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Distinct neutrophil subpopulations phenotype by flow cytometry in myelodysplastic syndromes

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Abstract
The cardinal feature of myelodysplastic syndromes (MDS) is dysplasia involving one or more myeloid cell lineages. In the present study, we used 4-color flow cytometric analysis to investigate dysgranulopoiesis in bone marrow specimens from 65 patients with MDS. The antigen expression patterns of total neutrophil granulocytes (TNG) and of the two distinct neutrophil granulocytic subpopulations (NGSs), NGS-1 (dimmer CD45 expression) and NGS-2 (stronger CD45 expression) identified on the side scatter (SS) *vs.* CD45-intensity plot, were studied. The neutrophil granulocytes from patients with MDS showed characteristic antigen expression aberrancies which were more pronounced in NGS-2 subpopulation. Studying separately the NGS-2 subpopulation with the CD16/MPO/LF combination, the low $CD16^+/MPO^+$ and low $CD16^+/LF^+$ percentages seemed to discriminate between lower-risk and higher-risk patients with MDS in most occasions. Furthermore, a detailed assessment of the NGS-1 and NGS-2 immunophenotypic patterns revealed early dysplastic changes, not otherwise observed by standard TNG analysis, especially in cases of lower-risk MDS.

Keywords: *Myelodysplastic syndrome, immunophenotype, flow cytometry, neutrophil granulocytes, myeloperoxidase, lactoferrin*

Introduction

The myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal hematological disorders characterised by dysplasia and ineffective hematopoiesis [1–3]. The majority of patients present with symptoms related to cytopenia(s), most frequently anaemia and less commonly neutropenia and thrombocytopenia. Patients with MDS carry an approximate 25% risk of progression to acute myeloid leukaemia [3]. The hallmark of MDS is morphologic dysplasia involving one or more bone marrow (BM) myeloid cell lineages. Although the diagnostic criteria for MDS are well established, a significant number of patients with MDS display peripheral blood (PB) and BM findings without robust diagnostic morphologic characteristics [4,5–8]. Recently, refined definitions and standards in the diagnosis and treatment of MDS were proposed at an International Working Conference in Vienna (2006). In the proposed minimal essential criteria to meet the diagnosis of MDS, BM flow cytometric analysis was introduced as a useful co-criterion in cases where morphology and cytogenetics are equivocal [9]. The use of flow cytometric data is not only limited to diagnostic purposes but also may have potential prognostic value for the patients.

In the present study, we used multicolor flow cytometry (MFC) to examine the expression of myeloperoxidase (MPO), lactoferrin (LF) and CD16

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antigen (CD16/MPO/LF combination) in the population of BM total neutrophil granulocytes (TNG) in marrow aspirates obtained from both patients with MDS and controls. Our aim was to assess the utility of the CD16/MPO/LF combination as a potential representative marker of granulocytic dysplasia. Moreover, we used an extensive monoclonal antibody combination panel for several granulocyte-associated antigens as well as staining for monocytic and lymphocytic antigens in order to obtain the most relevant analytical and precise information. It is known that total granulocyte-lineage cells can be divided into two major subpopulations on the basis of distinct CD45 fluorescence intensity in a CD45 *versus* side scatter (SS) dot plot. These phenotypic subpopulations (neutrophil granulocytic subpopulations, NGS-1 and NGS-2) are the sequel of the granulocyte series differentiation and maturation in the BM and can be identified in healthy subjects as well as in most patients with MDS. Herein, we report that the flow data obtained from the analysis of the NGS-1 and NGS-2 immunophenotypes might provide additional information in the diagnosis and prognosis of patients with MDS.

Materials and methods

Patients characteristics

BM aspirate specimens from 65 patients with MDS and MDS/myeloproliferative disorder (MDS/MPD) were studied using multiparameter flow cytometry. The specimens were referred to the Immunology-Histocompatibility Department of 'Evangelismos' Hospital from the Hematology and Lymphomas Department of 'Evangelismos' Hospital and the Hematology Department of the '251' General Hospital of Athens. All patients had a definite diagnosis of *de novo* primary MDS or MDS/MPD disease based on medical history, clinical data, cytomorphology of BM and PB smears, BM biopsy and cytogenetics. The patients were diagnosed according to the WHO classification standards with RA/RCMD $\pm$ RS ($n = 18$), RAEB-1 ($n = 14$), RAEB-2 ($n = 20$) and MDS/MPD ($n = 13$) (Table I). RCMD $\pm$ RS patients were grouped together with those belonging to the RA $\pm$ RS category because of similarities regarding the granulocytic immunophenotype characteristics. The WHO classification discriminates MDS/MPD from MDS in terms of clinical and pathological findings. However, after a detailed comparative analysis of the granulocytic immunophenotypes in patients with MDS/MPD and MDS no significant differences in antigen expression profiles were found between the two groups (data not shown) and patients with MDS/MPD were included in this study.

