

# Immune-monitoring of myelodysplastic neoplasms: Recommendations from the i4MDS consortium

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## Abstract

Advancements in comprehending myelodysplastic neoplasms (MDS) have unfolded significantly in recent years, elucidating a myriad of cellular and molecular underpinnings integral to disease progression. While molecular inclusions into prognostic models have substantively advanced risk stratification, recent revelations have emphasized the pivotal role of immune dysregulation within the bone marrow milieu during MDS evolution. Nonetheless, immunotherapy for MDS has not experienced breakthroughs seen in other malignancies, partly attributable to the absence of an immune classification that could stratify patients toward optimally targeted immunotherapeutic approaches. A pivotal obstacle to establishing “immune classes” among MDS patients is the absence of validated accepted immune panels suitable for routine application in clinical laboratories. In response, we formed International Integrative Immunology for MDS (i4MDS), a consortium of multidisciplinary experts, and created the following recommendations for standardized methodologies to monitor immune responses in MDS. A central goal of i4MDS is the development of an immune score that could be incorporated into current clinical risk stratification models. This position paper first consolidates current knowledge on MDS immunology. Subsequently, in collaboration with clinical and laboratory specialists, we introduce flow cytometry panels and cytokine assays, meticulously devised for clinical laboratories, aiming to monitor the immune status of MDS patients, evaluating both immune fitness and identifying potential immune “risk factors.” By amalgamating this immunological characterization data and molecular data, we aim to enhance patient stratification, identify predictive markers for treatment responsiveness, and accelerate the development of systems immunology tools and innovative immunotherapies.

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## INTRODUCTION

Myelodysplastic neoplasms (MDS) are a heterogeneous group of clonal hematopoietic stem and progenitor cell (HSPC) disorders arising in the bone marrow (BM),<sup>1</sup> characterized by ineffective hematopoiesis, dysplasia, peripheral cytopenias, and an increased risk of transformation to acute myeloid leukemia (AML).<sup>2,3</sup> MDS evolve from clonal HSPC outgrowth with the acquisition of genetic lesions and the implementation of genetic alterations into prognostic scoring systems has improved MDS risk stratification.<sup>4–6</sup>

In addition to cell-intrinsic lesions, increasing evidence suggests that immune disruption and tumoral BM microenvironment alterations occur during MDS pathogenesis.<sup>7–10</sup>

The association between inflammation and MDS<sup>11</sup> is reinforced by their linkage to an autoimmune disorder, present in 10%–20% of MDS patients.<sup>12–14</sup> MDS-associated autoimmune disorders are typically refractory to immunosuppressive agents and difficult to manage,<sup>12,15</sup> and are associated with adverse impact on both patients' quality of life and clinical outcome.<sup>16</sup> While immunomodulatory therapies may lead to a sustained response in some patients, universally accepted biomarkers that predict such a response are not yet established.<sup>17–19</sup>

Despite growing evidence highlighting the role of immune dysregulation in the pathogenesis and prognosis of MDS,<sup>20,21</sup> immune therapies remain underutilized in MDS management. Additionally, current diagnostic workups and prognostic models do not typically assess host immunity.<sup>5,22</sup>

The International Integrative Innovative Immunology for MDS (i4MDS) initiative, supported by the European Hematology Association (EHA), is a consortium of clinician scientists, biologists, and physicians actively engaged in MDS patient research and clinical care. We envision that incorporating immunological profiling alongside molecular data could enhance patient stratification, uncover predictive treatment response biomarkers, and steer the development of innovative immunotherapies.

In this position paper, we review the main immune cell dysregulation during the disease course and propose comprehensive guidelines to harmonize and routinely implement immune assessment in MDS.

## METHODS

Since its establishment in 2023, the i4MDS consortium has grown to encompass 27 centers across 10 countries, selected for their experience in the field of immunology in MDS.

The consortium has conducted several meetings to assess existing literature on immune dysregulation in MDS and to formulate recommendations for the monitoring of immune cells and cytokines.

As an initial step, i4MDS conducted a comprehensive literature search, including articles published between 1999 and 2023. This search focused on keywords such as “Myelodysplastic Syndromes” in conjunction with the name of specific immune cell types, including “Cytotoxic T lymphocytes (CTL),” “Regulatory T (Treg) cells,” “T helper (Th) cells,” “Gamma-delta ( $\gamma\delta$ ) T cells,” “B lymphocytes (Ly),” “Natural Killer (NK)

