Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working conference on flow cytometry in myelodysplastic syndromes

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ABSTRACT

The myelodysplastic syndromes are a group of clonal hematopoietic stem cell diseases characterized by cytopenia(s), dysplasia in one or more cell lineages and increased risk of evolution to acute myeloid leukemia (AML). Recent advances in immunophenotyping of hematopoietic progenitor and maturing cells in dysplastic bone marrow point to a useful role for multiparameter flow cytometry (FCM) in the diagnosis and prognostication of myelodysplastic syndromes. In March 2008, representatives from 18 European institutes participated in a European LeukemiaNet (ELN) workshop held in Amsterdam as a first step towards standardization of FCM in myelodysplastic syndromes. Consensus was reached regarding standard methods for cell sampling, handling and processing. The group also defined minimal combinations of antibodies to analyze aberrant immunophenotypes and thus dysplasia. Examples are altered numbers of CD34$^+$ precursors, aberrant expression of markers on myeloblasts, maturing myeloid cells, monocytes or erythroid precursors and the expression of lineage infidelity markers. When applied in practice, aberrant FCM patterns correlate well with morphology, the subclassification of myelodysplastic syndromes, and prognostic scoring systems. However, the group also concluded that despite strong evidence for an impact of FCM in myelodysplastic syndromes, further (prospective) validation of markers and immunophenotypic patterns are required against control patient groups as well as further standardization in multi-center studies. Standardization of FCM in myelodysplastic syndromes may thus contribute to improved diagnosis and prognostication of myelodysplastic syndromes in the future.

Key words: myelodysplastic syndromes, flow cytometry, standardization, ELN, consensus.

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Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid neoplasms characterized by dysplastic features of erythroid and/or myeloid and/or megakaryocytic lineages, a varying percentage of blast cells, progressive bone marrow failure and enhanced risk to evolve towards acute myeloid leukemia.[1] In 2007 refined definitions and standards in the diagnosis of MDS were reported.[2] Using the proposed minimal diagnostic criteria, additional tests (co-criteria) can be applied and may help to decide whether the patient has a myeloid neoplasm with bone marrow failure resembling (or highly suspicious of) MDS. This is of importance particularly in patients with only mild or absent dysplasia but otherwise typical MDS-related clinical findings (e.g. transfusion-dependent macrocytic anemia). Flow cytometry (FCM) analysis of bone marrow cells has been introduced as an important co-criterion.[2] In March 2008, the first International Workshop on Standardization of FCM in MDS convened in Amsterdam. Thirty participants from 18 institutes throughout Europe working within the European LeukemiaNet (ELN) and 3 experts from outside Europe (USA and Japan) joined this meeting. The group has a vast experience of integrating FCM in the work-up of patients with suspected MDS patients.[3] Recent studies conducted by members of the consortium indicate that the FCM approach is reproducible and can identify specific aberrations on both the immature and mature compartments among different bone marrow hematopoietic cell lineages. A more general application of FCM in the diagnosis and prognostication of MDS, especially in low and intermediate-I risk MDS, has been hampered by the lack of standardization of methods and interpretation of data obtained by FCM.

The major goals of the working conference were: (a) to define the role of FCM in diagnosis and prognostication of MDS related to the currently validated FAB, WHO, IPSS and WPSS systems; (b) to discuss the optimal methods of sample processing and handling; (c) to propose a consensual minimal set of monoclonal antibodies useful to assess dysplasia by FCM of bone marrow cells in known or suspected MDS; (d) to consider the specificity of FCM analysis of MDS related to a series of other hematologic benign and malignant diseases and (e) to suggest additional recommendations on FCM to further optimize analysis for future directions.

Role of flow cytometry in myelodysplastic syndromes in relation to diagnosis, prognosis and disease monitoring

Flow cytometry in myelodysplastic syndromes in relation to minimal diagnostic criteria and WHO classification

The previous use of FCM in MDS has been primarily restricted to the characterization of blast cells in secondary acute leukemia following MDS. However, it has been shown that myeloid and monocytic dyspoiesis identified as phenotypic abnormalities by FCM in MDS correlates with the International Prognostic Scoring System (IPSS), the WHO-adjusted prognostic scoring system (WPSS), transfusion dependency, and time-to-progression to advanced MDS/AML, as well as with outcome after hematopoietic stem cell transplantation.[4-6] In addition, immunophenotypic aberrancies of myeloblasts, e.g. over- or decreased expression of common myeloid antigens or expression of lineage infidelity markers, may have independent prognostic impact even if the percentage of blasts in the bone marrow is lower than 5%.[4-7] Aberrant expression of certain antigens (CD7 and/or TdT) on myeloblast cells has been shown to correlate with poor clinical outcome.[6,8,9] These studies indicate that FCM shows promise in contributing significantly to the diagnosis and prognosis of MDS.

