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Multiparameter Flow Cytometry: An Important Auxiliary Tool for The Diagnosis and Differential Diagnosis of Myelodysplastic Neoplasms

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Abstract: Multiparameter flow cytometry (MFC) shows promise in the diagnosis and differential diagnosis of myelodysplastic neoplasms (MDS). By identifying abnormal immunophenotypes of different cell lineages in bone marrow (BM) cells, MFC can aid in the differential diagnosis of some bone marrow failure diseases. Several flow cytometry scoring systems have been established based on current parameters, but there is still no specific immunophenotype available for the diagnosis of MDS. Morphological examination of cells remains the gold standard for MDS diagnosis. In the future, identifying specific cell immunophenotypes may improve MDS diagnosis and alter the use of MFC. MFC may also provide a solution for tracking the evolution of specific abnormal hematopoietic clones in MDS.

Keywords: Myelodysplastic neoplasms; Multiparameter flow cytometry; Immunophenotype; Diagnosis.

Introduction

MDS are a group of disorders that arise from clonal hematopoietic stem cells (HSCs). These diseases are marked by malformation and abnormal differentiation in the BM, resulting in low blood cell count and inefficient cell proliferation. This can lead to symptoms of BM failure and an increased risk of developing acute myeloid leukemia (AML). Therefore, it is crucial to diagnose and treat MDS patients promptly in clinical settings[1–6].

Under previous World Health Organization (WHO) standards, diagnosing MDS mainly relied on analyzing the shape of cells in peripheral blood and BM samples, as well as the presence of genetic alterations such as an elevated count of ring sideroblasts and abnormal chromosomes[5]. The 2016 version of the WHO standard includes dysplastic lineages and cytopenias as criteria for the morphologic subtyping of MDS[6–8]. However, the evolution of abnormal hematopoietic clones in different MDS patients is diverse, and the cell lineages affected by pathologic hematopoiesis are also diverse. Distinguishing between a single or multiple lineages of hematopoietic dysplasia to differentiate subtypes of MDS has limited clinical significance for treatment. The latest 2022 update of the WHO classification for MDS has streamlined the previous typology. This update emphasizes the significance of blast cells in determining the diagnosis and prognosis of MDS and supports the differentiation between low and high-grade MDS[9–11]. This highlights the necessity for supplementary testing methods, such as immunophenotyping of BM cells through flow cytometry, in order to track the progression of abnormal cell clones.

Although the morphological examination is still considered the most accurate method for diagnosing MDS, it is subjective in nature. There may be variations in the blood smear reading practices between different laboratories, and the quality and process of the blood smear preparation can significantly affect the results. Despite this, MFC has been shown to have a positive impact on the diagnosis and differentiation of MDS[12–16].

MFC can be utilized to detect abnormal expression patterns of multiple immune phenotypes in MDS patients on both BM and peripheral blood cells. Despite the lack of reports indicating the use of immunophenotypes as a standalone diagnostic tool for MDS, recent years have seen numerous studies aimed at discovering novel immunophenotypes that may indicate abnormal hemocytes development and developing MFC scores to aid in the diagnosis and prognosis of MDS, as well as inform treatment decisions. The integration of MFC has indeed enhanced the diagnosis of MDS, particularly in differentiating it from other disorders such as hypoplastic MDS (MDS-h), Paroxysmal nocturnal hemoglobinuria (PNH), Aplastic anemia (AA), and T-cell large granular lymphocyte leukemia (T-LGLL), all of which may present with symptoms of BM failure and ineffective hematopoiesis.

This article comprehensively discusses the application value of MFC in the diagnosis and differential diagnosis of MDS by summarizing the abnormal immunophenotypes and Flow Cytometry Scores (FCS) that are helpful for the diagnosis of MDS.

1. Flow Cytometry to Detect the Dysmaturation Hematopoietic Cells in MDS

The pathological diagnosis of MDS is based on hematopoietic dysplasia in BM smears. The use of flow cytometry to quantify and qualify the immunophenotype of BM blood cells can help identify cytopenia with no morphological abnormalities. This paper summarizes the immunophenotypes of blast cells (as shown

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in Table 1), granulocyte, monocyte, and erythrocyte lineages cells (as shown in Table 2) in the BM of MDS patients, as well as the flow cytometry scoring system. Additionally, a brief description of other abnormal cells associated with MDS in the BM microenvironment is provided to aid in the identification of immunophenotypic markers of MDS.

Table 1. Blast dysmaturation in MDS and cellular physiological significance.

