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CONCISE REVIEW

Revisiting guidelines for integration of flow cytometry results in the WHO classification of myelodysplastic syndromes—proposal from the International/European LeukemiaNet Working Group for Flow Cytometry in MDS

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Definite progress has been made in the exploration of myelodysplastic syndromes (MDS) by flow cytometry (FCM) since the publication of the World Health Organization 2008 classification of myeloid neoplasms. An international working party initiated within the European LeukemiaNet and extended to include members from Australia, Canada, Japan, Taiwan and the United States has, through several workshops, developed and subsequently published consensus recommendations. The latter deal with preanalytical precautions, and propose small and large panels, which allow evaluating immunophenotypic anomalies and calculating myelodysplasia scores. The current paper provides guidelines that strongly recommend the integration of FCM data with other diagnostic tools in the diagnostic work-up of MDS.

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INTRODUCTION

Since the publication of the World Health Organization (WHO) 2008 classification of myeloid neoplasms, multiple studies have corroborated the utility of flow cytometry (FCM) in the diagnostics and prognostication of adult patients with myelodysplastic syndromes (MDS), showing that FCM often is more sensitive than morphology in detecting dysplasia, especially of the myeloid lineage.[1–9] In a large cohort of 1013 patients evaluated by cytology, FCM and cytogenetics, 6.4% of all patients had FCM results that were in agreement with MDS without a clear diagnosis of MDS by cytology, and in 33% of those patients MDS diagnosis was corroborated by an aberrant karyotype.[1] Moreover, some studies have indicated that FCM can be successfully applied in individual risk assessment, choice of therapy and monitoring in adult MDS patients.[2,10–15] At present, clinical FCM laboratories all over the world apply various platforms, combinations of three to ten fluorochromes, and different analysis software.[16–18] The number of applied antibodies and/or fluorochromes may be limited by technical and economic constraints. Although standardization of FCM for hematological malignancies has been advocated,[16] the full standardization of the FCM methodology for MDS diagnosis and follow-up is not easily achievable and might not be possible in the near future. This is due to the fact that different FCM systems are applied in laboratories together with various types of analysis software, and also to different economic realities in different countries. However, with a better knowledge of normal bone marrow (BM) FCM features, it should be possible to harmonize the FCM methodology to the point that immunophenotyping results obtained in various laboratories could become a standard part of the integrated MDS diagnosis. Such an integrated diagnostic approach should include cytomorphological assessment of blood and BM smears, BM biopsy histopathology and immunohistochemistry, FCM analysis, cytogenetic analyses including standard karyotype and FISH, as well as molecular tests that search for mutations and single-nucleotide polymorphism that could allow for patients' stratification in clinical trials.[17–20]

This paper presents guidelines for a harmonized application of FCM in integrated MDS diagnostics, developed by the International/European LeukemiaNet (ELN) Working Group for Flow Cytometry in MDS (IMDSFlow) at a Workshop in Munich, 1–2 November 2013.

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HISTORICAL OVERVIEW

A number of previous studies have shown that FCM is a valuable approach to define immunophenotypic abnormalities in patients with MDS (reviewed in Bene,[21] Porwit,[22] and van de Loosdrecht and Westers[23]). Therefore, FCM has been proposed as a part of the diagnostic approach by a consensus group in 2007.[24] However, at the time of publication of the WHO 2008 classification of myeloid neoplasms, FCM procedures had not been fully developed for clinical use in the diagnosis of MDS. Therefore, FCM has not been recommended as a routine diagnostic procedure. It was stated that in cases where three or more immunophenotypic abnormalities are found, involving one or more of the myeloid lineages, the aberrant findings can be considered as suggestive of MDS. In the absence of conclusive morphologic and/or cytogenetic features, FCM abnormalities alone were not deemed sufficient to establish MDS diagnosis, and further follow-up of the patients with repeated BM studies was recommended until the morphological and/or cytogenetic criteria could be fulfilled.[25] Recent ELN recommendations for diagnosis and treatment of primary MDS in adults included FCM as a recommended diagnostic procedure[26] if performed according to published ELN guidelines.[27,28] However, it has also been mentioned that further standardization/validation in prospective multicenter studies is required.[28] Other clinical guidelines do not provide uniform recommendations for FCM use in the diagnostic work-up of MDS. The US National Comprehensive Cancer Network in 2011 recommended FCM mainly for estimation of the percentage of $CD34^+$ cells, paroxysmal nocturnal hemoglobinuria clones and presence of large granular lymphocytes, but warned against evaluating the percentage of blasts by FCM, which evaluates CD34+ progenitor cells, not cells with the typical morphologic features of blasts.[29] The 2013 National Comprehensive Cancer Network guidelines update included FCM as a useful adjunct procedure for diagnosing MDS in difficult cases.[30] The recent 2014 Continuous Education series publication in the *American Journal of Hematology* states that FCM can help in the identification of abnormal phenotypic patterns and in diagnosis of cases with minimal dysplasia.[31]