The WHO classification clearly distinguishes between patients with refractory cytopenia exhibiting unilineage dysplasia (RCUD) and refractory anemia with/without ring sideroblasts (RA+/-RS) from those with dysplasia in two or three cell lineages (refractory cytopenia with multilineage dysplasia, RCMD) with respect to overall survival (OS) and leukemia-free survival.[1] Patients with RCMD+/-RS show a shorter overall survival (OS) as compared to RA+/-RS.[10] It is of interest that the life expectancy of patients with unilineage dysplasia, e.g., RA above 70 years of age, does not differ from that of the normal population.[10] Therefore, a clear distinction between unilineage RA+/-RS and multi-lineage RCMD+/-RS is of importance for disease management. Flow cytometry might help to distinguish true RA from RCMD by identifying RA with immunophenotypic abnormalities in multiple compartments from RA with normal immunophenotype. Recently, patients with multi-lineage immunophenotypic aberrations were identified by FCM among cases displaying unilineage morphological dysplasia according to the WHO (RA and MDS-U).[6,11]

Flow cytometry may add significantly to the diagnosis of new subgroups of refractory cytopenias as defined by the new WHO classification of 2008, e.g. refractory anemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT), which will be studied prospectively. Also, the assessment of FCM aberrancies (qualitative and quantitative) on blasts may have additional diagnostic and prognostic impact in patients with excess of blasts. Therefore, in refractory anemia with excess blasts (RAEB-1 and RAEB-2), the extent of abnormalities in blast populations, as well as additional FCM aberrancies in the more mature myeloid or monocyte compartments, might be of importance.

Moreover, low-risk WPSS patients who are transfusion-independent and have a normal karyotype may be distinguished from patients with idiopathic cytopenia/anemia of undetermined significance (ICUS) based on immunophenotypic abnormalities.[2] In these patients, FCM may help in reaching the conclusion that the patient likely suffers from a clonal disease process (e.g. MDS) rather than ICUS which includes cases of sustained cytopenias (>6 months) in one or more myeloid lineages (erythroid, neutrophil and megakaryocytic) that do not meet the minimal criteria for MDS and cannot be explained by any other hematologic or non-hematologic

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disease.[2,12] In some of these patients, the type of cytopenia (e.g. transfusion-dependent macrocytic anemia) may point to the potential existence of an underlying MDS or a pre-MDS phase. It has been proposed that FCM contributes significantly to diagnosis and may distinguish early MDS from ICUS due to its accuracy in enumerating blasts, to assess abnormalities in the blast population (even <5%) and by documenting immunophenotypic abnormalities in maturing myeloid cells. Finally, the WHO classification separates MDS with isolated del(5q) as a distinct entity since these patients are characterized by specific morphological and clinical parameters and have a very low risk of evolving to AML.[13] Flow cytometry might help to identify 5q- patients with or without additional immunophenotypic abnormalities.

Flow cytometry in myelodysplastic syndromes in relation to risk assessment by cytogenetics, genomics, IPSS and WPSS

The IPSS represents the benchmark for clinical trials and the treatment-decision making processes in MDS.[14] In addition, the effects of the validated IPSS variables (morphological bone marrow blast cell percentage, number of peripheral cytopenias and karyotype) within WHO categories have been shown to correlate with outcome in several studies.[15-19] A major limitation of the IPSS is that newly defined prognostic parameters have not been included, e.g. LDH, bone marrow fibrosis, circulating blasts (normal or aberrant), as well as data on transfusion requirements. In addition, there is increasing evidence to suggest that the IPSS-defined cytogenetic subgroups should be reclassified.[17,20] In that context, FCM may also be of importance in those patients with a normal karyotype and an equivocal morphology (e.g. not fulfilling the minimal diagnostic criteria as discussed above) and in patients with a normal karyotype but with less than 20 metaphases analyzed with no proof of clonality.[21,22] Emerging evidence from single nucleotide polymorphisms (SNP) array genotyping studies suggests that clonal selection and expansion is present in some cases where morphological evidence for MDS is equivocal. Prospective studies will be required to determine whether conventional karyotyping, SNP karyotyping and FCM are able to further distinguish MDS subgroups. First data are currently being reported on microRNA expression profiling in MDS.[23-29] Although the WPSS provides a superior prognostic algorithm compared to IPSS, FCM may further improve diagnosis and prognostication. Prospective studies are now beginning to further assess the relevance of WPSS and FCM in MDS. Yet, the significance of morphological classification and prognostic scoring systems in MDS is not defined for patients treated with currently available drugs and therefore may not adequately predict disease outcome. Newly designed prospective studies are warranted to include old and new methods, e.g. FCM, to accurately investigate the prognostic significance.

Flow cytometry in monitoring treatment and disease progression in myelodysplastic syndromes

Disease monitoring in MDS implies sequential analysis of characteristic aberrancies. Once certain immunophenotypic aberrancies are defined, patients can also be monitored by FCM to determine disease progression (increased abnormalities) or response to therapeutic interventions. Preliminary studies indicate that MDS-related FCM abnormalities in bone marrow cells are no longer detectable or decrease in number in responding patients when compared to pre-treatment results.[30] Response to intensive therapy may lead to reconstitution of normal cells after neoplastic cells have been eliminated which can be nicely demonstrated by FCM. A caveat is that therapeutic drugs can alter aberrancies or even introduce new ones. Stable FCM aberrancies during treatment may spare patients long-term treatment with ineffective drugs with potential toxicity. Flow cytometry monitoring may be important especially when no other disease parameters, such as molecular and cytogenetic parameters, are available. Finally, FCM may contribute significantly in those patients in complete remission (CR) after induction therapy for AML with persistence of cytopenias that suggest a pre-phase of MDS. The clinical outcome of these patients is very poor.