Abnormal Immune Phenotypes	Indicate
CD34 decreased	Dysplasia precursors[17]
Low CD38 and CD19 Expression in B-Cell Progenitor Cells	Abnormal B-cell progenitor development or increased poorly differentiated stem cells, lead to developmental issues[18]
High levels of immature hematopoietic progenitor cells	$CD34^+$ cell differentiation is impaired, mainly in secondary AML and high-risk MDS[19]
CD2, CD5, CD7, and CD56 are expressed abnormally	Whether the expression of CD7 has an impact on a worse prognosis is somewhat debatable[20,21]
The maturation process is characterized by asynchronous expression of CD10, CD15, and CD11b	Maturation process hindered[17,20,22]
Increased expression of CD117	This may suggest high proliferation rate of clonal cells in MDS and advancement of the disease[23]

Table 2. Immunophenotypes of normal versus MDS myeloblast, granulocyte, monocyte, and erythrocyte.

	Granulocyte	Monocyte	Erythrocyte
BM of MDS (abnormal expressions)	▪Abnormal CD15 expression[12,13,17,22,24–27] ▪No re-expression of CD33 on mature granulocytes[12,13,17,22,24–27] ▪CD5, CD7, CD19, and CD56 are expressed across multiple lineages[21,28–30]	▪Reduced SSC with abnormal cell granularity formation[31] ▪The frequency of mature monocytes expressing CD300e is abnormally low[24] ▪Reduced expression of CD13, CD14, CD15, CD36, CD64, CD11b[22,24,31–33] ▪CD56, CD5, CD2, and CD7 are expressed across various lineages[34]	▪Increase in the number of immature cells in the erythroid lineage[35–42] ▪Reduced erythroid progenitor cells[35–43] ▪Aberrant expression pattern of CD235a and CD71[12,31] ▪Decrease in CD71±CD36-expressing MFI and increase in CV[40–43] ▪CD105 overexpression[36–42]
Normal BM (differentiation process)[25,31,44–52]	1. Granulocyte progenitor cells: $cyMPO^+$, $CD34^+$, $CD13^{hi}$, $CD33^{hi}$ 2. Gradual increase in CD15 and CD65 expression 3. CD34, HLADR, CD117, CD13, CD15 gradually downregulated 4. Access to specific functional molecules: CD11b, CD16, CD13 and CD10 $CD11b^{hi}$, $CD16^{hi}$, $CD13^{hi}$, $CD10^+$	1. Monocyte lineage progenitor cells: $CD34^+$, $CD64^+$ 2. CD64 expression upregulated, CD117 and CD34 expression gradually downregulated 3. Appearance of granulocyte/monocyte lineage antigens such as CD11b, CD11c, CD312, cyCD68, CD36, CD35, CD14, CD300e, and cyLysozym 4. Both immature monocytes (e.g., CD64CD14) and mature monocytes (e.g., CD300ehi) express L-selectin (CD62L+)	1. Erythroid lineage progenitor cells: $CD34^+CD105^+CD36^+$ 2. Gradual loss of CD34, HLA-DR, CD117, CD13 and CD33 3. Began to gradually express CD173, CD238 and CAR 4. CD105 expression gradually decreases while the expression of CD71, glycoprotein A, CD173, CD233, CD238, and CD239 begins 5. Loss of CD36 and CD71 begins during nucleus spitting

1.1. Blast dysmaturation in MDS

Blast count is a critical factor in morphologically diagnosing MDS. MFC can further analyze abnormalities in the immunophenotype of blasts, which is helpful for risk stratification and detecting minimal residual disease (MRD) of MDS.

In MFC, the $CD34^+$ $CD45^{dim}$ population is commonly used to identify blasts, including HSCs and HPC. The expression of CD7, CD19, CD38, and CD117 on $CD34^+$ cells is indicative of the differentiation, proliferation, and transformation of hematopoietic stem and progenitor cells[53–56].

Rigorously designed experiments have analyzed $CD34^+$ cell subpopulations and the immunophenotype of more than 90% of $CD34^+$ HPCs in normal human BM, which showed progressive differentiation. HPC primarily matures into neutrophils, erythroid cells, and lymphocyte lineages, with median values of 33%, 35%, and 23%, respectively. Approximately 10% of HPCs do not have a specific antigen expression spectrum and are not classified. Elevated levels of erythroid and neutrophil profile $CD34^+$ cells were found in low-grade MDS, whereas high-grade MDS displayed an increased proportion of $CD34^+$ plasmacytoid dendritic cells and B-cell precursors[31,44].