Standardization efforts concerning FCM diagnostics in MDS were started under the auspices of ELN's Work Packages (WP) 8 (MDS) and 10 (Diagnostics) (www.leukemia-net.org). The IMDSFlow consists of ~30 participants from 26 institutes (21 participating in ELN, and representatives from Australia, Canada, Japan, Taiwan and USA (Supplementary Appendix). The IMDSFlow published guidelines concerning the recommended methods for cell sampling, handling and processing.[27] Subsequently, a minimum consensus panel of antibody combinations was published, aimed at providing effective immunophenotypic characterization of different cell subsets, cell maturation disorders, lineage infidelity and the presence of aberrant progenitor cells.[28] A rationale for clinical applications of FCM in MDS patients has been described in detail.[32] The group has also published a multicenter study, in which such a minimum screening panel for MDS-related features was successfully applied in various laboratories using different FCM platforms.[33]

PROPOSED GUIDELINES FOR INTEGRATION OF FCM IN THE WHO CLASSIFICATION OF MDS

1. While developing an FCM application for MDS diagnostics, it is recommended that the recently published general guidelines for FCM test development[34] and pre-analytical recommendations of IMDSFlow[27] be applied.
2. For screening purposes, a mini-panel based on the so-called 'Ogata score' can be applied. This mini-panel, which could also prove useful in settings with limited resources, includes four parameters: percentage of $CD34^+$ myeloid progenitor cells in BM, frequency of B-cell-related precursors within the $CD34^+$ subset, CD45 expression on myeloid progenitors by comparison to lymphocytes and orthogonal light scatter (side scatter, SSC) of granulocytes by comparison to lymphocytes.[9] High scores are associated with a high probability of MDS. However, it has to be pointed out that high scores can sometimes be seen in reactive conditions and that some MDS BM samples can have low FCM scores with this screening panel. These limitations in specificity and sensitivity have to be taken into consideration in the interpretation of results and in the integration of limited FCM testing in the final diagnosis. For BM samples from patients where clinical data provide strong suspicion of MDS, more comprehensive panels are recommended. Moreover, due to hypocellular BM samples the 'Ogata-score' was difficult to apply in samples with suspicion of pediatric MDS.[35]
3. In laboratories where comprehensive immunophenotyping can be performed, an MDS immunophenotyping panel including the parameters listed in Table 1 is recommended. Some of the characteristic aberrant patterns are illustrated in Figure 1. Panels should include antibody combinations allowing the evaluation of all listed features and all listed cell compartments. Aberrant findings in at least three tested features comprising at least two cell compartments have been reported to be highly associated with MDS or MDS/MPN diagnosis in several studies.[1,13,36,37] Thus, we recommend application of this definition to determine aberrant FCM results. High numbers of FCM abnormalities in MDS have been associated with cytogenetic abnormalities, transfusion dependency, progressive disease and risk of transformation to acute myeloid leukemia (AML).[1,2,22] Moreover, high FCM scores in MDS patients have been related to shorter overall survival and worse outcome after stem cell transplant.[38,39]
4. Reporting FCM findings in MDS should be done in an integrated diagnostic report, together with morphological, cytogenetic and/or molecular findings. Integration can be performed either in one report or through multidisciplinary conferences. If the FCM analysis is part of an integrated report, an interpretative comment stating whether the results are consistent with MDS, shows limited number of changes seen in MDS or does not show MDS-related features should be added.[23] If the FCM report is released independently of other diagnostic reports, it should be descriptive and final conclusions regarding MDS diagnosis should be avoided. In various published reports, scoring of FCM myeloid aberrancies has been reported with a sensitivity for MDS diagnosis between 59 and 98% and a specificity of 93–100%.[1,2,33,39–42] As is the case for morphology and cytogenetics, there is only a limited negative predictive value when FCM is used as a sole diagnostic technique. Any of the diagnostic techniques may fail to show informative results in individual patients, because of the heterogeneity of MDS. Indeed, approximately half of the MDS patients have normal cytogenetics.[43] Moreover, MDS-related cytogenetic findings are found in ~10% of patients with minimal dysplasia by cytological evaluation of BM smears, that is, dysplasia, which would not be sufficient for an unequivocal MDS diagnosis according to the WHO criteria.[1] Minimal dysplasia is also a characteristic feature of a subset of patients with del(20 q) MDS.[44] As stated above, the diagnosis should be an integrated process. However, adding FCM to morphology in the minimal samples obtained for instance after dry taps can support a diagnosis of MDS or suggest close clinical follow-up and repeated tests at a later time. Since MDS-related FCM changes can also be detected in peripheral blood, FCM can provide valuable information for morphologically uninformative samples.[45]