Optimal methods for processing and handling of samples for flow cytometry in myelodysplastic syndromes

Published studies regarding the immunophenotypic characteristics of erythroid, precursor and maturing myeloid cells in MDS patients employed variable methodologies and different reagents. In virtually all studies, diverse panels of antibodies, different anticoagulants, time to FCM, and storage/transport conditions were used. Moreover, information about instrument settings and quality control is usually limited. In previous immunophenotypic studies of hematologic malignancies, many of these variables have been shown to dramatically influence the results of FCM analysis.[31] In MDS, such technical issues are particularly relevant, since they can affect the number of immunophenotypic abnormalities detected and the magnitude of such abnormalities. Thus, standardized application of FCM in the diagnosis and monitoring of MDS requires a minimal variability introduced by different sample processing, antibody combinations, data acquisition and interpretation of data.

We documented the variations in these technical parameters in 15 participating institutes through a survey of practice. A summary of the questionnaires (set out in part in Tables 1-3) was a starting point of discussions with the aim of reaching a consensus on how to standardize these parameters. The final recommendations for the various steps involved in FCM in MDS are discussed below.

Sample preparation, handling and processing

Samples

Analysis of FCM aberrancies in MDS should be performed preferably in bone marrow samples; only sparse data are available on FCM in MDS in peripheral blood samples. Bone marrow aspirates should be collected using either heparin or, less preferable, EDTA as anticoagulant. Delayed processing of specimens collected in

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EDTA may influence the expression of certain antigens such as CD10, CD11b, CD16 and CD64.[11,32] Sample processing should preferably be performed within 24 h after aspiration. The sample should be stored at room temperature until processed in the laboratory. Samples processed at later time points may still be evaluable, but extra controls should be included to evaluate the quality of the sample. Addition of glucose containing tissue culture media (1:1) may be helpful in preserving cell viability.

Red blood cell lysis

Flow cytometry in MDS mainly concerns analysis of white blood cells and nucleated red blood cells. For lysis of the non-nucleated red blood cells, ammonium chloride (either home-made or commercially available) should be used. The lysing solution should not contain a fixative, since this may result in selective cell loss or may change light scatter properties of the cells. Other (commercial) lysing solutions, not containing a fixative, may be used after parallel testing. The lysis of red blood cells is recommended prior to antibody staining for two main reasons. First, bulk-lysis will result in fully identical cell suspensions in the separate analysis tubes, thereby facilitating comparison between tubes. Second, this approach allows the use of a fixed cell concentration for the stainings. Red blood cell lysing can be performed by mixing a maximum of 3 ml whole bone marrow with ammonium chloride (total volume: 50 mL); incubation for 10 min at room temperature while mixing gently. Cells should be washed using phosphate buffered saline (PBS) with 0.5% bovine or human serum albumin (hereafter referred to as washing buffer). Centrifugation should be performed at 300g or less. If, after washing, macroscopic evaluation shows that the cell pellet still contains red blood cells, a second lysing step may be performed. Otherwise, the cell pellet can be resuspended in washing buffer. The use of mononuclear cells after density separation is not recommended due to selective loss of cells (particularly mature granulocytes). It should be noted, however, that density separation may be preferable for specific applications such as assessment of the erythroid lineage.[24]

Antibody staining

Dilution of antibodies and washing of cells should be performed using PBS/0.5% bovine or human serum albumin. The addition of serum albumin prevents non-specific binding of antibodies and therefore reduces background staining. To assure sufficient and constant numbers of analyzed cells, it is recommended to use 500,000 cells per tube. Optimally titrated antibodies should be added to the cell suspension and after gently mixing, cells should be incubated for 15 min at room temperature in the dark. Special care should be taken if tandem-fluorochromes are used, since these fluorochromes may be highly sensitive to light exposure (such as PE-Cy5, PE-Cy7, APC-H7 and Horizon™ V450 conjugates). It must also be pointed out that compensation settings frequently vary from batch to batch. After incubation, cells should be washed at least once. In addition to surface staining, intracellular staining may be informative. If intracellular staining is performed, the membrane-bound antigens should be stained first, followed by fixation and permeabilization using well evaluated commercial reagents and subsequent intracellular staining and washing. Since many fixation and permeabilization reagents may alter the light scatter characteristics of the cells, it is not recommended to use the scatter characteristics for identification and gating of cells. In addition, since fixation and permeabilization

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Table 1. Parameters scored as aberrant in the immature compartment.

Myeloblasts	%[1]	B cell progenitors	%
Increased percentage[2]	91	Decreased number/percentage† increased number/percentage	82 45
Abnormal granularity (SSC)[2]	73		
Abnormal expression of CD45[2]	100		
Abnormal expression of CD13	100		
Abnormal expression of CD33	100		
Abnormal expression of CD34[2]	91		
Abnormal expression of CD117[2]	82		
Expression of CD11b[2]	82		
Expression of CD15[2]	64		
Abnormal expression of HLA-DR[2]	55		
Expression of CD36	45		
Abnormal expression of TdT	36		
Expression of CD41/CD42b/CD61	36		
Expression of CD38	27		
Expression of CD4	18		
Expression of lineage infidelity marker CD2[2]	82		
Expression of lineage infidelity marker CD5[2]	82		
Expression of lineage infidelity marker CD7[2]	91		
Expression of lineage infidelity marker CD19[2]	91		
Expression of lineage infidelity marker CD56[2]	91		

[1]percentage of survey participants who analyzed a given aberrancy; [2]considered as relevant finding.