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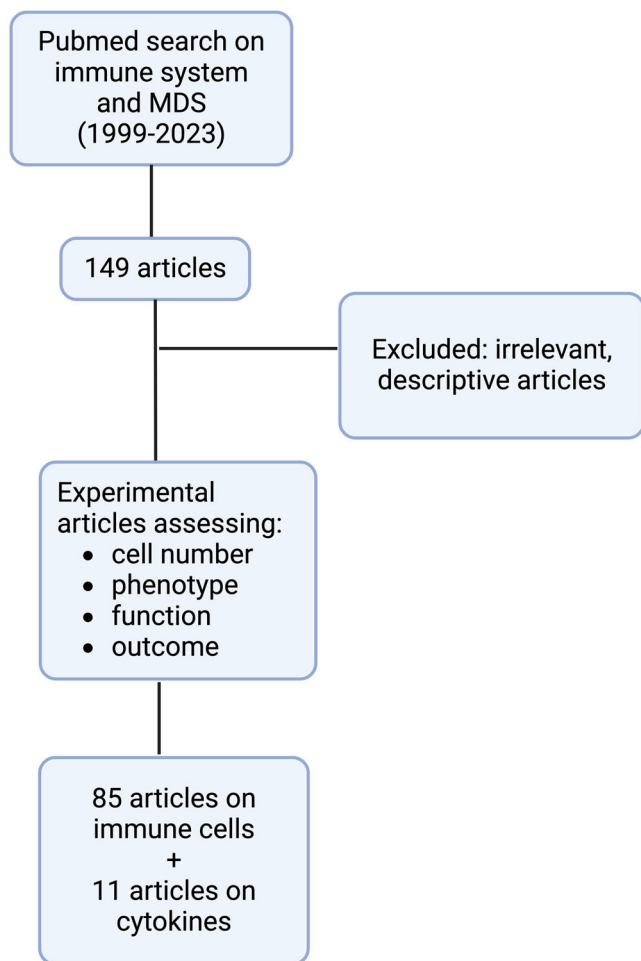
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**FIGURE 1** Flow diagram for literature review regarding immune cell type alterations in myelodysplastic neoplasms.

cells,” “Dendritic cells (DC),” “Monocytes,” “Macrophages,” “Myeloid-derived suppressor cells (MDSC),” “Mesenchymal Stem cells (MSC),” and “Cytokines.” Articles selected for the review of each immune cell type were included if they explored variations in cell number, phenotype, and/or function among MDS patients (Figure 1).

Second, i4MDS conducted a survey involving eleven hemato-immunological clinical centers with expertise in the field: Leipzig University Hospital (Germany), MLL Munich Leukemia Laboratory (Germany), The National Heart, Lung and Blood Institute (US), Rigshospitalet, University of Copenhagen (Denmark), UMC of Amsterdam (Netherlands), University Hospital of Dresden Carl Gustav Carus (Germany), Vall d’Hebron Hospital (Spain), HMDS of Leeds (UK), Saint-Louis Hospital of Paris (France), and Cochin Hospital of Paris (France). The aim was to identify a core set of immune cell markers for clinical flow cytometry and clinically relevant cytokines for routine assessment in clinical care. The proposed recommendations underwent an iterative panel review process aimed at achieving consensus.

## IMMUNE CELL DYSREGULATIONS IN MYELODYSPLASTIC NEOPLASMS

Low-risk (LR) MDS are characterized by a “proinflammatory state” with a higher prevalence of effector cells (e.g., Th17 and CTL)<sup>8,23</sup> whereas high-risk (HR) MDS are dominated by an “immunosuppressive state” with

increased regulatory T cells (Tregs).<sup>7</sup> We highlight the complex modifications in cell repertoire that occur during MDS progression (Figure 2 and Table 1). Additionally, as malignant stem cells can differentiate into mature immune cells, MDS-related genetic lesions transmitted to downstream myeloid and lymphoid progeny can trigger aberrant inflammatory signaling and contribute to maturation defects and immune dysregulation.<sup>106,107</sup>

## CHANGES IN INNATE IMMUNITY

### Neutrophils

Functional deficits, including reduced phagocytosis<sup>108</sup> and decreased bactericidal and fungicidal activities,<sup>109</sup> have been described in MDS neutrophils. Moreover, neutrophils have been found to display an aberrant phenotype with reduced expression of GM-CSF receptor<sup>26</sup> and CD43 adhesion molecule,<sup>25</sup> correlating with defective chemotaxis mostly in HR-MDS.<sup>110</sup> A recent study demonstrated a higher frequency of low-granule neutrophils in individuals with clonal hematopoiesis related to *TET2* mutations.<sup>111</sup> *TET2*-inactivated neutrophils had more compact neutrophil extracellular traps (NET) and decreased flow cytometer (FCM) median side scatter (SSC)-assessed granularity. Consistently, decreased granularity has also been reported in MDS patients.<sup>112,113</sup>