MDS patients may have low expression of CD19 and CD38 on $CD34^+$ blasts, indicating abnormal B-lymphatic progenitor cell development or an increased proportion of early or hypofractionated HSCs[18]. The increase in high-risk MDS and immature HPCs ($CD34^+$ $CD38^{dim}$) secondary to AML may indicate a hindrance in the differentiation of $CD34^+$ cells[19]. In MDS patients, CD34 expression may be absent in some dysplastic precursors. $CD34^+$ cells may also exhibit mature asynchronous expression of CD10, CD11b, and CD15, as well as abnormal expression of CD2, CD5, CD7, and CD56[17,20,22]. Elevated levels of CD117 on $CD34^+$ blasts may suggest that MDS clonal cells are

proliferating rapidly, which could also indicate disease progression[34,56,57].

In contrast, the relationship between CD7 expression and prognosis is somewhat controversial. It is now recommended that a small proportion of normal precursors in some post-treatment recovery periods may also express CD7[20,21]. The Flow cytometry (FCM)scores for CD34, CD19, CD38, CD117, and CD7 have been considered late scores as these antigens are abnormally expressed in high-grade MDS[23].

The latest classification of MDS, MDS-LB (MDS with low blasts), refers to low-grade MDS without ringed sidero blasts and encompasses previous classifications of refractory anemia, refractory cytopenia with multispectral heteroplasia, MDS untyped, and MDS associated with isolated Del (5q).

The scoring system proposed by Ogata et al. in 2006 uses four main core parameters to diagnose MDS: (1) the percentage of $CD34^+$ myeloid blasts; (2) the proportion of B lineage progenitor cells that express CD34; (3) the CD45 mean fluorescence intensity ratio of lymphocytes and myeloblasts; and (4) the Side Scatter (SSC) ratio of granulocyte to lymphocyte. Each parameter is assigned a weight of 1 point, with 1 point recorded above the threshold. A score of 0-4 points is given, with a score greater than 2 points being diagnostic of MDS. Della Porta et al. found that this scoring system is highly specific for diagnosing low-risk MDS and although slightly less sensitive, it is still useful for diagnosis[58–60].

Kiyoyuki O et al. enhanced the sensitivity of the Ogata score by including CD33 expression on $CD34^+$ cells[61]. In their study, Nikolaos et al. identified three $CD34^+$ cell populations that had a high diagnostic value for MDS: $CD34^+$ $CD45^{weak}$ cells, CD117-TdT $^+$ $CD34^+$ $CD45^{weak}$ cells, and $CD33^+$ MPO^- $CD45^{int/dim}SSC^{int}$ cells. These cell populations increased the specificity of the Ogata score. An elevated proportion of late apoptotic $CD34^+/CD117^+$ cells was found to be a robust indicator of a positive outcome in MDS, according to their findings[62].

According to the revised International Prognostic Scoring System (IPSS-R), cases of low-grade MDS with BM blasts ≤ 2% are associated with a lower risk of progression to AML and a better overall prognosis. In the analysis of MDS patients with BM blasts ≤ 2%, there was limited agreement among the different cytomorphologic evaluations of the blast count, even with high-quality BM smears[63–65]. The accuracy and reliability of immature cell counts in low-risk MDS cases with <5% BM blasts can be enhanced by adhering to stringent experimental conditions that involve acquiring at least 50,000 events and maintaining the presence of dead cells, cell debris, and cell clumps in BM samples to less than 5% by using a combination of CD45, CD34, CD117, and HLA-DR monoclonal antibodies, even though MFC has the potential to undercount primary cell blasts[66,67]. Subsequently, van der Velden et al. found that a minimum of 100,000 leukocytes should be obtained per tube to assess the number of BM blasts better[68]. Kern et al. conducted a multi-center study as part of the European LeukemiaNet international MDS Flow Working Group (ELN iMDS Flow WG), and confirmed that a population of 3% $CD45^{dim}/SSC^{low/int}$ in myeloblasts is a critical threshold for the diagnosis of MDS or MDS/MPN using MFC. It's important to exclude acute leukemia in these cases[24].