Table 2. Parameters scored as aberrant in the maturing myeloid and monocytic compartment.

Granulocytes (maturing myeloid cells)	%[1]	Monocytes	%[1]
Decreased myeloid *vs.* lymphoid ratio (<1)	36	Decreased/increased as compared to lymphocytes[2]	45
Abnormal granularity (SSC)[2]	100	Abnormal granularity (SSC)	45
Abnormal expression of CD45[2]	64	Abnormal expression of CD45	55
Asynchronous shift to the left	82	Abnormal distribution of immature/mature cells	
Abnormal CD16/CD13 pattern[2]	82	Abnormal expression of CD33[2]	73
Abnormal CD11b/CD13 pattern[2]	73	Expression of HLA-DR	73
Abnormal expression of CD33	73	Abnormal CD11b/HLA-DR pattern[2]	73
Expression of CD34[2]	73	Expression of CD34	73
Expression of HLA-DR	73	Abnormal expression of CD14	73
Abnormal expression of CD15	55	Abnormal expression of CD13[2]	73
Abnormal/lack of expression of CD10	45	Abnormal expression of CD36[2]	45
Abnormal expression of CD36	45	Abnormal expression of CD64	36
Abnormal expression of CD64[2]	36	Abnormal expression of CD11c	18
Abnormal expression of CD66	27	Abnormal expression of CD15	9
Abnormal expression of CD16	9		
Expression of lineage infidelity marker CD2[2]	73	Expression of lineage infidelity marker CD2[2]	73
Expression of lineage infidelity marker CD5	73	Expression of lineage infidelity marker CD5[2]	73
Expression of lineage infidelity marker CD7[2]	82	Expression of lineage infidelity marker CD7[2]	73
Expression of lineage infidelity marker CD19[2]	82	Expression of lineage infidelity marker CD19[2]	73
Overexpression of CD56	45	Overexpression of CD56[2]	91

[1]*percentage of survey participants who analyzed a given aberrancy;* [2]*considered as relevant finding.*

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may result in selective cell loss, this procedure should not be used for enumeration of cell populations.

Cell fixation

To stabilize cell membranes, prevent possible dissociation of antibodies and reduce biohazard, it is recommended to fix the cells after staining and washing.[33] Commercially available 0.5% paraformaldehyde (PFA) in PBS (pH 7.4) is recommended. Washed cells should be resuspended in fixation buffer (PBS/0.5% PFA). Cells should not be stored after this fixation step, but immediately processed for sample acquisition on the flow cytometer. Of note, some of the newer tandem conjugates are subject to partial degradation when PFA is added. To minimize variations in sample processing, the use of automated sample preparation platforms would be preferable.

Instrument setup and data acquisition

The number of lasers, the number of detectors, the setup of the filters, and digital or analog data processing will logically affect the number of antibodies and the types of fluorochromes that can be assessed simultaneously and will have significant impact on the fluorescent patterns obtained. The flow cytometer should be set up and calibrated according to previously published recommendations.[34]

Table 3. Parameters scored as aberrant in the erythroid compartment.

Erythroid cells	%[1]
Increased percentage after lysis	45
Abnormal granularity (SSC)	45
Abnormal expression of CD45	64
Abnormal expression of CD71	73
Abnormal CD71/CD235a pattern[2]	64
Abnormal expression of CD235a	55
Abnormal expression of CD117	55
Abnormal expression of CD34	45
Abnormal expression of CD36	36
Abnormal expression of CD105[2]	36
Abnormal expression of H-ferritin	18
Abnormal expression of M-ferritin	18

[1]*Percentage of survey participants who analyzed a given aberrancy;* [2]*considered as relevant finding.*

Application of multiparameter flow cytometry analysis in myelodysplastic syndromes

Application of FCM in the diagnosis, prognostication and monitoring of MDS requires consensus on a minimal panel of antibodies necessary to classify specific bone marrow subpopulations, enumerating blast cells and detecting immunophenotypic lineage-associated dysplasia. The workshop participants agreed on the characterization of the immature blasts as well as the maturing monocyte, myeloid and erythroid lineages. Analysis of dysplasia of megakaryopoiesis is excluded since current knowledge of dysplastic marker expression in this cell type is limited. The same holds true for basophils, eosinophils, dendritic cell populations and mast cells, although one can expect that also these cells are sometimes involved in the neoplastic process and thus display aberrant phenotypes in MDS.[7,3]

Recommended combinations of monoclonal antibodies

A simple and robust set of markers to analyze dysplasia in the erythroid and myeloid lineages is desirable. A minimal panel for FCM analysis of MDS should allow the analysis of abnormal expression of selected antigens and the relation between antigens of relevance in specific subpopulations of cells. A 4-color panel is recommended in multicenter studies. A panel larger than 4-colors is currently regarded as being too technically and analytically challenging for standard clinical analysis of MDS bone marrow samples in daily clinical practice in all centers. Table 4 summarizes recommended antibody combinations in which consensus could be reached, CD45 being ubiquitous in every combination. Experience of workshop participants and data from the literature were helpful in deciding which of the parameters would be most informative and important in the FCM analysis of MDS. An example of a 4-color antibody panel for FCM in MDS is shown in Table 5. Recommended antibody combinations per specific subpopulation will be discussed below.