### Natural killer (NK) cells

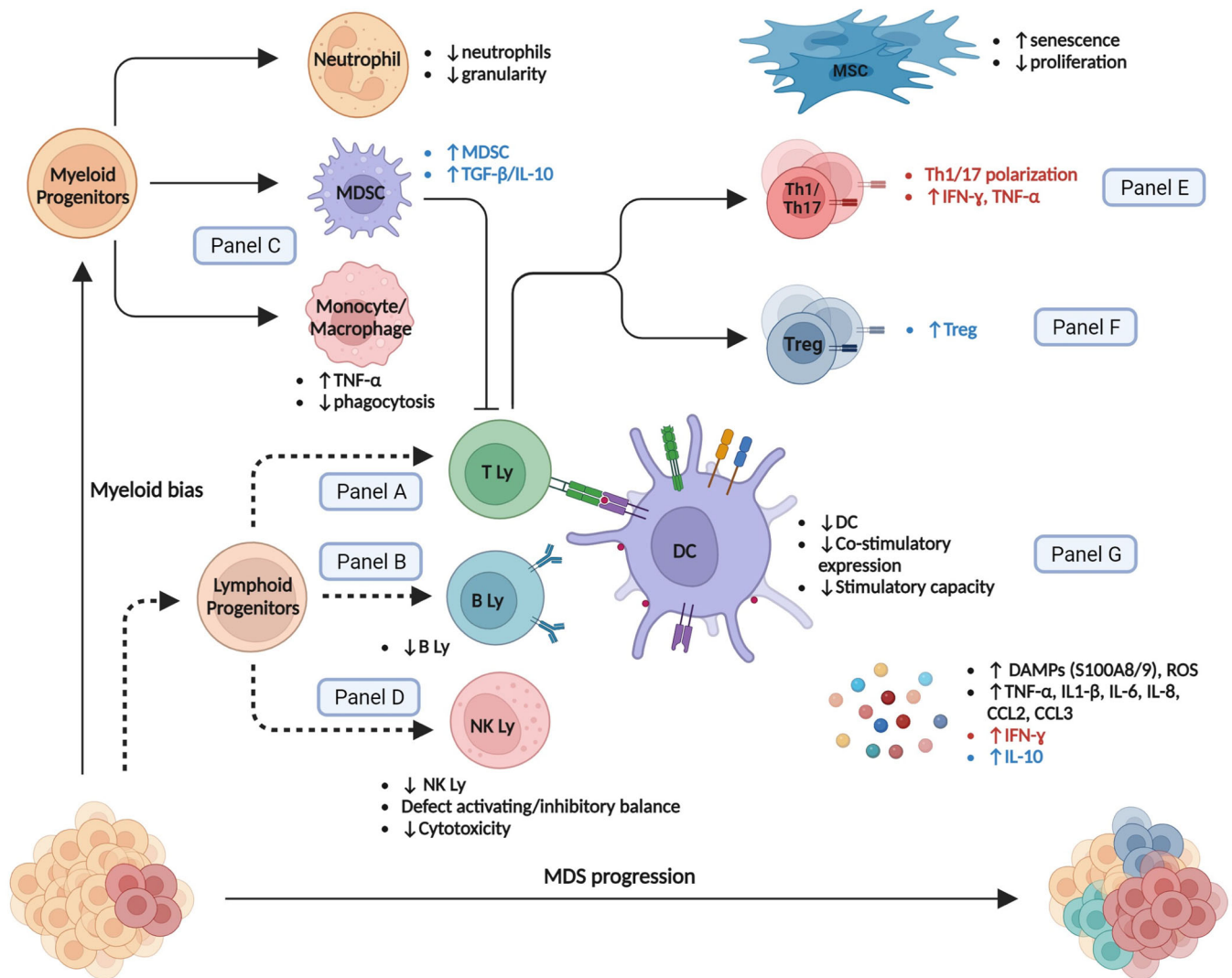
MDS are characterized by a decrease in NK cells, mostly the mature cytotoxic CD56<sup>dim</sup> subset, and numbers inversely correlate with disease severity.<sup>29,30,32</sup> MDS NK cells further show a “less activated” phenotype, reduction of lytic perforin/granzyme granules, and licensing NK Killer Ig-Like Receptors (KIRs) molecule expression.<sup>29,32,37</sup> These phenotypic alterations further correlate with reduced tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) secretion following interleukin-2 (IL-2) stimulation,<sup>29,33,38</sup> defective NK in vitro degranulation,<sup>33</sup> and reduced cytotoxicity capacities,<sup>33,37,38</sup> which have been associated with adverse prognosis.<sup>42</sup> A recent study correlated the presence of *TET2* mutations with reduced KIR expression and impaired NK functions, which were restored with hypomethylating agent (HMA) therapy.<sup>114</sup>