In conclusion, the primary abnormality in MDS-IB (MDS with increased blasts) patients is an increase in the number of blasts, but the increase in $CD34^+$ cells may not be observed during flow cytometry diagnosis due to hemodilution in MFC specimen. This typically occurs when a second aspiration of the BM puncture mixed with more peripheral blood is used for MFC (the first aspiration is used for morphology examination only), resulting in differences in the percentage of BM cell populations obtained through morphological analysis and FCM analysis. Therefore, it's important to note the abnormal immunophenotype of blasts with abnormal differentiation, which has significant implications for the prognosis and detecting MRD of these patients.

In MDS-LB patients, the abnormality in blasts is primarily related to progenitor cell differentiation, with a predominant abnormality in the percentage of B-lineage progenitor cells and myeloid progenitor cells. The Ogata score, which takes this as the main parameter, and the modified flow scoring system based on it are widely used and have shown great utility in differentiating MDS-LB from non-clonal cytopenia.

1.2. BM granulocyte and monocyte immunophenotypes in MDS

Normal BM contains hematopoietic cells that are constantly proliferating and differentiating, resulting in a variety of cell maturity levels and immunophenotypes across different lineages. MDS is a heterogeneous disease without specific diagnostic antigens, and the WHO recommends a diagnosis based on the presence of three or more immunophenotypic abnormalities, such as differences in cell size and granularity, abnormal antigen expression, cross-stage or cross-lineages antigen expression, and expression of cytokine receptors[6,17,69,70]. Due to the widespread use of MFC for leukemia immunophenotyping and MRD detecting, granulocyte and monocyte immunophenotyping has become more accessible and well-established.

In terms of cytomorphology, neutrophil hypogranulation is a key feature of myeloid dysplasia, which can be demonstrated as a downward shift in granulocyte scatter in CD45/SSC plots obtained by MFC. Ogata et al. first proposed a strict definition of this parameter using the SSC ratio of mature granulocytes to mature lymphocytes as an internal standard. This definition has since been widely adopted in clinical practice[58]. In normal humans, the expression of CD45 on mature granulocytes is moderately high (as measured by mean fluorescence intensity, MFI) compared to mature lymphocytes. In MDS patients, however, it is reduced[28]. A low number of neutrophils shows that the BM cells have limited ability to differentiate into granulocytes. This is included in the MFC scoring system. However, it can also occur in BM failure disorders, so the ELN iMDS Flow WG suggests using caution when applying this parameter[68].

Normal human neutrophil lineage cells typically account for 46-74% of the total number of nucleated cells in BM. As the granulocyte progenitor matures, there is a decrease in the expression of CD34, HLADR, CD117, and CD13, and an increase in the expression of CD15/CD65, and decreasing expression of CD34, HLADR, CD117, and CD13 as it matures. Mature neutrophil precursors then acquire surface membrane expression of specific functional molecules, including CD11b, CD16, CD13 (re-expressed on metamylocytes), and CD10. Finally, $CD11b^{hi}CD16^{hi}CD13^{hi}CD10^+$ segmented granulocytes and a few band granulocytes reach the peripheral blood[25,31,44].

Many teams have started to study the differences in antigen expression and differentiation patterns of granulocyte populations in MDS patients. They use combinations of markers, such as CD13/CD16/CD11b, CD13/CD33, CD15/CD33, and CD64/CD10, to examine irregularities in granulocyte maturation patterns, such as a lack of correlation between reduced CD15 expression and CD13/CD16/CD11b expression, decreased CD10 expression in conjunction with high CD15 expression, and absence of CD33 expression on mature granulocytes[12,13,17,22,24–27,29].

The expression of lymphoid antigens across different cell lineages during granulocyte maturation is crucial for evaluating MFC in MDS, such as the expression of CD5, CD7, CD19, and CD56[21,28,30,71]; However, it has been demonstrated that the expression of CD56 by granulocytes can also occur in the presence of inflammatory diseases, diabetes, and post-treatment conditions. As a result, the ELN iMDS Flow WG does not endorse the use of CD56 expression as a diagnostic tool for post-treatment MDS[72,73].

In a normal human BM, monocytes usually constitute 3-8% of the total nucleated cells. Additionally, monocyte lineage progenitor cells commonly express CD34 and CD64[31,44,45]. Progenitor cells originating from the monocyte lineage exhibit varying levels of CD64 expression along with decreased CD34 and CD117 expression at the outset. During the maturation process of monocyte lineage cells, a progressive expression of granulocyte/monocyte lineage antigens such as CD11c, cyLysozym, cyCD68, CD36, CD35, CD11b, CD14, CD300e, and CD312 is observed. Immature monocytes (e.g., $CD64^+$ $CD14^+$) and mature monocytes (e.g., $CD300e^+$) in the BM both express L-selectin (CD62L), although there can be some variation in expression among different monocyte subpopulations in peripheral blood and lymphocytes[25,45,46].

