use of CD14 alone may underestimate the percentage of monocytic cells, particularly when immature forms are present.
Notably, CD14 recognizes a GPI-anchored protein that can be absent because of coexistence of PNH.

Maturing neutrophils with an abnormally low SSC can interfere in the analysis of the monocytic population. The previous paragraph describes how these subpopulations might be separated. Aberrancies of interest in the monocytic lineage are the proportion of an abnormal distribution of maturation stages, abnormal relationships of HLA-DR versus CD11b, abnormal intensity of CD13, and overexpression of CD56 (recommended aberrancies in Table 3).

CD56 (and CD2) may even contribute to discriminate chronic myelomonocytic leukemia from MDS/myeloproliferative neoplasms. A caveat is that CD56 expression is frequently seen on hematopoiesis regeneration, activation, and inflammatory responses, and therefore the clinical context is of utmost importance. Activation often coincides with upregulation of HLA-DR and CD64.

**Evaluation of Dysplasia in the Erythroid Compartment**

The erythroid population can be defined by its very dim to negative CD45 expression and low FSC and SSC properties. Commonly applied markers are CD45, CD71, CD235a, CD36, CD117, and less frequently the endoglin marker CD105. One of the most frequently observed aberrancies (80% of low-risk MDS) is an increased number of erythroid progenitors associated with a larger proportion of immature erythroid cells (CD117+). Otherwise, a decrease in erythroid progenitors from apoptosis or erythropoietin deficiency can be observed. In approximately 70% of the patients, asynchronous expression of CD71 versus CD235a and/or decreased CD36 have been reported.

Examples are displayed in Figure 4. Note that the nucleated erythroid cells are generally studied after lysis of mature erythrocytes with ammonium chloride; this procedure may affect features of nucleated erythroid cells that may falsely be assigned to dysplasia.
Figure 2  Expression patterns of several antigens in the immature and maturing myelomonocytic compartment. The graphs in the upper row show forward light scatter/side light scatter (FSC/SSC) profiles of 3 bone marrow control samples and 2 myelodysplastic syndromes (MDS) cases (unique patient number 1 [UPN1], UPN2, and UPN3, respectively). Same cases are shown in Figure 1. (A) FSC is depicted on x axes and SSC on y axes. (B–D) Only the progenitor compartment (myeloid and if applicable B cell progenitors in dark blue and pale blue, respectively), maturing neutrophils (pink), and monocytes (orange) are displayed. (B) CD34 versus CD117 profiles. (C) HLA-DR versus CD117 profiles. (D) HLA-DR versus CD11b profiles. (E) CD16 versus CD13 profiles. (B) Myeloid progenitor (dark blue) differentiation toward neutrophils shows retention of CD117 while CD34 is rapidly lost, then cells become CD34− and CD117−. Monocytic differentiation is characterized by simultaneous loss of CD34 and CD117 toward double-negative cells. CD34+/CD117− progenitors (in pale blue) are lacking in both MDS cases (UPN2 and UPN3). (C) Myeloid progenitor differentiation is characterized by a decrease in HLA-DR followed by loss of CD117. On monocytic differentiation, CD117 decreases while HLA-DR is increased; then CD117 is lost, and monocytes remain HLA-DR+. (D) The clockwise differentiation of myeloid progenitors toward neutrophils is demonstrated: first HLA-DR is lost, then CD11b is gained; monocytes differentiate anticlockwise. HLA-DR is slightly increased on gain of CD11b. Mature monocytes have slightly lower HLA-DR expression than their precursors. (E) Finally, the characteristic pattern of neutrophil differentiation is displayed in CD16 versus CD13 plots. CD13 increases on differentiation of progenitor cells toward the most immature granulocytes, then CD13 declines and inclines again on further maturation. Monocytes have CD13 expression comparable to most immature granulocytes; a subpopulation of monocytes can express CD16. Aberrancies in UPN2 are: the absence of progenitor B cells; CD117 is partly increased but does not reach 0.5 log (Figure 3); neutrophil SSC is decreased; aberrant neutrophil CD16 versus CD13; and monocytes show increase in HLA-DR, CD16, and CD13. Aberrancies in UPN3 are: the absence of progenitor B cells; CD13 is absent on progenitor cells; neutrophil SSC is decreased; aberrant neutrophil CD16 versus CD13; and monocytes are increased in percentage and show decrease of CD11b and partly lack CD13. FSC/SSC profiles are in linear scale; antibody expression levels in log scale.
Another well-known feature of erythroid dysplasia is the presence of ring sideroblasts. These cells are definitely of diagnostic relevance; currently, however, FC has no additional value for quantifying ring sideroblasts.

**FC Within an Integrated Diagnostic Report**

WHO 2008 recommendations recognize the presence of 3 or more FC aberrancies in maturation patterns as indicative of MDS. Furthermore, increased percentages of progenitor cells and aberrant marker expression indicate dysplasia. In line with this, it is recommended that the results of analysis of dysplasia with FC be reported in a descriptive manner. A report should contain the following:

1. Percentage of myeloid progenitors, including description of aberrancies when present
2. Absence or presence of dysgranulopoiesis (either decreased SSC and/or 2 other aberrancies)
3. Absence or presence of dysmonocytopenia (either CD56 expression and/or 2 other aberrancies)

These results are translated into a conclusion that elucidates whether the immunophenotypic profile is “A: not-fitting,” “B: suggestive of,” or “C: indicative of” MDS taking into account the diagnostic score based on the 4 cardinal parameters (Table 1). Note that FC results should be part of an integrated diagnostic approach, including cytology, bone marrow immunohistochemistry, cytogenetics, and fluorescence in situ hybridization analysis in selected cases (Table 4).