### Dendritic cells (DC)

In MDS, all BM DC subsets show significantly lower frequencies *ex vivo* or following *in vitro* generation from DC precursors.<sup>43,46,48,115</sup> MDS patients also have reduced numbers of DC progenitors,<sup>21</sup> and the reduction in DC further correlates with higher blast percentage and IPSS risk.<sup>46</sup> Additionally, CD141<sup>high</sup> conventional myeloid DC numbers are decreased in MDS patients and correlate with worse overall survival (OS).<sup>21</sup> Functionally, MDS DCs have reduced T-cell priming capacities, with clear T helper 1 skewing,<sup>48</sup> together with a reduction of their endocytic ability.<sup>43</sup> These functional defects are accompanied by a reduction of HLA-DR, CD11c, and costimulatory molecule expression after activation.<sup>47,49</sup>

### Myeloid-derived suppressor cells (MDSC)

The fundamental MDSC function is to secrete immunomodulatory molecules (arginase-1, indoleamine 2,3-dioxygenase, NADPH-oxidase-2), which suppresses T-lymphocyte proliferation while transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-10 promote NK-cell energy and the development of suppressive Treg.<sup>116,117</sup>



**FIGURE 2** Immune and bone marrow environment changes during the MDS course. Main alterations in low-risk, high-risk, or all MDS patients are specified in red, blue, and black, respectively. DC, dendritic cell; IL, interleukin; Ly, lymphocyte; MDS, myelodysplastic neoplasms; MDSC, myeloid-derived suppressor cell; MSC, mesenchymal stromal cell; NK, natural killer; Th, Helper CD4<sup>+</sup> T cell; Treg, regulatory T cell. Dashed lines represent impaired differentiation.

HR-MDS patients have increased MDSC numbers that correlate with increased Treg levels.<sup>10</sup> Consistently, levels of TGF-β and IL-10 are elevated in HR-MDS patients.<sup>52</sup> MDSC from MDS patients also display an aberrant chemokine receptor profile with increased CXCR4 and CX3CR1 expression.<sup>118</sup>

## Monocytes and macrophages

While an increase in classical monocytes ≥94%<sup>119</sup> is highly suggestive of chronic myelomonocytic leukemia,<sup>120</sup> monocyte subset composition in the context of MDS remains controversial. Velegriaki et al. reported a higher proportion of CD14<sup>+</sup>/CD16<sup>+</sup> intermediate monocytes in MDS patients,<sup>55</sup> while Talati et al. reported increased CD14<sup>+</sup>/CD16<sup>-</sup> classical monocytes in *SF3B1*-mutated MDS.<sup>62</sup> Further studies may help elucidate the role of monocyte repartition in MDS and its distinction from chronic myelomonocytic leukaemia, especially in light of the most recent international classifications.<sup>2,3</sup>

In MDS, macrophages exhibit an altered phenotype characterized by reduced levels of CD206 and SIRPα,<sup>53</sup> along with

heightened expression of CD68, CD86, and CD163.<sup>121</sup> Functionally, Meers et al. demonstrated an increase in the CD40 (on monocytes)/CD40L (on T Lymphocytes) axis in MDS patients, resulting in elevated TNF-α secretion by monocytes and subsequent hematopoiesis suppression.<sup>56</sup> Furthermore, MDS monocytes display impaired ability to differentiate in macrophages and reduced in vitro phagocytic abilities.<sup>53,55</sup>

## CHANGES IN ADAPTIVE IMMUNITY

### T cells

The current body of literature strongly indicates the presence of clonal or oligoclonal T-cell expansion as a distinctive feature in MDS.<sup>122,123</sup> These observations support the hypothesis of a dominant clonal T lymphocyte population suppressing hematopoietic precursors, ultimately leading to BM failure.

In one of the largest studies conducted on T cells in MDS patients,<sup>61</sup> the numbers of peripheral blood (PB) CD4<sup>+</sup> and CD8<sup>+</sup> T cells