Table I. Patient information.

MDS classification	RCMD/ RA $\pm$ RS	RAEB-1	RAEB-2	MDS/ MPD
Number of cases	18	14	20	13
Normal: abnormal karyotype*	12:1	6:3	13:6	6:3
Sex (male: female)	10:8	8:6	13:7	9:4
Age (years)	67–80	28–82	50–82	66–80

*Karyotypic data available only in 50 cases (data not shown).
MDS, myelodysplastic syndromes; MPD, myeloproliferative disorder.

Patients were grouped as 'lower-risk MDS' and 'higher-risk MDS'. Patients with 'lower-risk MDS' includes relatively low-risk patients who are in the international prognostic scoring system (IPSS) low/intermediate-1 prognostic category, whereas patients with 'higher-risk MDS' fit in the intermediate-2/high IPSS category. These two major prognostic risk groups have been recently proposed by the National Comprehensive Cancer Network (NCCN) expert panel to guide therapeutic options [10].

BM specimens from 19 patients with non-myeloid disorders were used as controls. The control group included one healthy BM donor, one patient with chronic renal disease, eight patients with iron deficiency anemia, one patient with essential thrombocythemia, seven individuals with limited-stage non-Hodgkin lymphoma and one patient with stage I Hodgkin lymphoma. The marrows in the lymphoma cases were performed for staging purposes prior to any therapy and no dysplastic changes were detected by cytomorphology or histopathology.

Specimens preparation

A standardised methodology was used for the preparation of specimens. EDTA anticoagulated BM aspirate specimens were collected and processed within 4–6 h using (a) Immunoprep™ Reagent System [Beckman Coulter (BC), Miami] for red cell lysing and surface markers staining and (b) IntraPrep™ Permeabilisation Reagent (BC) for cytoplasmic markers staining. Four-color MFC was performed on an EPICS Coulter® XL-MCL™ Flow Cytometer (BC) using commercially available reagents and the appropriate staining procedures recommended by the manufacturing company.

The panel of monoclonal antibodies (clones in parenthesis) included: (a) fluorescein isothiocyanate (FITC)-conjugated specific clones: CD10 (J5, BC), CD15 (MSC-1, Cytognos, SL, Salamanca, Spain), CD16 (3G8, BC), CD34 (8G12, BDB), CD64 (MCA756F, AbD Serotec, Oxford, UK), HLA-DR

(L243, BDB), KORSA (KOR-SA3544, BC) and anti-MPO (CLB-MPO-1, BC), (b) Phycoerythrin (PE)-conjugated specific clones: CD11b (D12, BDB), CD13 (L138, BDB), CD33 (D3HL60.251, BC), CD117 (95C3, BC) and anti-LF (CLB13.17, BC), (c) PE-Texas Red-x (ECD)-conjugated with panleucocyte common antigen CD45 (J33, BC) and (d) PE-Cyanin 5 (PC5)-conjugated specific clones: CD14 (RM052, BC), CD16 (3G8, BC), CD33 (D3HL60.251, BC) and CD34 (581, BC). MPO and LF molecules were detected by intracellular staining. All the other molecules were detected by surface staining.

Flow cytometric analysis

CD45-ECD was used in all tubes in order to gate TNG using SSc and CD45 expression. TNG were identified as CD45(dim-bright)/SS(int-high), as defined by Van de Loosdrecht *et al*. [10]. The two subpopulations within the TNG gate were analysed in the gates of dimmer CD45 (NGS-1) and stronger CD45 (NGS-2) signal in a CD45-SS-plot. The NGSs gating is illustrated in Figure 1. At least 100 000 events were acquired in most occasions and at least 30, 000 in cases with limited specimens volume or marked marrow hypocellularity. In the $CD45^{weak}/SS^{low}$ gate, 1000–10, 000 events were required in order to ensure good sensitivity and an adequate detection limit.

Discriminating between the two granulocytic subpopulations on the basis of a SS-*vs*-CD45 dot plot is not always easy. This depends not only on the type of the MDS but also largely on the quality of the specimen. In our exprerience, about 15% of patients with MDS did not have 'clear-cut' granulocytic subpopulations. In the cases in which only one population existed we could consider it as belonging to one of the two subpopulations depending on the dominant expression pattern of maturational antigens.