Analysis of immature myeloid and lymphoid compartment (myeloblasts and lymphoblasts)

Definition and enumeration of blasts

The combination of CD45 and SSC provides a means to identify myeloblasts (CD45^{dim} and SSC$^{low/int}$).[36] The blasts observed in MDS are usually restricted to this region; however, maturation along the monocytic or neutrophilic lineages may place the abnormal blasts outside this region. Occasionally, abnormal myeloblasts in MDS can lack CD45 expression altogether.[37] The region where myeloblasts reside by CD45/SSC includes not only myeloblasts, but B-cell precursors (lymphoblasts or *hematogones*), monoblasts, basophils, erythroblasts, dendritic cell precursors, and hematopoietic stem cells (HSC) in varying proportions. The myeloblasts present in MDS should be distinguished from these other populations upon analysis by proper gating strategies using antibody combinations such as CD45/CD34/CD117/HLA-DR and CD45/CD34/CD123/HLA-DR.

The enumeration of blasts may be of critical importance, though there is a caveat about sample quality. Differences are seen in blast counts as assessed by morphology and FCM. Bone marrow aspirates for FCM analysis often contain fewer bone marrow spicules than the fraction used to prepare smears. Moreover, samples can be diluted by peripheral blood.[38] Another important issue is the terminology of what constitutes a blast: the definition of blasts by FCM (myeloid as well as lymphoid), inclusion or exclusion of granular blasts, and the resemblance of monocytes and blasts should be defined and/or corrected in a uniform manner.[3] Myeloblasts may

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Table 4. Proposed marker combinations for flow cytometry analysis of dysplasia in myelodysplastic syndromes.*

	Erythroid	Immature myeloid	Immature lymphoid	Maturing granulocytes	Monocytes	Mature lymphoid
CD71, CD235a, CD117	X					
CD105	(x)					
CD34,CD117	(x)	X				
CD11b, CD117				X		
CD11b, HLA-DR		X		X	X	
CD117, HLA-DR		X				
CD123, HLA-DR		(x)†		(x)‡		
CD34, CD15		X		X		
CD34, CD5		X		X	X	
CD34, CD7, (CD13§)		X		X	X	
CD34, CD56		X		X	X	
CD34, CD19		X	X	X	X	
CD10, CD19			(x)	X		
CD10, CD38			(x)			
CD11b, CD13, CD16				X		
CD65				(x)		
CD64, CD14					X	
CD64, CD36					(x)	
CD33, CD14		X		X	X	
CD33, CD36	(x) CD36	X			X	
TdT		(x)	(x)			
CD79a			(x)			
CD19 κ,λ						(x)**
CD3, CD4, CD8						(x)**

**CD45 is ubiquitous in every combination. X: minimally required, (x): supplementary. †Analysis of plasmacytoid DC (precursors), ‡analysis of basophils, §CD7 expression on myeloblasts can be normal, e.g. monocytic/dendritic precursors are CD13dim/CD7dim, **to complete immunological differential.*

be defined as $CD45^{dim}SSC^{low}CD34^{+}$ (in combination with a myeloid marker, negative for lymphoid markers) and denotes the earliest stage of the myeloblast. The most mature myeloblast in normal bone marrow expresses HLA-DR and CD117 in addition to CD34. The CD34 antigen is expressed on all hematopoietic precursors, but is lost relatively early in the maturation of hematopoietic cells. The level of CD34 expression is highly dependent on the CD34 antibody used since there are three different epitopes of the CD34 antigen, and on the fluorochrome coupled to the antibody.[3] Due to the variability of the myeloblast position in CD45/SSC plots and the heterogeneity of the CD34 positive fraction, multiple or redundant methods are applied to identify and count the myeloblast cells present and to analyze aberrancies on blast cells. The percentages of myeloblasts obtained with redundant antibodies should correlate, unless the aberrant myeloblasts lack a particular antigen. An important caveat is that myeloblasts in MDS can be CD34 negative. Therefore, simply counting CD34 positive cells may be inappropriate in studies of this disease. If cells are CD34 negative, CD117 can be chosen as an alternative progenitor-marker, and myeloblasts can be defined as $CD45^{dim}$ $SSC^{low/int}HLA^{-}DR^{+}CD11b^{-}$. The most accurate method to enumerate myeloblasts by FCM requires elimination of the erythroid cells in the analysis, e.g. by electronically gating them out. Therefore, the denominator will be total non-erythroid cells. Flow cytometry in MDS should not be considered an analysis to exactly replicate myeloblast counts by morphology. The cut-off level for abnormally increased blast counts by FCM in MDS may be closer to 3% of total nucleated cells in contrast to the 5% currently used by morphology.[4,6,35,39] In addition, the detection and enumeration of circulating (aberrant) blasts is of increasing interest since some data suggest that the presence of blasts in peripheral blood in RA+/-RS may influence OS or may be associated with an increased risk of leukemic transformation and post-transplant relapse.[5,40,41] The effect of secondary myelofibrosis, which may an independent risk factor, on the presence of circulating blasts or myeloid progenitor cells is not yet clear. Circulating blasts in myelofibrosis are part of the natural course of the disease. Within the current WHO proposal of 2008, the presence of circulating blasts is discussed and seems to be associated with a worse clinical outcome, with overall survival identical to patients with MDS RAEB-1.[5,40,41]