**TABLE 1** Summary of major innate and adaptive immune cell dysregulation in MDS (continued on next page)

Cell type	Number	Phenotype	Function	Outcome	Other
Neutrophils	<ul style="list-style-type: none"> <li>↓ Neutrophils<sup>24</sup></li> <li>↓ CD54<sup>high</sup>/CD181<sup>low</sup> subset, especially in HR-MDS<sup>7</sup></li> </ul>	<ul style="list-style-type: none"> <li>↓ CD43<sup>55</sup> and GM-CSF receptor expression<sup>26</sup> (PB)</li> <li>↓ Phagocytosis<sup>7</sup></li> <li>↓ Bactericidal and fungicidal activities<sup>7</sup></li> </ul>	<ul style="list-style-type: none"> <li>↓ Granularity<sup>25,26</sup></li> <li>↓ Phagocytosis<sup>7</sup></li> <li>↓ Bactericidal and fungicidal activities<sup>7</sup></li> </ul>	<ul style="list-style-type: none"> <li>Neutropenia correlated with reduced LFS and OS<sup>22,27</sup></li> </ul>	<ul style="list-style-type: none"> <li>↓ CD54<sup>high</sup>/CD181<sup>low</sup> correlated with response to decitabine<sup>28</sup> (PB)</li> </ul>
Natural killer cells (NK cells)	<ul style="list-style-type: none"> <li>↓ Total NK<sup>29-31</sup> (PB + BM)</li> <li>↑ CD56<sup>high</sup>/ ↓ CD56<sup>dim</sup> NK Cells ratio<sup>32</sup> (PB + BM)</li> </ul>	<ul style="list-style-type: none"> <li>↓ NKG2D, DNAM1, NKP30, NKP46 (activation)<sup>32-35</sup> (PB + BM)</li> <li>↑ TIGIT (inhibitory)<sup>36</sup> (PB)</li> <li>↓ Perforin/Granzyme B (lytic granules<sup>29</sup> (PB)</li> <li>↓ KIR (inhibitory/licensing)<sup>32</sup> (PB + BM)</li> </ul>	<ul style="list-style-type: none"> <li>↓ Cytotoxicity<sup>29,33,36-39</sup> (PB + BM)</li> <li>↓ Cytokine secretion<sup>36,38</sup> (PB + BM)</li> <li>↓ Endocytosis<sup>45</sup> (PB)</li> <li>↑ IL-10 secretion<sup>49</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>↓ CD56dim correlated with reduced OS<sup>40</sup> (BM)</li> <li>KIR haplotype correlated with reduced LFS and reduced OS<sup>41</sup></li> <li>↓ cytotoxicity correlated with reduced OS<sup>42</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>NK cells derived from MDS clone<sup>38</sup> (PB)</li> <li>↓ function associated with higher IPSS, abnormal karyotype, excess blasts, and age-adjusted BM hypercellularity<sup>37</sup> (PB + BM)</li> <li>↓ activating receptor expression correlated with elevated BM blasts<sup>33</sup> (BM)</li> </ul>
Dendritic cells (DC)	<ul style="list-style-type: none"> <li>↓ Total DC<sup>21,43-45</sup> (PB)</li> <li>↓ BM DC progenitors<sup>21</sup> (PB)</li> <li>↓ Myeloid cDC<sup>46</sup> (BM)</li> <li>↓ pDC<sup>46</sup> (BM)</li> </ul>	<ul style="list-style-type: none"> <li>↓ CD1a, CD54, CD80 and MHC class II<sup>44</sup> (PB)</li> <li>↓ HLA-DR, CD11c, CD80 and CD86 costimulatory molecules expression<sup>47</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>↓ Allostimulatory capacity<sup>44,45,48,49</sup> (PB + BM)</li> <li>↓ Endocytosis<sup>45</sup> (PB)</li> <li>↑ IL-10 secretion<sup>49</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>↓ CD141<sup>High</sup> cDC correlated with reduced OS<sup>21</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>DC derived from MDS clone<sup>43-45</sup> (PB)</li> </ul>
MDS	↑ in HR-MDS <sup>10,50</sup> (PB + BM)		<ul style="list-style-type: none"> <li>↑ Production of S100A9<sup>51</sup> (BM)</li> <li>↑ IL-10/IL-12 and TGF-β/TNF-α ratio in HR-MDS<sup>52</sup> (BM)</li> </ul>	<ul style="list-style-type: none"> <li>↑ TGF-β/TNF-α ratio correlated with increased blast %<sup>52</sup> (BM)</li> </ul>	<ul style="list-style-type: none"> <li>Expansion driven by S100A-CD33 interaction<sup>50</sup> (BM)</li> </ul>
Monocytes/macrophages	<ul style="list-style-type: none"> <li>↑ Total<sup>53</sup> notably in HR-MDS<sup>54</sup> (BM)</li> <li>↓ Classical monocytes<sup>55</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>↑ CD40, <sup>56</sup>HLA-DR expression<sup>57</sup> (PB + BM)</li> <li>↑ Thrombomodulin expression in LR-MDS<sup>58</sup> (PB)</li> <li>↓ CD206 and SIRPα expression<sup>53</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>↑ TGF-β<sup>54</sup> and TNF-α<sup>56,59</sup> production (PB + BM)</li> <li>↓ Phagocytic function<sup>53</sup> (PB)</li> <li>↑ Apoptotic index<sup>59</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>↑ BM monocytes correlated with adverse prognosis<sup>60</sup> (BM)</li> <li>↑ CD300E correlated with higher MDS risk and adverse LFS<sup>60</sup> (BM)</li> </ul>	
T lymphocytes		<ul style="list-style-type: none"> <li>↑ Surface expression of activation markers (HLA-DR+, CD57+, CD28-, CD62L-<sup>β1</sup> (PB + BM)</li> </ul>			<ul style="list-style-type: none"> <li>T clonality restriction,<sup>3,62</sup> loss of clonal T-cell populations in responders to ATG-based treatment<sup>3</sup></li> </ul>
Cytotoxic T cells (CTL)	<ul style="list-style-type: none"> <li>↑ Effector CTL (CD57+)<sup>63-65</sup> (PB + BM)</li> <li>↓ Total CTL<sup>66</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>Exhausted (expression of TIM3, CD39, PD1, CTLA4)<sup>32,52,66,67</sup> (PB + BM)</li> <li>WT-1 specific cytotoxic T cells have a unique phenotype with coexpression of CD39 and CXCR4<sup>68</sup> (PB + BM)</li> <li>Contracted TCR-V β repertoire (clonal/oligoclonal)<sup>64,69</sup> (PB + BM)</li> </ul>	<ul style="list-style-type: none"> <li>↓ Cytotoxicity<sup>65,66,70</sup> (PB + BM)</li> <li>Effector CTL inhibits bone marrow hematopoiesis<sup>71</sup> (BM)</li> <li>↑ Tc1/Tc2 ratio<sup>72</sup> (PB)</li> </ul>	<ul style="list-style-type: none"> <li>In patients with abnormal karyotype deprivation of effector T cells increased the proportion of abnormal cells<sup>71</sup> (BM)</li> </ul>	
T regulatory cells (Treg)	<ul style="list-style-type: none"> <li>↑ In HR-MDS<sup>7,73-76</sup> (PB + BM)</li> <li>↑ Higher even in LR-MDS<sup>7,77,78</sup> (PB)</li> </ul>		<ul style="list-style-type: none"> <li>In LR-MDS dysfunctional and impaired BM homing, in HR-MDS they retain their function and migratory capacity<sup>74</sup> (PB + BM)</li> </ul>	<ul style="list-style-type: none"> <li>↑ Treg in LR-MDS correlated with adverse prognosis<sup>79</sup> (PB + BM)</li> <li>↑ CD4+ Treg correlated with adverse prognosis<sup>7</sup> (PB)</li> <li>In LR-MDS Treg subset CD4(+)/FOXP3(+)/CD25(+)/CD127(low)/CD45RA(-) CD27(-) (effector memory Treg) correlated with adverse prognosis<sup>80</sup> (PB)</li> <li>↑ Naive Treg/induced Treg ratio correlated with adverse prognosis<sup>81</sup> (BM)</li> </ul>	<ul style="list-style-type: none"> <li>↓ In patients responding to treatment (induction ch1)<sup>74</sup> (PB + BM)</li> </ul>