The use of MFC with an extensive monoclonal antibody combination panel was considered a requisite for obtaining the most detailed analytical information. Moreover, staining for monocytic specific antigens (CD14 and CD64) was important for the exclusion of monocytes from the granulocytes gate. Within each granulocytic compartment, the expression of several antigens and the phenotypic patterns of maturation were analysed and the results were compared with the BM control specimens.

Flow cytometry analysis quality assurance

Quality Assurance Programs regarding instrument and specimens as well as isotypic controls and internal controls were used in most specimens to confirm the good performance of the technique. Instrument set-up was performed weekly in order to check and adjust photomultiplier tubes and compensation settings for the acquisition of comparable results. Method validation (data not shown) was accomplished in order to give reliable results.

Data collection analysis

Data (collected in list mode) were analysed using XL2 (System II^{TM} Software Version 3.0) and CXP Software of Beckman Coulter.

Statistical analysis

Statistical analysis was carried out with the statistical program SPSS 12.0. In the first part of the analysis, (a) non-parametric tests for two independent specimens were used for all variables concerning 'controls' and 'patients with MDS' and (b) non-parametric tests for K-independent specimens were used for all variables concerning 'controls', patients with 'lower-risk MDS' and 'higher-risk MDS'. In the second part of the analysis, the NGS-1 and NGS-2 subpopulations from patients with MDS and controls were analysed by non-parametric two related specimens test. Non-parametric test for K-independent specimens was used for all parameters and groups under study.

Results

Investigation of total neutrophil granulocytes phenotype in myelodysplastic syndromes

Comparing the TNG antigen expression patterns from patients with MDS with the control group, the most important findings were as follows: low mean SSc, low CD16 expression as well as low $CD11b^+/CD16^+$, MPO^+/LF^+, $CD16^+/MPO^+$ and $CD16^+/LF^+$ percentages. The high $HLA\text{-}DR^+$ and $HLA\text{-}DR^+/CD13^+$ percentages in MDS TNG proved also statistically important. No important differences were noted between patients and controls regarding all the other antigens studied.

The immunophenotype of granulocytes from MDS cases was further investigated and a comparison between lower-risk MDS ($n = 26$) and higher-risk MDS ($n = 32$) was accomplished. Lower MPO^+/LF^+, $CD16^+/MPO^+$ and $CD16^+/LF^+$ percentages were found in lower-risk than in higher-risk MDS. A number of aberrant phenotypic patterns including reduced expression of CD16 and low $CD16^+/CD11b^+$ co-expression percentages were similarly observed in lower-risk and higher-risk