Similar to the granulocyte lineage, monocyte lineage cells from MDS patients also exhibit a decreased SSC compared to normal controls, indicating abnormal cell granulometry formation[21] and an abnormally low frequency of mature monocytes expressing CD300e. Furthermore, MDS monocyte lineage cells show abnormal patterns of differentiation antigen formation during maturation, such as abnormal combinations such as CD11b/HLA-DR, CD13/CD16, CD15/CD64. Cross-lineage expression of CD2, CD5, CD7, and CD56 may be observed in mononuclear cells from the BM of MDS patients[22,24,31–33].

In 2003, Wells et al. introduced the Wells flow cytometric scoring system (FCSS), which was based on a MFC phenotype assessment of primary cell blasts, granulocytes, and monocytes. The Wells FCSS assigns numerical values to various abnormalities and classifies results into three categories: normal/mild (0-1), moderate (2-3), or severe ($\geq$ 4) grade. Analysis of the results showed that the granulocyte, monocyte, and blast characteristics of the majority of MDS patients were different from those of normal individuals and were significantly different from those found in patients with other BM disorders. The Wells FCSS can effectively distinguish MDS patients, who have a score greater than or equal to 3, from the control group[21].

With ongoing research, the number of abnormal immunophenotypes in MDS is increasing, but quantifying these abnormal phenotypic results remains challenging.

In 2019, Barreau Sylvain et al. introduced the Diff score, which measures the differences in the differentiation pathways of granulocyte and monocyte populations between a sample from a suspected MDS patient and a normal reference database[28]. The Diff score is determined using a flow cytometry panel that includes CD34/CD45/CD13/CD14/CD16/CD33/CD10/CD11b/CD56/CD64. The MFI and standard deviations (SD) for each marker at different stages of granulocyte and monocyte maturation are calculated and compared to a reference threshold. Scores above the threshold are considered abnormal. Combining the Diff score with the well-established Ogata score significantly enhances the accuracy of MDS detection by improving both specificity and sensitivity.

Generally speaking, compared to other lineage cells, normal granulocyte and monocyte lineage cells can produce internal granules during their development, which can be evaluated by the SSC value using MFC and can serve as a useful indicator for the diagnosis of MDS. The abnormalities in the flow phenotype of MDS cells focus on quantifying cell populations (abnormal percentage), evaluating the reduced SSC values, characterizing the reduced number of mature antigen-expressing cells, and abnormal expression of immunophenotypes. Some institutions have developed different flow cytometry scoring systems to identify the most effective and practical panels for MDS detection, but these scoring systems still need to be validated by further research from multiple centers and standardized normal reference ranges.

1.3. Expression of BM Erythrocyte immunophenotypes in MDS

Most MDS patients exhibit anemia symptoms that are primarily associated with dysplasia of the erythroid lineage cells, which is characterized by an increased mean red blood cell volume, presence of basophilic spots, multinucleation, nuclear outgrowth, nuclear fragmentation, irregular chromatin clumping, and asynchronous maturation of the nucleoplasm[74].

The examination of cellular immunophenotypes during different stages of the erythroid lineage is highly relevant for the diagnosis of MDS. Current studies have centered on the alterations in immunophenotype in nucleated erythroid cells (NEC) to enhance the accuracy of MDS diagnosis through MFC[8,12,31,35,75]. Due to the vast quantity of erythrocytes, multi-parametric flow experiments typically need to restrict the number of cells that are detected. The MFC procedure involves lysing mature red blood cells. Variations in lysis reagents and sample collection sites can result in varying flow cytometric outcomes, thus limiting the detection of the immunophenotype of erythroid lineage progenitor cells. These primary methods for standardizing the test involve creating an appropriate hemolytic agent, establishing peripheral blood contamination index (PBCI), and continuously refining MFC protocols[47,76–78].

Early erythroid lineage progenitor cells in the BM have a phenotype of $CD34^+$ $CD105^+$ $CD36^+$ and progressively lose expression of CD34, HLA-DR, CD117, CD13, and CD33 during their maturation process. Additionally, they start to express lower levels of CD173, CD238, and the coxsackie-adenovirus receptor protein. Meanwhile, the expression of CD71 (transferrin receptor) gradually increases. These erythroid precursor cells are morphologically similar to basophilic erythrocytes, and they begin to express CD235a, CD173, CD233, CD238, and CD239 as they mature further, with a gradual decline in CD105 expression. This

stage corresponds to the morphological transition from polychromatic to normochromic erythrocytes. Finally, erythrocytes start to lose CD36 and CD71 as they shed their nuclei, and the CD36-CD71- phenotype is produced for reticulocytes and mature erythrocytes[47–52,79].