Table 1. Aberrant FCM features to be included in the diagnostic work-up of patients with myelodysplastic syndromes (modified from Westers *et al.*[28])

Markers	*Progenitor myeloid*	*Neutrophils*	*Monocytes*	*Progenitor B*	*Erythroid*
SSC	Increased SSC	**Low ratio to lymphocytes**	Decreased SSC		
CD45	Decreased expression	Decreased expression	Decreased expression		
CD117	Decreased frequency	Increased expression			Increased frequency of positive precursors
CD34	**Increased frequency of $CD34^+/CD19^-$ (>2%) Increased proportion of $CD38^{-/dim}/CD34^+$**	**Asynchronous expression**	**Asynchronous expression**	$CD19^+/CD34^+$ $\leqslant$5% of $CD34^+$ cells	
HLA-DR	Increased proportion of $HLA\text{-}DR^{-/dim}/CD34^+$ cells	Increased expression	Decreased expression		
CD11b	Increased expression on $CD34^+$ cells		Decreased expression		
HLA-DR/CD11b		Aberrant pattern	Aberrant pattern		
CD11b/CD16		**Aberrant pattern (most often due to low CD16)**	**Abnormal expression of CD16 on $CD11b^+$ monocytes**		
CD13/CD11b		Aberrant maturation pattern			
CD13/CD16		Aberrant maturation pattern			
CD13/CD33	Increased number of $CD33^+/CD13^-$ or $CD33^-/CD13^+$ cells	Increased number of $CD33^+/CD13^-$ or $CD33^-/CD13^+$ cells	Increased number of $CD33^+/CD13^-$ or $CD33^-/CD13^+$ cells		
CD14			Decreased expression		
CD15	Asynchronous expression on progenitors	Asynchronous expression together with CD34			
CD15/CD10		Aberrant pattern Lack of CD10 on mature neutrophil granulocytes			
CD19	Decreased $CD34^+/CD19^+$ lymphoid progenitors	Abnormal expression			
CD19/CD10				Decreased frequency	
CD36		Increased expression	Abnormal Expression		**Abnormal heterogeneous and/or low expression**
CD36/CD14			Aberrant pattern		
CD5	**Abnormal expression on $CD34^+$ and/or $CD117^+$ cells**	**Abnormal expression**	**Abnormal expression**		
CD56	**Abnormal expression on $CD34^+$ and/or $CD117^+$ cells**	**Abnormal expression**	**Abnormal expression**		
CD7	**Abnormal expression on $CD34^+$ and/or $CD117^+$ cells**	**Abnormal expression**	**Abnormal expression**		
CD71					**Abnormal heterogeneous and/or low expression**
CD71/CD235					Aberrant pattern

Abbreviation: SSC, side scatter. Items in boldface have been reported to have strong value in supporting MDS diagnosis. Aberrant pattern indicates a difference from the pattern seen in normal bone marrow. Abnormal expression indicates that the relevant marker is not present on this cell type in normal bone marrow. Increased/decreased expression is to be considered in comparison to normal bone marrow counterparts.