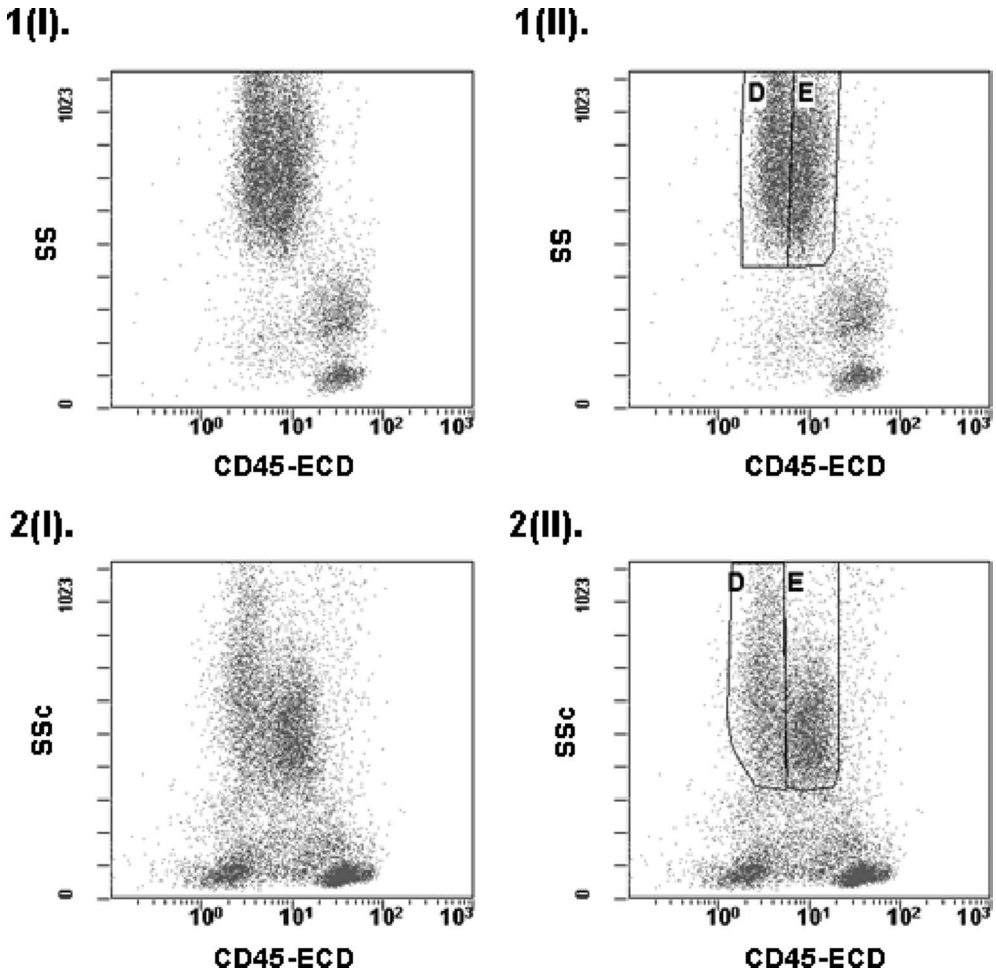

Figure 1. Analysis of the two neutrophil granulocytic subpopulations, NGS-1 (gate D) and NGS-2 (gate E) using the SSc = f(CD45 expression) dot plot (NGS-1 = dimCD45 and NGS-2 = strongCD45 fluorescence intensity). Dot plots 1 represent the control specimen and dot plots 2 are derived from patient with MDS.

MDS. Higher HLA-DR expression as well as higher $HLADR^+/CD13^+$ co-expression percentages were observed in higher-risk *versus* lower-risk MDS (Table II) (Figures 2 and 3). The TNG phenotypic findings did not show any correlation with the cytogenetic data.

Neutrophil granulocytic subpopulations

In both patients with MDS and control group, similar CD45 mean fluorescence intensity (MFI) values were detected, allowing thus for a reliable NGSs discrimination.

Phenotypic identification of neutrophil granulocytic subpopulations in control group. The analysis of granulocytic subpopulation-1 (NGS-1) *versus* granulocytic subpopulation-2 (NGS-2) showed significantly higher SSc, lower CD16, CD10 and CD13 expressions as well as lower $CD16^+/CD11b^+$, $CD16^+/MPO^+$ and $CD16^+/LF^+$ percentages in NGS-1. Stable well-defined gates for CD16/CD11b, MPO/LF, CD16/MPO and CD16/LF were set according to the MFC analysis of the control specimens and kept throughout the subsequent analyses of all the patients' specimens.

In the control group, the NGS-1 represents a cell 'mixture' of different maturational stages such as promyelocytes ($CD16^-/CD11b^-$), myelocytes ($CD16^-/CD11b^+$), metamyelocytes ($CD16^+$dim/$CD11b^+$) and bands ($CD16^+$strong/$CD11b^+$) as presented in Figure 2 [dot-plot 1(III)], whereas control NGS-2 consists mainly of mature neutrophil granulocytes expressing $CD11b^+$, $CD16^+$, MPO^+, LF^+ and $CD13^+$ in high percentages [Figure 2, dot-plots 1(IV) and 3(IV)]. Both control, NGS-1 and NGS-2, expressed high percentages of MPO and LF and a very low percentage of HLA-DR. Important phenotypic findings of control NGS-1 and NGS-2 are presented in Table III.

Table II. TNG analysis of lower-risk and higher-risk MDS cases.

Parameters	TNG mean values ± standard deviation			*p*
	Control	Lower-risk MDS	Higher-risk MDS	
SS	678 ± 117	625 ± 163	656 ± 174	NS
$CD16^+$ (%)	75.2 ± 7.8	63 ± 13	57 ± 20	<0.001
$CD16^+/CD11b^+$ (%)	72.1 ± 6.7	58 ± 17	56 ± 22	0.005
MPO^+/LF^+ (%)	72 ± 20	26 ± 25	41 ± 22	<0.001
$CD16^+/MPO^+$ (%)	56 ± 16	19 ± 19	24 ± 25	<0.001
$CD16^+/LF^+$ (%)	52 ± 16	15.3 ± 9.6	23 ± 20	<0.001
$HLA\text{-}DR^+$ (%)	3.4 ± 2.6	7.6 ± 5.5	14.3 ± 8.2	<0.001
$HLA\text{-}DR^+/CD13^+$ (%)	1.7 ± 1.7	6.3 ± 4.6	11.9 ± 8.2	<0.001

MDS, myelodysplastic syndromes; TNG, total neutrophil granulocytes; NS, not statistically significant; SSc, side scatter; MPO, myeloperoxidase; LF, lactoferrin.