Studies that have reported abnormal immunophenotypes associated with impaired erythropoiesis in MDS have revealed the following findings:1. An increased proportion of NEC in BM nucleated cells; 2. Increased numbers of immature erythroid lineage cells ($CD117^+ \pm CD105^+$); 3. Reduced erythroid lineage progenitor cells; 4. Abnormal expression patterns of CD71 and CD235a; 5. Reduced MFI of CD71 ± CD36 expression and their increased coefficient of variation (CV); 6. Overexpression of CD105[12,31,35–41,43]. Previous MFC protocols for the diagnosis of MDS have not included a thorough analysis of abnormal erythroid proliferation due to the ongoing debate regarding the significance of MFC data from the erythroid lineages[17].

In 2013, Mathis et al. suggested a new method for diagnosing MDS by combining the CV values of CD71 and CD36 expressing MFI with hemoglobin levels to form a "RED score"[80]. The RED score was used in conjunction with the Ogata score to improve the accuracy of MDS diagnosis. Later, a multicentre study conducted by the ELN iMDS Flow WG combined CD36 and CD71-expressing MFI and CV, CD71 and CD117 erythroid progenitor cell ratios, and Ogata scores and FCSS to form integrated flow cytometry scores (iFCs). This combination of scores improved the diagnostic sensitivity of MDS and allowed differentiation between MDS-associated erythroid dysplasia and nonclonal hematocrit[30].

In a few words, the diagnosis of MDS using MFC based on erythrocyte surface antigens is grounded in established experimental practices. Although there are limited phenotypes available, mostly restricted to nucleated immature erythrocytes, the analysis of these phenotypic changes has enhanced the sensitivity of MDS diagnosis by MFC. As technology in MFC continues to advance, it is worth considering exploring the phenotype of mature red blood cells as well.

1.4. Immunophenotypes on the remaining cells of the MDS BM

Immune dysregulation is a key factor in the development of MDS and has been thoroughly documented in numerous review articles[42,81,82]. The quantity and functionality of NK cells, T cells, and dendritic cells are related to the severity of the MDS condition[83,84]. These include the changes mentioned above in the proportion of B-lineage progenitor cells, the increased proportion of inflammation-promoting T cells in low-risk MDS[85], elevated immunosuppressive cells such as regulatory T cells (Tregs), and decreased dendritic cells and NK cells in high-risk MDS[86–88]. Basophilia may occur during the progression of MDS disease, associated with a poor prognosis[89]. An abnormal expression of CD25 in mast cells has also been linked to MDS. Testing the immunophenotypes of mast cells using MFC can help identify and diagnose conditions such as MDS with mast cell involvement[90].

The examination of lymphocyte subgroups in MDS BM samples can provide valuable insights into the diagnosis and risk evaluation of MDS patients. Despite its potential benefits, widespread agreement on its use has yet to be established and further validation through large-scale, multi-center studies is necessary. The growth and development of myelopoietic cells may be influenced by the cells present in the BM microenvironment of MDS, as demonstrated in Table 3.

Table 3. Phenotypes and significance of cellular abnormalities in the BM microenvironment related to the diagnosis of MDS.

Cell Type	Anomalies Related to MDS Diagnosis	Indicate
Lymphocyte	Reduced proportion of B-lineage progenitors[18]	The correlation between the number and functional status of NK cells, T cells and dendritic cells with the severity of the disease[83,84]
	Increased proportion of T cells promoting inflammation in low-risk MDS[85]	
	Decreased dendritic cells and natural killer cells in high-risk MDS[86–88]	
Basophilic granulocyte	Increased in MDS disease progression[89]	Related to poor prognosis[89]
Mast cell	Abnormal expression of CD25[90]	Identifying and Detecting Conditions such as MDS with Mast Cell Disease[90]

In order to optimize the use of MFC in the diagnosis of MDS, further research and exploration must be conducted to build a comprehensive understanding of the normal immunophenotype of myeloblasts. To improve the diagnostic capabilities of MFC in MDS, additional research and exploration is necessary, with a focus on gaining a comprehensive understanding of the normal immunophenotype of myeloblasts.