Since a decreased percentage of progenitor B cells is frequently observed in MDS, enumeration of progenitor B cells, identified in the $CD45^{dim/low}SSC^{low}$ region and by their $CD34^{+}CD19^{+}CD10^{+}$ phenotype, is recommended as a useful parameter.[6,7,42,43] The relevance of this observation is not yet known, since a decrease in progenitor B cells is also seen in the elderly population without MDS and may be a non-specific finding in other diseases such as myeloproliferative disorders (MPD) and immunodeficiencies. Recently, it was reported that, unlike CMML and MDS patients, most MPD patients maintained an adequate B-cell development except for those with chronic myeloid leukemia (CML).[44,45] To circumvent problems regarding hemodilution in the enumeration of progenitor B cells, it was recommended that these cells are expressed as a fraction of all ($CD34^{+}$) blast cells.[39]

Identification of flow cytometry aberrancies in blasts

Neoplastic myeloblasts in MDS may have an aberrant phenotype that distinguishes them from normal blasts. The most widely recognized aberrancies in the immature myeloid compartment in MDS, next to an increased percentage, concern abnormal intensity of CD34, CD45,

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Table 5. Example of a screening panel for 4-color flow cytometry of dysplasia in myelodysplastic syndromes.

	FL-1	FL-2	FL-3	FL-4
1			CD45	
2	CD71	CD235a	CD45	CD117
3	CD36	CD64	CD45	CD14
4	CD10	CD33	CD45	CD14
5	CD16	CD13	CD45	CD11b
6	HLA-DR	CD117	CD45	CD11b
7	CD13+CD33	CD117	CD45	CD34
8	CD13	CD7	CD45	CD34
9	CD2	CD56	CD45	CD34
10	CD5	CD19	CD45	CD34
11	CD15	CD11b	CD45	CD34

This panel was modified from a panel defined by the Dutch working party "FCM in MDS" of the Dutch Society for Cytometry (NVC). It should be of note that the construction of a panel depends on the available fluorochrome conjugates, antibodies and instrument performance characteristics.

Table 6. Proposed list of pathological controls to determine the specificity of flow cytometry aberrancies in myelodysplastic syndromes.

AML, hypoplastic AML, CML,
Chronic myeloproliferative disease (e.g. PV, ET),
Aplastic anemia, PNH,
Myelofibrosis (p/s MPD and/or MDS), systemic mastocytosis, chronic eosinophilic leukemia
Langerhans cell histiocytosis, hemaphagocytosis, anemia of
Chronic diseases, anemia in autoimmune diseases (AITP, Rheumatoid Arthritis, SLE, SS), anemia due to renal insufficiency
Secondary MDS, post-chemotherapy, drugs interfering with angiogenesis,
Post-stem cell transplant settings (autologous or allogeneic stem cell Tx)

AML: acute myeloid leukemia; CML: chronic myeloid leukemia; PV: polycythemia vera; ET: essential thrombocytosis; PNH: paroxysmal nocturnal hemoglobinuria; MPD: myeloproliferative disorders; AITP: autoimmune thrombocytopenic purpura; SLE: systemic lupus erythematosus; SS: Sjögren syndrome; MDS: myelodysplastic syndromes.

CD13 or CD33 and/or the expression of lineage infidelity markers, as discussed below.[4,6,8,11,35,46-48] Aberrancies evaluated by the participants of the workshop are listed in Table 1. The consensus group regarded the following features of progenitor cells as most relevant: an increased percentage of myeloblasts, abnormal granularity (SSC), abnormal intensity of CD45, CD34, or CD117, or expression of CD11b or CD15, abnormal intensity (or lack) of HLA-DR and the expression of lineage infidelity markers such as TdT, CD7, CD19 or CD56. CD7 can be expressed on a small subset of early, normal myeloblasts. Antibody combinations that enable detection of these parameters are included in Table 4. To ensure that lineage infidelity markers are expressed on myeloblasts, a myeloid marker such as CD13 or CD33 can be added in the antibody combination. One must also take into account that certain aberrancies may appear following growth factor stimulation or regeneration, such as expression of CD56.[4,49]

Analysis of the maturing myeloid and monocytic compartment

Definition of and aberrancies in the maturing myeloid compartment

Maturing neutrophils, hereafter referred to as granulocytes, are most often defined by their CD45 expression (intermediate)[36] and SSC profile (bright); CD33 may be added to distinguish granulocytes from monocytes in case of overlapping populations (dim and bright expression of CD33, respectively). Hypogranularity of granulocytes is a well-known phenomenon in MDS.[46] Hence, an abnormal SSC is amongst the most frequently observed FCM aberrancies within this compartment.[3] SSC of granulocytes is often expressed as ratio relative to that obtained for lymphocytes. Abnormal granulocytes can also be recognized by aberrant expression of antigens. This includes an altered expression, e.g. an increased or decreased expression of CD45, CD13, CD33, CD11b, CD16, and CD64, lack of CD10 on mature granulocytes, expression of CD34 or CD14, and expression of the lineage infidelity markers CD2, CD7 or CD19.[4,6,11,46-48,50] Due to genetic polymorphisms the expression of some antigens, e.g. CD33, may be non-specifically decreased. Aberrant expression of such antigens should be interpreted in relation to other myeloid subsets as well as related to extensive normal and pathological bone marrow samples.