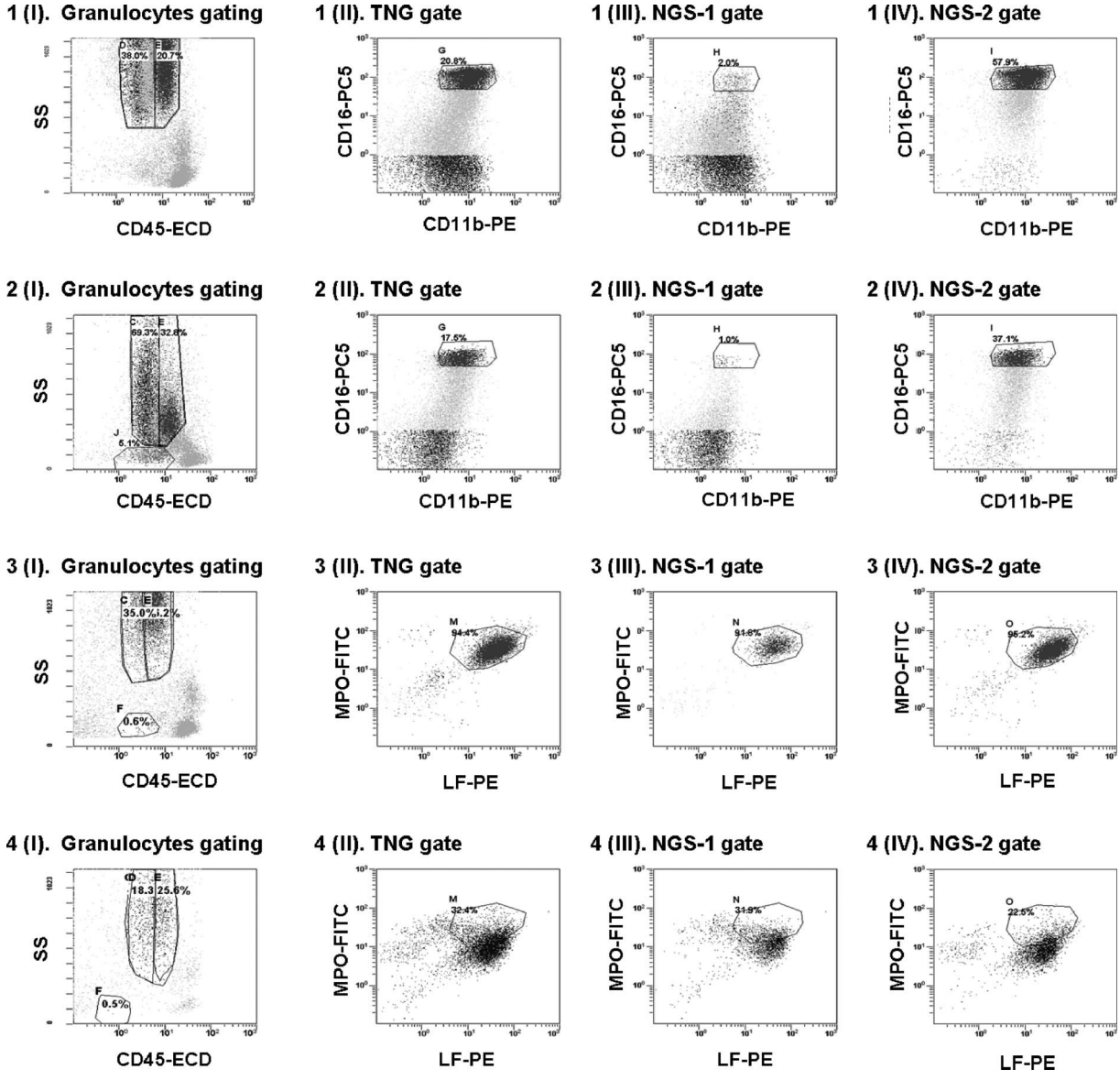

Figure 2. Examples of total neutrophil granulocytes (TNG) and neutrophil granulocytic subpopulations (NGSs) phenotypic analysis. Examples 1 and 3 represent control specimens whereas 2 and 4 represent lower-risk MDS patients. Dot plots 1 and 2 show the expression pattern of CD16 and CD11b. Dot plots 3 and 4 show MPO/LF expression patterns.