2. The use of MFC in distinguishing MDS from other conditions

The latest edition of the WHO classification of myeloid tumors recognizes the significance of immune system anomalies in the blood cell deficiency observed in MDS, and includes MDS-h as a new type within the classification.

In the past, MDS-h was considered to be a transitory phase to other forms of MDS and accounted for approximately 10-15% of all MDS diagnoses. Its defining features included myelodysplasia, a slower progression to acute leukemia, and a poor response to typical MDS treatments. MDS-h was also classified as a specific subtype between AA and MDS[11,91–95]. Differentiating MDS-h from AA can be difficult and the diagnosis often relies on examination of the BM histopathology and complementary tests, including cytogenetics and molecular tests[93,96–100]. Given that the pathogenesis of hypocellular BM failure diseases, such as MDS and AA, is immune-related, MFC-based detection of immune phenotypes in the BM can also aid in their diagnosis and differen-

tial diagnosis. For instance, Cytotoxic T Cells (CTLs) are more abundant and polyclonal in AA than in MDS-h[101]. Both AA and MDS are characterized by an increase in the proportion of Th1 cells and a decrease in Tregs. These changes have been quantified by measuring transcription factors, which are more pronounced in AA[102,103].

Large Granular Lymphocytes (LGL) are a subpopulation of peripheral blood lymphocytes accounting for 2−6% of peripheral leukocytes and 10−15% of peripheral blood lymphocytes. They are slightly larger in diameter (10−12 μm) than typical lymphocytes and contain a large number of peroxidase-negative granules in the cytoplasm[104]. An amplification of LGLs may indicate oligoclonal disease[105], with T-LGL leukemia being the most common form[106].

BM failure in T-LGL leukemia can be as severe as that observed in MDS[107]. The immunophenotype of T-LGL is diverse, however, the most commonly observed is $CD3^+CD8^+CD57^+CD16^+CD4^-$. The presence of amplified $CD3^+CD8^+$ T-LGL is frequently seen in BM failure syndromes such as AA, PNH, and MDS[107−110]. The rearrangement of LGL cells into a clonal population takes place in the region of the mature T-cell receptor (TCR) gene, and the determination of clonality of the TCR gene can aid in the identification of patients with both MDS and T-LGL and the differentiation between the two[107,110]. This information may help in distinguishing MDS-h from other types of MDS and AA, as a higher proportion of both $CD3^+$ T-LGL and CD3-$CD16^+CD56^+$ NK-LGL cells are present in MDS-h compared to other forms of MDS and AA[111].

MFC panels specifically designed for determining the subpopulations of lymphoid cells and the clonality of TCR receptors are available. These panels can be utilized to create a more informed and systematic approach for analyzing and confirming the presence of immune cells in both the BM and peripheral blood using flow cytometry techniques[112].

PNH is a clonal disorder of HSCs that can manifest as hemolytic anemia, thrombosis, smooth muscle dystrophy, and BM failure in some cases. The BM image in individuals with PNH may typically be normal or hyperproliferative, but some may display hyperproliferation. This condition is characterized by a somatic PIGA mutation leading to a deficiency of GPI-anchored proteins, resulting in the absence of CD55 and CD59[113]. Due to the similarities in clinical presentation and myelography between PNH and other hematological disorders such as MDS and AA, PNH was previously thought to be a late-onset clonal complication of these conditions.

Additionally, PNH has been known to be associated with the use of immunosuppressive treatments[114]. The assessment of CD55, CD59, and Flare levels using MFC is a common method for diagnosing PNH. With advancements in flow cytometry technology, the differentiation between PNH, AA, and MDS has been improved. It has been found that up to 10−20% of patients diagnosed with MDS and as many as 60% of patients diagnosed with AA have a concurrent presence of PNH clones at the time of diagnosis[115], and detection of PNH clones by MFC at the time of diagnosis can aid in more precise treatment of affected individuals.

The diagnosis of MDS is made by excluding other conditions and must be differentiated from other diseases that cause BM failure and cytopenia in clinical practice. AA has a similar underlying mechanism to MDS and analysis of lymphocyte subpopulations by MFC is crucial in understanding its immunopathogenesis and making an accurate diagnosis for appropriate treatment. The study of abnormal lymphocytes, particularly T-large granular lymphocytes (T-LGL), is currently a highly researched area. The use of MFC enables further categorization and confirmation of T-LGL, which enhances the diagnosis of this disease and reduces the occurrence of misdiagnosis and underdiagnosis by accounting for the expansion of these cells in the context of oligoclonal T-LGL leukemia in MDS management. Our understanding of PNH has advanced with the discovery that PNH clones may be present in other hematological disorders, and can be rapidly detected using MFC. As such, routine screening for PNH clones should be performed when diagnosing MDS to improve the management of affected individuals.