5. The added value of FCM results in the diagnosis and classification of MDS varies depending on MDS category and other diagnostic results. It can be summarized as follows:

   - In cases with minimal morphological dysplasia and no detected cytogenetic/molecular abnormalities, aberrant FCM findings may support MDS diagnosis. Conversely, normal FCM findings should prompt further investigation for other causes of cytopenias, close follow-up and retesting when clinically indicated.[1,11,37,41]
   - In patients with cytological findings suggesting MDS of RCUD (refractory anemia subtype) or refractory anemia with ringed sideroblasts categories, aberrant FCM findings in the granulopoietic or myelomonocytic lineages may indicate multilineage dysplasia, which is of prognostic significance.[2] Morphological findings in these cases should be thoroughly re-evaluated to avoid misclassification.

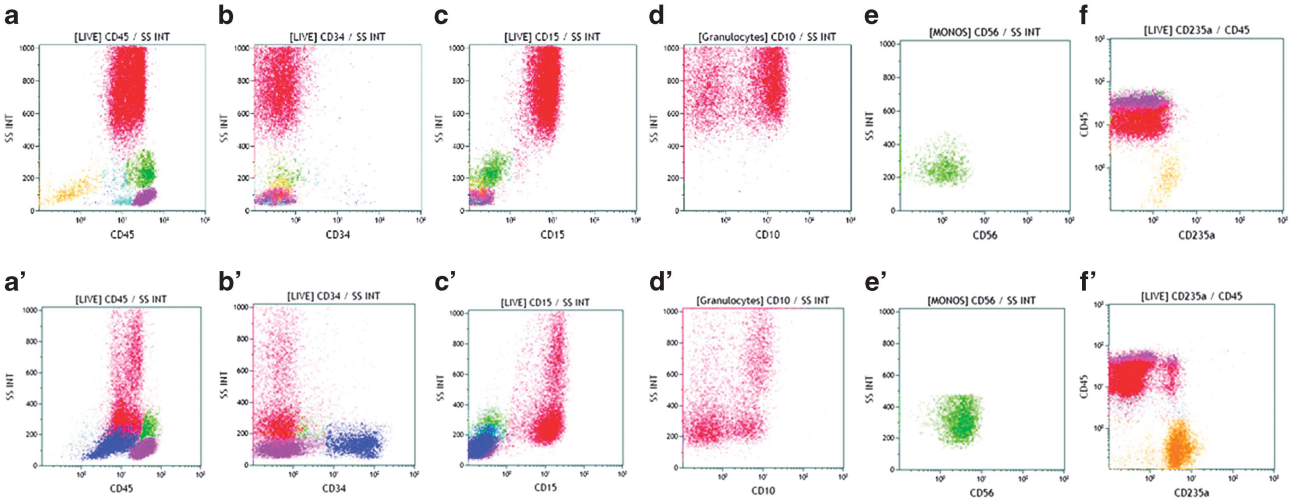

Figure 1. Examples of flow cytometry plots illustrating some aberrant phenotypes (see Table 1) by comparing a normal (top) and MDS (bottom) BM sample. (**a** vs **a**′) Summarizing CD45/SSC plots with color coding according to Arnoulet *et al*,[62] lymphocytes in magenta, monocytes in green, granulocytes in red, erythroblasts in orange, progenitors in cyan (sc. bermudes). Backgating of $CD34^+$ (**b** vs **b**′) immature progenitors in dark blue. The decreased 90° scatter (SSC) of granulocytes, also shown in **c**′, is already clearly visible on the **a**′ plot. (**b** vs **b**′) Increased proportion of $CD34^+$ immature progenitors in MDS (dark blue dots 13% vs 0.3% in **b**). (**c** vs **c**′) Decreased 90° scatter (SSC) of $CD15^+$ granulocytes in MDS (red dots). (**d** vs **d**′) Increased proportion of $CD10^{dim}$ granulocytes in MDS (red dots). (**e** vs **e**′) Abnormal expression of CD56 in MDS monocytes (green dots). (**f** vs **f**′) Increased erythroblasts in $CD235^+$ compartment (orange dots).