**Current Flow Cytometric Scoring Systems in the Diagnosis of MDS**

Current guidelines were developed when 4-color FC was the mainstay. Nowadays, many institutes apply 6- to 8-color FC. Recommended antibody combinations remain similar; however, some can be combined, thereby revealing new information on relationships between different cellular compartments (Figure 2). Despite this, it remains a struggle to dissect MDS from other conditions with cytopenia and normal karyotype in some cases. Numerous FC

---

**Figure 3** Application of coefficient of variation (CV) to evaluate homogeneity or heterogeneity of marker expression. Aberrant homogeneity or heterogeneity of antigen expression can be analyzed easily through applying CV values of that particular antigen compared with reference ranges in a control group. For instance, CD117 expression can be aberrantly homogeneous; CV below the determined reference range of CD117 indicates an aberrant homogeneous CD117 expression. (A) Histograms showing some examples of CD117 expression (control: unique patient number 1 [UPN1]; myelodysplastic syndromes cases: UPN2 and UPN3). Conclusions on aberrant homogeneity in myeloid progenitors (dark blue line) in UPN2 and UPN3 cannot be drawn from one single reference (UPN1). Arrows indicate the width of the histogram in UPN1; green vertical lines show the expression of the particular antigen in control UPN1. (B) Examples for CD13 in monocytes. Histograms for CD71 in the erythroid lineage are depicted in Figure 4.
markers are being explored by several centers. These markers are used in numerous flow scoring systems (partly summarized by Westers et al. and Tang et al.). Multicenter analyses of retrospective data sets should elucidate the most important markers and their combinations in multiparameter analyses. The only scoring systems currently applied by multiple centers are the 4-parameter diagnostic score by Della Porta et al. and the more extensive multiple-parameter FC scoring system designed by Wells et al.6

**FC and Prognosis of MDS**

WHO classification, International Prognostic Scoring System (IPSS and IPSS-Revised [IPSS-R]), and WHO-based Prognostic Scoring System (WPSS) are all well validated and applied routinely in clinical practice.5-44 Thus far, the only validated prognostic FC score is the FC scoring system by Wells et al.8,9,27,45 This score weighs the percentage of progenitor cells and their aberrancies and the amount of aberrancies in the maturing compartment. The flow scores are rather heterogeneous within the subgroups or risk groups of the WHO, IPSS-R, and WPSS. This suggests that FC can identify patients with worse prognosis within validated risk categories; this concept is currently under evaluation.

**FC in Predicting and Monitoring Treatment Response**

Furthermore, FC analysis of bone marrow cells in low and intermediate-1 (int-1) risk MDS is instrumental to identify subgroups with distinct clinical behavior regarding transfusion dependency and progression. A clinical decision model was designed using the presence of aberrant myeloid progenitors determined by FC in the bone marrow in combination with endogenous erythropoietin levels to predict response to growth factor treatment. Patients with low and int-1 risk MDS with normal myeloid progenitors and low endogenous erythropoietin have the highest probability of responding to growth factor treatment.46 In addition, aberrant marker expression on myeloid progenitors cells by FC identifies patients who may not benefit from treatment with hypomethylating agents such as

---

**Figure 4** Expression patterns of erythroid antigens in the myeloid progenitor and erythroid compartment. (A) Histograms showing examples of CD71 expression (control: unique patient number 1 [UPN1]; myelodysplastic syndromes [MDS] cases: UPN2 and UPN3); arrows indicate the width of the histogram in UPN1. (B) Profiles of CD71 versus CD235a (glycophorin A) expression in the erythroid and myeloid progenitors (red and blue populations, respectively). An increased amount of erythroid cells is apparent in both MDS cases; CD71 expression is mildly heterogeneous but cannot be compared with UPN1 alone. Comparison with reference ranges in a control group will elucidate this matter.
as azacitidine in int-2 and high-risk MDS (C. Alhan, MD, unpublished data, 2013). Studies are currently ongoing to implement FC by intensive monitoring in selecting patients who may benefit from prolonged treatment with azacitidine.

**Conclusions**

In MDS, FC is regarded as a new key player, and is therefore a recommended tool for diagnosing MDS if performed according to the ELNet guidelines. However, it should only be applied as a part of an integrated diagnostic approach. Repeated FC assessments are highly recommended, not only in inconclusive cases but also as part of new prognostic scoring systems. Finally, current investigations focus on the role of FC in monitoring the disease course in patients with untreated, mainly lower-risk MDS, and during treatment with emerging new drugs to identify patients who may benefit from prolonged treatment of established and new drugs in low- and high-risk MDS.

**Acknowledgments**

The authors would like to thank all participants of the ELNet and Dutch MDS FC working groups for providing input in the development of the ELNet guidelines for the assessments of FC in MDS.

**References**