Conclusion

With increasing focus on studying normal myeloblast immunophenotypes and the identification of more markers in the myeloblast lineages, MFC has become increasingly valuable in the diagnosis of blood disorders and has become an indispensable tool in diagnosing MDS. A number of FCSS for diagnosing MDS have been developed, offering the advantage of quicker and more reproducible results that can be performed in various clinical settings.

While there is no specific flow phenotype that directly confirms the diagnosis of MDS, multiple composite scores based on abnormal immunophenotypes of MDS are available to assist in diagnosis and risk assessment. The use of various composite scores (as shown in Table 4), such as the Ogata score, RED score, Wells FCSS, and iFCs, helps to identify and differentiate MDS from other hematologic disorders, particularly in cases of hypocellular MDS. However, we need more standardized, large-scale normative studies to establish a range of normal values and further improve the accuracy of MFC in diagnosing MDS.

In conclusion, MFC is an essential tool in the diagnosis and differential diagnosis of MDS. As MFC continues to evolve, it may also provide a solution for tracking the evolution of specific abnormal hematopoietic clones in MDS.

Abbreviations

AA, Aplastic anemia; AML, Acute myeloid leukemia; BM, Bone marrow; CTL, Cytotoxic T cells; CV, Coefficient of variation; ELN iMDSFlow WG, International/European LeukemiaNet Working Group for FC in MDS; FCS, Flow Cytometry Score; FCSS, Flow cytometric scoring system; HPC, Hematopoietic progenitor cell; HSC, Hematopoietic stem cells; iFCs, Integrated flow cytometry scores; IPSS-R, Revised international prognostic scoring system; LGL, Large granular lymphocytes; MDS, Myelodysplastic neoplasms; MDS-h, Hypoplastic MDS; MDS-IB, MDS with increased blasts; MDS-LB, MDS with low blasts; MFC, Multiparameter flow cytometry; MFI, Mean fluorescence intensities; MRD, Minimal residual disease; NEC, Nucleated erythroid cells; PBCI, Peripheral blood contamination index; PNH, Paroxysmal nocturnal hemoglobinuria; SD, Standard devi-

Table 4. Comparison of various scoring systems.

Scoring System	Markers	Strengths	Weaknesses	Recommended Cell Lineage
Ogata Score[58]	CD10, CD34, CD45, CD15, CD11b, CD56	Widely used, validated in multiple studies	The sensitivity and specificity of low risk MDS were 69% and 92% respectively[60]	Blast
Wells FCSS[21]	CD16, CD15, CD34, CD7, CD5, CD38, HLA-DR, CD33, CD11b, CD13, CD33, CD14, CD19, CD34, CD56, CD45	It is useful in assessing marrows for diagnosis of MDS and in determining the prognostic outcome in patients with this disorder.	Limited data on its diagnostic accuracy	granulocyte and monocyte
Diff[28]	CD34, CD45, CD13, CD14, CD16, CD33, CD10, CD11b, CD56, CD64	The combination of the Diff score with the concomitantly calculated Ogata score increased the sensitivity to 74.2% and 78.3% respectively.	Limited data on its diagnostic accuracy, not as widely used	granulocyte and monocyte
Red Score[80]	CD117, CD71, and hemoglobin	When combined with the flow score described by Ogata et al., this strategy allowed to reach a very high sensitivity of 88% of patients correctly classified	Not as widely used or validated	Erythrocyte
iFCs[29]	CD45, CD235a, CD71, CD36, CD105, and intracellular markers such as cytosolic H-ferritin, cytosolic L-ferritin and mitochondrial ferritin	More sensitive and specific than Ogata score, validated in multiple studies	Requires specialized software, may be time-consuming	Erythrocyte

ation; SSC, Side Scatter; TCR, T-cell receptor; T-LGL, T-large granular lymphocytes; T-LGLL, T-cell large granular lymphocyte leukemia; Tregs, Regulatory CD4 + T cells; WHO, World Health Organization.

Conflict of interest

All authors declared that there are no conflicts of interest.

Authors' contributions

SfL, SqL and RH responsible for the article idea design, SqL responsible for the paper writing, collection and summarizing the literature. All authors received, revised and edited the manuscript.

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