- It is important to note even small populations of myeloid progenitors with multiple immunophenotypic aberrant features (such as aberrant expression of CD7, CD56 or CD11b, see Table 1), since they indicate a higher risk of progression to AML.[14,41,46–50] FCM findings in these cases should be included in the individual risk assessment.
- Enumeration of the progenitor cell compartment by FCM has shown significant positive correlation with cytomorphology but yields lower counts (on average by 1%, but more striking differences may be noticed).[1] Enumeration of these cells by FCM should be compared with the morphological enumeration performed on BM smears to detect any major differences due to the quality of the sample. The most frequent cause of hemodilution of the FCM sample is that the first draw is most often used for morphology and the second draw for FCM. Whenever possible, the results should also be assessed together with BM biopsy evaluation to exclude the impact of possible fibrosis. Enumeration of CD34+ cells by FCM has been reported as more relevant for prognosis than the percentage of blasts evaluated by morphology, and a limit of >2% CD34+ cells within nucleated CD45+ cells has been reported to be significant.[7,12] Of note, revised IPSS uses >2% of morphologically determined blasts as the first limit for significant blast percentage categories.[51]

DISCUSSION

Incorporation of FCM in the diagnostic work-up of MDS brings additional information not provided by morphology, cytogenetics or molecular data. Multiple cell subsets can be analyzed by FCM in the BM. Immunophenotypes of various cell subsets and maturation patterns can be assessed individually. Of note, FCM can also be of value in better appreciating aberrancies in the erythroid and/or megakaryocytic lineages.[47,52–55] Since FCM provides the immunophenotypic features of individual cells, this method may provide information on aberrant features not necessarily identified by morphology or molecular studies. However, further studies are necessary to establish whether in patients with unilineage dysplasia, normal karyotype and no detected mutations definitive aberrant FCM findings will provide an added value in the diagnostic work-up.

Similar to clinical scoring systems, different scoring systems for FCM have been proposed by various groups.[2,7,9,13,39,40,48] All these studies have shown that higher numbers of FCM aberrancies correlate with high-risk IPSS in adult MDS. In low-risk IPSS, higher numbers of FCM aberrancies indicate an increased risk of progression. Low scores have been found mainly in patients with RCUD (refractory anemia, refractory neutropenia, refractory thrombopenia), refractory anemia with ringed sideroblasts, myelodysplastic syndrome unclassified, and in rare patients with Refractory Cytopenia with Multilineage Dysplasia and low IPSS risk score.[2] Independent of the scoring system used, it should be possible to classify FCM findings as consistent with MDS, showing a limited number of changes seen in MDS or not showing MDS-related features.[23] However, no definitive MDS diagnosis should be given if the FCM report is not integrated with the other diagnostic information provided by clinical information, blood and BM smear cytology, BM biopsy morphology, cytogenetics and molecular genetics.

Some FCM aberrancies have also been seen in patients with conditions other than MDS. A multicenter study where 380 control BM samples from patients with cytopenia were included showed that the myeloblast-related cluster size and the granulocyte/lymphocyte SSC ratio could be significantly reduced in patients with idiopathic/iatrogenic hypoplasia.[33] The lymphocyte/myeloblast CD45 ratio was also significantly lower in patients with cytopenia associated with BM infiltration than in those with other pathological conditions, while no significant difference was noted in the size of the B-progenitor-related cluster in various hospital control groups. CD56 can be expressed on monocytes in autoimmune diseases[56] but should not be found in reactive states on granulocytes or myeloid progenitor cells. Moreover, in patients with AML, MDS-associated findings may be suggestive of pre-existing dysplasia.[36]

Besides their obvious value in MDS diagnosis, there is already evidence that high FCM scores indicate adverse prognosis and can be used in individual risk assessment of MDS patients. High-risk pediatric MDS seems to show similar FCM abnormalities as adult MDS.[57] However, refractory cytopenia of childhood is clinically

different from adult MDS.[58] Thus, the guidelines proposed here only relate to adult MDS and guidelines specific for pediatric MDS should be further developed.

It has also been reported that FCM can be included in therapeutic decisions. Patients with aberrant FCM findings indeed have lower sensitivity to treatments with growth factors.[10,59] Moreover, patients who respond to azacitidine treatment have been shown to display a lower FCM score and normalized FCM features after treatment.[60]

Similar to the characteristic immunophenotypes found in AML with specific translocations,[61] characteristic patterns of immunophenotype changes in MDS may be in the future related to specific molecular mutations, which may provide guidance for choice of molecular analyses in MDS patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)