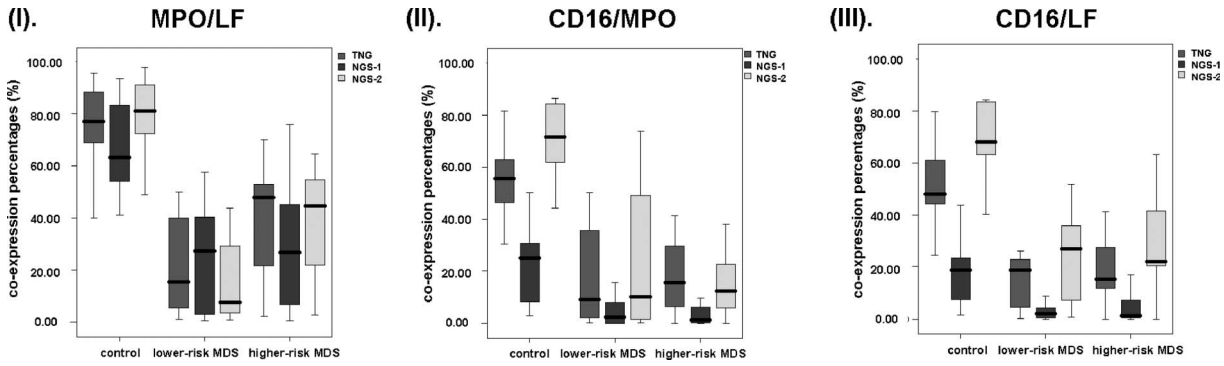

Figure 3. Statistically significant findings of (I) MPO/LF, (II) CD16/MPO and (III) CD16/LF co-expression in TNG, NGS-1 and NGS-2 of controls, lower-risk and higher-risk patients with MDS.

Table III. NGS-1 and NGS-2 findings of controls and patients with MDS.

Parameters	Control	MDS	*p* (control *vs.* MDS)	Lower-risk MDS	*p* (control *vs.* lower-risk MDS)
NGS-1 mean values ± standard deviation					
CD45 MFI	3.8 ± 2.5	5.2 ± 2.4	NS	4.5 ± 1.9	NS
SS	709 ± 124	662 ± 169	NS	647 ± 154	NS
$CD16^+$ (%)	54 ± 15	36 ± 22	0.004	46 ± 19	NS
$CD10^+$ (%)	13 ± 13	20 ± 17	NS	20 ± 17	NS
$CD13^+$ (%)	89 ± 12	81 ± 19	NS	78 ± 22	NS
$CD16^+/CD11b^+$ (%)	49 ± 10	30 ± 16	<0.001	37 ± 14	0.013
HLA-DR^+ (%)	3.9 ± 4.9	13 ± 13	<0.001	6.4 ± 6.5	0.047
MPO^+/LF^+ (%)	67 ± 18	31 ± 25	<0.001	25 ± 21	<0.001
$CD16^+/MPO^+$ (%)	22 ± 15	7.5 ± 14.2	<0.001	5.6 ± 8.7	<0.001
$CD16^+/LF^+$ (%)	17 ± 12	6.4 ± 13.6	<0.001	3.4 ± 4.4	<0.001
NGS-2 mean values ± standard deviation					
CD45 MFI	9.8 ± 6.6	12.2 ± 5.0	NS	11.0 ± 5.0	NS
SS	677 ± 128	616 ± 170	NS	576 ± 148	0.045
$CD16^+$ (%)	92.7 ± 3.7	78 ± 16	<0.001	85.6 ± 9.9	0.009
$CD10^+$ (%)	67 ± 24	53 ± 19	0.006	54 ± 17	0.033
$CD13^+$ (%)	99.57 ± 0.40	96.7 ± 5.1	0.016	96.7 ± 6.3	NS
$CD16^+/CD11b^+$ (%)	89.0 ± 9.7	75 ± 17	<0.001	85.6 ± 6.0	0.012
HLA-DR^+ (%)	4.0 ± 5.3	10.2 ± 7.5	<0.001	7.1 ± 4.4	0.002
MPO^+/LF^+ (%)	78 ± 19	32 ± 26	<0.001	17 ± 16	<0.001
$CD16^+/MPO^+$ (%)	71 ± 15	25 ± 25	<0.001	26 ± 29	<0.001
$CD16^+/LF^+$ (%)	69 ± 16	28 ± 19	<0.001	25 ± 18	<0.001

*NS: Not statistically significant; MDS, myelodysplastic syndromes; NGS, neutrophil granulocytic subpopulations; MPO, myeloperoxidase; LF, lactoferrin.