TABLE 1 (Continued)

Cell type	Number	Phenotype	Function	Outcome	Other
T helper cells (Th)	<p>↑ Th1/Th2 ratio especially in LR-MDS<sup>72,82</sup> (PB + BM)</p> <p>↔ Th1/Th2 ratio<sup>83</sup> (PB)</p> <p>↑ Th17 in LR-MDS<sup>8,24,84,85</sup> (PB + BM)</p> <p>↑ Th22 higher in HR-MDS<sup>24</sup> (PB + BM)</p>	<p>↑ CD40L expression<sup>56</sup> (PB + BM)</p>	<p>High Th1 correlated with levels of INF-gamma/TNF-α and apoptotic index of nucleated cells<sup>82</sup> (BM)</p> <p>↑ Th17 function in LR-MDS<sup>84</sup> (PB + BM)</p>	<p>↑ IL-17 expression associated with more severe anemia<sup>81</sup> (BM)</p> <p>Th17 positive relation with normal karyotype, ANC, and Hb concentration; negative relation with morphologic blast count<sup>84</sup> (PB + BM)</p>	<p>Abnormalities in the Vdelta receptor repertoire<sup>87,88</sup> (PB)</p>
Gamma-delta T cells	<p>↓ Total gamma delta T cells<sup>86</sup> (PB)</p>	<p>↔ Function<sup>86</sup> (PB)</p>			
B cells	<p>↓ Increase in apoptosis<sup>89</sup> and reduced progenitors in LR-MDS<sup>90</sup> (BM)</p>			<p>↓ B progenitors correlated with adverse prognosis<sup>79</sup> (PB + BM)</p>	
MSC	<p>↓ Proliferation<sup>91-93</sup> and increased senescence<sup>93-95</sup> (BM)</p>	<p>↔ Phenotype<sup>96</sup> (BM)</p> <p>Altered expression of osteopontin, Jagged1, Kit-ligand and angiopoietin<sup>91</sup> (BM)</p>	<p>↓ Supporting hematopoiesis function<sup>97,98</sup> (BM)</p> <p>↓ Osteogenic differentiation<sup>91,93,95,99-102</sup> (BM)</p> <p>↑ Inhibition of DC differentiation and function in HR-MDS<sup>103</sup> (BM)</p> <p>↑ Production of IL-6 and TNF-α<sup>59</sup> (BM)</p> <p>↑ TGF-β, apoptosis, and immunosuppression in HR-MDS<sup>97</sup> (BM)</p>	<p>Abnormal karyotype<sup>104,105</sup> (BM)</p>	