Evaluation of aberrancies may be hampered by contamination of the population of interest. For instance, when the SSC of neutrophils is low, granulocytes often overlap monocytes; these cells should be electronically excluded by monocyte specific antigens such as CD14, CD36 and CD33^{hi}. Eosinophils should be excluded as well; this can be done by an additional gate on CD45, SSC and/or CD16. Loss of CD16 is observed in paroxysmal nocturnal hemoglobinuria (PNH) and in patients with a genetic polymorphism.[51-53] Loss of CD16 has also been reported to coincide with apoptosis, at least in cell culture.[54] It must also be taken into account that use of the anticoagulant EDTA may influence the expression of certain calcium dependent antigens, e.g. CD11b, and that cell activation can induce increased expression of HLA-DR and CD64, if samples are not prepared rapidly and certain antibody clones are used.[3,11,32] Therefore, immunophenotypic differences from normal patterns may not be specific for MDS.

Definition of and aberrancies in the monocytic compartment

Monocytes can be defined based on their CD45 expression (intermediate-bright)[36] and SSC (intermediate); additional markers are CD36, CD14, CD64 and CD33. The proportion of monocytes in a sample can be expressed as ratio to the total non-erythroid cells or to lymphocytes. It was recommended to quantify monocytes not only by CD14 alone but also by CD36 and CD64, since the use of CD14 alone may underestimate the percentage of monocytes, particularly if immature forms are present.

The following aberrancies in maturing monocytes are considered as relevant: decreased or increased proportion of monocytes as compared to lymphocytes, abnormal intensity of CD13 or CD33, an abnormal CD11b/HLA-DR pattern, abnormal intensity of CD14, CD36 or CD64, overexpression of CD56 and expression of lineage infidelity markers CD2, CD7 or CD19 (Table 2).[4,6,11,46-48,50] As noted above, granulocytes with a decreased SSC can interfere in the analysis of monocytic aberrancies. It should be noted that the adhesion molecule CD56 is not truly a lineage infidelity marker since it can be expressed on either lymphoid or myeloid cells; only overexpression (>1 log) is considered as aberrant.[4,49] CD56 is frequently seen in regenerating bone marrow after chemotherapy or stem cell transplant, on granulocytes and monocytes during G-CSF primed stem cell collections and during infections.[4,49,55,56] Nevertheless, it was recently reported that CD56 expression on monocytes was the only discriminating marker between chronic myelomonocytic leukemia (CMML) and MDS (p=0.007); differences relating to CD56 expression were even more significant between CMML and MPD (p=0.0002).[45] In addition, differential expression of IREM2 and CD14 might distinguish between normal and leukemic monocytes; in a normal differentiation route CD14 is expressed before IREM2, while in some leukemic monocytes IREM2 precedes CD14.[57] However, additional studies are needed to confirm this observation.

Antibody combinations to enable analysis of aberrancies on granulocytes and monocytes as discussed above are presented in Table 4.

Erythroid compartment

Flow cytometry patterns of normal erythroid development were originally described in 1987.[58] Based on the knowledge of normal patterns, FCM aberrancies in the erythroid lineage in MDS were also reported in 2001.[46] At present, only limited numbers of antibodies are available to study erythroid dysplasia, therefore the spectrum of abnormalities in the erythroid compartment cannot be fully assessed. The erythroid population can be defined by lack of CD45 expression and light scatter.[58] The expression profile of glycophorin A (CD235a) and CD71 within this subpopulation is most widely analyzed. For immature erythropoietic cells, endoglin (CD105) and CD117 as well as CD36 can be employed as markers in normal marrow and probably also in MDS.[7,35] Analysis of the expression of CD105 and CD117 may thus also help in the assessment of erythroid dysplasia in MDS,

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although this has not been formally established. In addition, assessment of intracellular expression of H-ferritin and M-ferritin (MtF) may add information on erythroid dysplasia. M-ferritin expression is closely related to the presence of ringed sideroblasts in bone marrow (98% sensitivity, 100% specificity); and a strong correlation between MtF and Perls staining was reported (r=0.89).[59] However, these antibodies are not commercially available at present. Consensus has been reached that at least the expression of CD45, CD71, CD235a, CD117 and CD105 should be analyzed with emphasis on an abnormal pattern of CD71 in relation to CD235a (Table 3).

Validation of specificity of flow cytometry analysis in myelodysplastic syndromes

In addition to consensus on antibody combinations, agreement on interpretation of antigen expression profiles and expression patterns is necessary to enable solid information on diagnosis, prognosis and treatment monitoring in MDS. Multi-variate analysis of retrospective data-sets may clarify what is important to analyze and how to evaluate the abnormalities for prognosis.