Phenotypic investigation of neutrophil granulocytic subpopulations in myelodysplastic syndromes. The NGS-1 in MDS is a cell 'mixture' of different maturational stages with abnormalities such as a higher percentage of myelocytes and an absence of metamyelocytes and bands as revealed from the patterns presented in Figure 2 [dot-plot 2(III)]. The NGS-2 in MDS is a mature population having high $CD10^+$ and $CD13^+$ percentages but with dysplastic features such as abnormal expression of HLA-DR^+ and lower $CD16^+$, LF^+, $CD16^+/CD11b^+$, MPO^+/LF^+, $CD16^+/MPO^+$, $CD16^+/LF^+$ percentages, compared with the control NGS-2 [Figure 2, dot-plots 2(IV) and 4(IV)] (Table III).

We studied separately the NGS-1 phenotypic changes and found that $CD16^+$ and $CD16^+/CD11b^+$ percentages seem to decrease progressively moving from controls to lower-risk and to higher-risk MDS whereas the percentages of cells expressing HLA-DR seem to increase in parallel. These differences between the three groups were more pronounced in NGS-1 than in TNG or NGS-2. The MPO^+/LF^+, $CD16^+/MPO^+$, $CD16^+/LF^+$ co-expressions were more decreased in lower-risk than

in higher-risk MDS, and that could be demonstrated in both NGS-1 and NGS-2 subpopulations (Table III and Figures 2 and 3).

When the aforementioned variables were analysed in the setting of different MDS WHO categories, the MPO^+/LF^+, $CD16^+/MPO^+$ and $CD16^+/LF^+$ co-expression percentages did not seem to discriminate between the different MDS categories. The NGSs phenotypic findings did not show any correlation with the cytogenetic data.

Discussion

Many studies have addressed the role of flow cytometry immunophenotyping in the study and diagnosis of MDS [4–7,11–27]. In general, flow cytometric analysis should not be restricted to the calculation and characterisation of BM blast cells but must also include the evaluation of the granulocytic surface markers expression [4–7,11–28]. Several authors comment on the aberrant antigen expression patterns of the granulocytes in cases of MDS and how these phenotypic abnormalities correlate with the cytomorphologic features [4,5,13–16,18,20,21]. For that reason, the assessment of the granulocytic phenotype is of great value in the immunophenotypic evaluation of dysplasia.

Normal BM phenotypic characteristics have been well described by several investigators in the past [29–31]. Stetler-Stevenson *et al.* showed that flow cytometric immunophenotyping was highly sensitive in detecting myeloid abnormalities such as granulocytic hypogranulation, abnormal co-expressions, asynchronous expression of antigens and deranged maturation in challenging cases of equivocal or indeterminate morphology [4]. The most important diagnostic findings in assessing dysgranulopoesis in were proved to be: (i) side scatter (SS) variability and (ii) asynchronous expression of CD11b and CD16 antigens. Several other reports have confirmed these observations [5–7,24].

The investigation of TNG population from MDS cases in comparison to the control group in our series showed the typical flow cytometric findings of dysplasia such as decreased SS and asynchronous expression of CD16 and CD11b antigens. Taking a step forward, our findings revealed additional phenotypic patterns of dysplasia such as the decreased MPO/LF, CD16/MPO and CD16/LF co-expression percentages in MDS TNG. These aberrancies reflect the maturational abnormalities of the granulocytic series in the BM of patients with MDS and can be used as additional markers for diagnostic purposes (Figure 2).