Abbreviations: BM, bone marrow; HR, high risk; IPSS, international prognostic scoring system; KIR, killer-cell immunoglobulin-like receptor; LFS, leukemia-free survival; LR, low-risk; MDS, myelodysplastic neoplasms; NK, natural killer; OS, overall survival; p/cDC, plasmacytoid (pDC)/conventional myeloid (cDC); PB, peripheral blood.

in MDS patients did not significantly differ from those in age-matched healthy individuals. However, there appeared to be a reduction in CD8<sup>+</sup> T cells in patients with high-risk (HR) disease. Additionally, the authors verified that most T cells exhibited an activated phenotype, both in PB and BM. This activation was characterized by heightened expression of HLA-DR and CD57, coupled with diminished expression of CD28 and CD62L. Importantly, the presence of this activated phenotype did not appear to influence symptoms, prognosis, or risk of disease progression.<sup>61</sup> Furthermore, T cells derived from MDS patients exhibited elevated expression of chemokine receptors CCR3, CCR5, and CX3CR1, while showing reduced expression of CCR7. This pattern suggests that T cells in MDS tend to possess a more mature chemokine receptor profile.<sup>124</sup> Hypocellular MDS seem to be characterized by clonal T and NK cell expansions, respectively in HR and LR MDS.<sup>125</sup>

## Cytotoxic CD8<sup>+</sup> T cells

Multiple studies have consistently depicted a cytotoxic environment in low and intermediate-risk MDS. This is evidenced by several factors, including the expansion of mature effector CTLs (CD8<sup>+</sup>, CD28<sup>-</sup>, CD57<sup>+</sup>), heightened expression of granzyme B and perforin, alterations of TCR-Vβ repertoire, and reduced frequencies of Tregs when compared to healthy individuals.<sup>63-65</sup> This augmented cytotoxicity has a dual impact: first, it suppresses BM hematopoiesis and contributes to cytopenias; second, CTLs exhibit heightened activity against MDS cells, underscoring their role in restraining the malignant clone.<sup>71</sup>

With the progression of MDS alterations in the immune micro-environment hinder effective anti-tumor responses. Yang et al. revealed that PD-1 and its two ligands, PD-L1 and PD-L2, along with CTLA-4, were aberrantly upregulated in BM cells from patients with myeloid disorders. This upregulation predisposes individuals to an exhausted CTL phenotype, allowing the malignant clone to evade immune surveillance.<sup>67</sup> This finding aligns with another study that reported reduced cytotoxic capacity in high-risk MDS patients.<sup>65</sup>

## CD4<sup>+</sup> T helper cells (Th)

In MDS, there is an elevation in the Th1/Th2 ratio, characterized by a Th1 bias in patients with LR-MDS. Two studies have demonstrated a correlation between Th1 bias and the presence of proapoptotic cytokines, such as IFN-γ and TNF-α. This suggests that Th1 cells may contribute to nucleated cell apoptosis, and therefore BM failure, by overproducing proapoptotic cytokines.<sup>72,82</sup>

Th17 cells represent a distinct subset of CD4<sup>+</sup> T cells, characterized by the production of IL-17. Kordasti et al. found a significantly elevated Th17 population in LR-MDS compared to HR-MDS and controls, together with elevated proinflammatory cytokines and a higher apoptotic index.<sup>8</sup> Similarly, Zhang et al. showed elevated levels of IL-17/IL-17R as well as increased levels of the potentially pathogenic cytokines IFN-γ and TNF-α in BM supernatants. Li et al. found a positive correlation between the percentage of Th17 cells, neutrophils, and hemoglobin levels, and a negative correlation with morphologic blast percentage, as well as a significantly lower Th17 frequency in unfavorable karyotype groups. The authors speculate that Th17 may have a protective role in MDS that contributes to malignant clone inhibition as well as sustaining normal hematopoiesis.<sup>84</sup>