To provide additional insights in the specificity and sensitivity of the observations in MDS with respect to controls, all aberrancies should ideally be analyzed versus (age-matched) normal and pathological controls of benign, stressed marrows and malignant hematologic diseases. A list of proposed controls is depicted in Table 6. Using this approach more insights will be gained dealing with disease-specific FCM aberrancies. To illustrate the issue of specificity: in case of hypogranularity, other disorders have to be ruled out. For instance, as a stressed bone marrow recovers the neutrophils reappear as hypogranular, illustrating the importance of obtaining concurrent clinical data. Further prospective (preferably multi-center) studies need to be completed to address these issues, and to learn what aberrancies are indeed MDS-related or even MDS-specific. Sensitivity of the method is another issue that raises many questions. How many cells in a defined cellular compartment are aberrant? Can FCM help in cases in which cytological analysis detects e.g. 9% dysplasia in one or more lineages? Moreover, MDS is a clonal disease; a small clone may not be recognizable since intramedullary apoptosis of progenitor cells may hamper appropriate detection, specially in low-risk MDS.

Future directions for flow cytometry in myelodysplastic syndromes

Flow cytometry data analysis is a complex issue and consensus has not yet been reached. The inherent variability of instrument set-up and standardization can give significant differences in the data collected between and within institutions. Similarly, differences in reagents, especially the fluorochromes used, will result in inconsistent sensitivity and specificity of data. Definitions must be generated that are explicit for what is considered normal or aberrant. Several flow scoring systems have been validated that distinguish MDS from reactive/normal controls.[4,7,11,35,47,50,60] The type of FCM analysis may be particular to any given institution and still remains valid. Several points of discussion remain.

- Analysis of the number of aberrancies in multicenter studies requires definition of a fixed number of aberrancies to be scored.
- In case of aberrancy (altered expression or altered patterns of antigen expression) the degree of deviation from normal has to be defined. In some laboratories, comparison of percentage or mean fluorescence intensity of individual antigens with normal controls has been applied.[7,35] Difference from normal can be described as >0.5 log, 1 log, 2 log or as >1SD, >2SD, or >3SD. This allows ranking of the aberrancy in a relatively simple way. However, this analysis is dependent on a confidence interval of expression of antigens on reference normal samples for each institute. Matarraz *et al*. showed in this way that FCM was able to discriminate between normal or reactive bone marrow samples and MDS cases; their scoring system even discriminated low- and high-grade MDS patients.[7,35]
- The interpretation of expression patterns, i.e. the relation between two individual antigens, can be definitive or equivocal. A calculation of the degree of deviation from a normal pattern might improve evaluation. Currently, software programs are available that can merge numerous normal expression patterns. MDS samples could, therefore, be compared to normal samples and the program might be able to compute the deviation from normal, not only for individual antigens but probably even for a pattern.[61,62]
- Lineage infidelity marker expression may differ between blasts, granulocytes and monocytes. Clearly abnormal expression of certain antigens must take into account autofluorescence of each individual cellular compartment.
- Weighing of aberrant parameters in the design of an FCM scoring system must be clinically driven. This can be determined based on parameters from retrospective data (multivariate analysis). Some abnormalities may be informative for diagnosis, while others for prognosis.

Expression of lineage infidelity markers on (im)mature myeloid or monocytic cells might carry more weight than altered expression of myeloid and monocytic antigens and has been used in scoring systems for outcome.[4] For instance, expression of CD7 on myeloblasts was shown to correlate with poor clinical outcome, and CD15 expression on myeloblasts was associated with good prognosis.[6,8,9] Another issue related to interpretation of data regards the minimal number of abnormalities by FCM necessary to diagnose a patient as having MDS. The presence of multiple aberrancies has a higher predictive value for MDS than single aberrancies.[4,5,21,47] The weight of a parameter in a scoring system must be validated in a prospective study. Of note, retrospective analysis of markers and marker combinations can only be performed after this list has been defined.

Conclusions

Flow cytometry is an increasingly important technology in the diagnosis and prognostication of hematopoietic neoplasms. In MDS, FCM is also regarded as a new forthcoming standard, although several questions

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remain to be solved. The consensus working group has started to address these issues in Amsterdam, and made significant progress in proposing standards for adequate sampling and processing of bone marrow cells for FCM. In addition, the group proposed antibody combinations to define dysplasia as well as diagnostic and prognostic FCM patterns. The group also concluded that FCM reports should always be descriptive in nature, with a statement that findings could be consistent with MDS. The working group is dedicated to initiate further studies to establish commonly accepted standards and to establish robust diagnostic and prognostic markers and marker-patterns in MDS, with the ultimate goal being to refine/improve diagnosis and prognostic scoring systems.

Authorship and Disclosures

All persons listed as co-authors contributed to pre-conference and post-conference discussions (January until November, 2008) and actively participated in the Standardization Conference (Amsterdam, 2008, March 27-28). All co-authors contributed equally by discussing criteria, standards, algorithms, and recommendations at the Working Conference.

AAvdL was chair and MRL and TMW were co-chairs of the conference.

In addition, all persons listed as co-authors provided essential input by drafting parts of the manuscript and by approving the final version of the document.

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