The disturbed expression of MPO in the neutrophil granulocytes from MDS has been reported in the past [32]. The reduced staining of neutrophil granulocytes for MPO by standard cytochemistry and immunohistochemistry has been long considered a marker of functional defect (acquired MPO deficiency) and therefore a sign of dysplasia. The investigation of LF expression in MDS using immunocytochemistry did not show significant disturbances and was not considered an important marker in previous studies [32]. In this report, the MFC analysis of 4-color combination of CD16/MPO/LF and CD45 yielded significant results. Decreased co-expression of MPO/LF, CD16/MPO and CD16/LF indicates maturational abnormalities of the granulocytes. The CD16 antigen is expressed preferentially in neutrophils whereas MPO and LF are specific markers for primary (azurophilic) and secondary (neutrophilic) granules, respectively [32]. We believe that by using more than one specific markers for mature granulocytes the gating and the identification of these cells is ensured, especially in MDS cases where the dysplastic granulocytes display phenotypic characteristics of other lineages such as the bright CD45 and the SS of monocytes. Staining for LF is very important because it can distinguish monocytes with aberrant CD16 expression from neutrophils with abnormally strong CD45 expression. MDS cases are sometimes difficult to diagnose especially in early stages. In these situations, we suggest that the MPO/LF combination pattern may reveal important differentiation abnormalities of the neutrophil granulocytes and provide ancillary information indicative of dysplasia.

The comparison of TNG immunophenotypes between lower-risk and higher-risk patients with MDS demonstrated interesting findings. The different phenotypic patterns of dysplasia in lower-risk and higher-risk MDS should correspond to the maturation disturbances that are expected to be more prominent in the higher-risk than in the lower-risk MDS. Taking this into consideration, lower SSc and lower percentages of MPO^+/LF^+, $CD16^+/MPO^+$ and $CD16^+/LF^+$ were supposed to characterise higher-risk MDS. However, our findings did not confirm this hypothesis because the more pronounced disturbances concerning MPO and LF expression were found in lower-risk patients with MDS. Nevertheless, this finding offers help in the diagnosis of lower-risk MDS by discriminating them from controls. The population of TNG from higher-risk MDS cases displayed abnormal expression of HLA-DR and the aberrant $HLA\text{-}DR^+/CD13^+$ combination, a finding that has also been reported by Karmon *et al.* [18]. It should be noted that $CD16^+/CD11b^+$ co-expression does not discriminate between lower-risk and higher-risk patients with MDS.

Our findings emphasise the importance of identifying the two distinct NGSs on the basis of a SS-*vs*-CD45-fluorescence intensity dot plot in patients with MDS. The two NGSs have been mentioned before [17] but without an extensive phenotypic analysis. Instead, we used an extensive monoclonal antibody combination panel in order to get adequate information as well as to confirm the accuracy of the flow cytometric analysis (by excluding non-granulocytic myeloid cells). Analysis and comparison of the two NGSs in controls and in patients showed that the two subpopulations comprise of cells in different maturational stages; NGS-2 is more mature (having a higher percentage of last maturational granulocytes i.e. strongCD16/strongCD11b and higher expression of CD16, LF, CD11b antigens) than NGS-1.

The NGS-1 in MDS displays certain maturational disturbances such as the higher percentage of promyelocytes, myelocytes and the absence of metamyelocytes and bands, as revealed by the antigen expression patterns presented in Figure 2. These findings underline the prominent dysplastic changes and the 'left-shift', typical of the higher-risk MDS. Therefore, the analysis of CD16, HLA-DR and CD16/CD11b expressions in the NGS-1 gate offers a clearer distinction between controls, lower-risk and higher-risk MDS than gating TNG. The NGS-2 in MDS is a mature population having high CD10, CD13 but with dysplastic features such as aberrant expression of HLA-DR, and lower CD16, LF, CD16/CD11b, MPO/LF, CD16/MPO, CD16/LF percentages. It should be mentioned that very low $CD16^+/MPO^+$ and $CD16^+/LF^+$ co-expression percentages were identified in some individuals with lower-risk MDS especially when the NGS-2 was separately studied. We think that these findings are consistent with the dysplastic characteristics of these patients.

Our work indicates that the multicolor flow cytometric analysis of NGSs is a useful aid in diagnosing MDS, especially regarding the lower-risk patients with MDS. This observation is important because sometimes the immunophenotypic disturbances in TNG are minor and not obvious, whereas the separate examination of the two NGSs (NGS-1 and NGS-2) might reveal the dysplastic features that otherwise would have been missed. Therefore, the NGSs phenotypic analysis (especially of the NGS-2 subpopulation) using specific markers and patterns of expression, as described, seems to offer a clear advantage for dysplasia screening. By studying NGSs, 'advancing degrees' of dysplasia can be identified among the lower-risk patients with MDS that frequently have few dysplastic features in their TNG. This observation might be of particular interest if the BMs of patients with MDS are serially assessed. It is conceivable that our ability to estimate and follow-up minor and advancing dysplasia by flow cytometry could help substantially in the development of a diagnostic and prognostic tool for patients with MDS.

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