Th22 cells, which mainly secrete IL-22, TNF-α, and IL-13, are markedly elevated in MDS compared with healthy donors, especially in HR-MDS.<sup>24</sup> There may be a role of Th22 cells in the immune evasion of MDS clone(s) and disease progression. However, their role may be more complex. Current studies on Th22 in autoimmunity and

cancer show a potential biphasic activity, with both inflammatory and immunosuppressive activity, based on the focal microenvironment.<sup>24</sup>

## Regulatory T cells (Tregs)

In HR-MDS, Tregs are increased compared to LR-MDS and controls.<sup>7,73,126,127</sup> The expansion is typically polyclonal and involves a predominantly naïve subset.<sup>7</sup> High levels of Tregs have been significantly correlated with MDS with excess blasts, high IPSS score, and disease progression.<sup>7</sup> In early-stage disease, published results are inconclusive. Some studies show normal or near-normal Treg frequencies,<sup>7</sup> whereas others show an increased number compared to healthy controls.<sup>77,78</sup> This may indicate that even LR disease can harbor a dysregulation of immune tolerance, which can negatively affect OS.<sup>79</sup> Kotsianidis et al. showed that early and advanced-stage diseases are associated with differential Treg activity.<sup>74</sup> In early-stage MDS, Tregs' anti-inflammatory potential is compromised by a suppressed CXCL12/CXCR4 axis due to CXCR4 downregulation and impaired BM homing. As a result, the authors showed a reduction of Tregs in the BM microenvironment, which could be a significant mechanism supporting the autoimmunity and BM failure observed in LR-MDS. In late disease stages, Tregs retain their function and homing and can expand locally and systemically.<sup>74</sup> In both early and late-stage disease Tregs show an inverse correlation with the numbers of Th17 and CTL,<sup>8,75</sup> which could permit the emergence of the autoimmune responses observed in the context of LR-MDS.

## B lymphocytes

Literature on the role of B cells in MDS pathogenesis is limited. Meers et al. showed that MDS patients had lower B cell counts compared to age-matched controls.<sup>61</sup> B-cell progenitors are reduced in MDS patients compared with healthy controls,<sup>90</sup> and BM B cells display increased apoptosis. The increase of B progenitors has been shown to correlate with adverse prognosis in LR-MDS.<sup>79</sup>

## CHANGES IN BONE MARROW MICROENVIRONMENT

Smoldering inflammation, particularly driven by NLRP3 inflammasomes and damage-associated molecular pattern (DAMP) molecules (e.g., S100A8/A9) via Toll-like receptors (TLR), is a main feature in MDS.<sup>128,129</sup> Constitutively activated TLR-signaling with downstream mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) activation further induces the release of proinflammatory cytokines propagating the vicious inflammatory loop, increasing pyroptosis and consequently cytopenias.<sup>129</sup> TLR activation promotes the secretion of several proinflammatory cytokines, including IL-1, IL-6, IL-8, TNF-α, INF-γ, and GM-CSF, and leads to the priming of inflammasome components and ASC (apoptosis-associated speck-like

protein containing a caspase-recruitment domain) protein aggregation into large cytoplasmic aggregates (ASC specks). These large complexes act as adaptors to promote pro-caspase1 activity leading to the conversion of pro-IL1β and pro-IL18 into their active form, further amplifying the inflammatory loop.<sup>129</sup> As a result, pyroptosis, a proinflammatory lytic form of cell death, participates in ineffective hematopoiesis.

Kornblau et al. found several upregulated (CSF3, IL-1RA, IL-8, IL-12, IL-15, and CXCL10) and downregulated (IL-4, IL-6, IL-7, IL-10, IFN-γ, CCL3, PDGF-BB) cytokines in PB from MDS patients compared to age-matched controls<sup>130</sup> (Table 2). This points toward a profound cytokine dysregulation in MDS patients, which differs according to the MDS risk group. Kordasti et al. reported higher serum levels of proinflammatory molecules, including IL-7, IL-12, RANTES, and IFN-γ in LR-MDS patients, whereas HR-MDS showed higher levels of suppressive cytokines IL-10 and soluble IL2R.<sup>8</sup> Feng et al. also observed differential cytokine profiles according to IPSS risk stratification, with decreased levels of CCL5, CXCL5, CD40L, VEGF, and EGF in HR-MDS compared to LR-MDS.<sup>131</sup>

Interestingly, some studies reported a correlation between cytokine levels with clinical outcomes. Tsimberidou et al. found that higher TNF-α levels (>10 pg/mL) were associated with lower rates of responses and significantly lower event-free survival and OS.<sup>134</sup> Additionally, increased serum CXCL10, IL-7, and IL-6 levels were independently associated with adverse OS in a retrospective series of 79 patients.<sup>135</sup>

## Mesenchymal stromal cells (MSC)

Numerous reports found that MDS MSC exhibit functional defects in vitro,<sup>91</sup> such as increased senescence, a decline in proliferation ability,<sup>136,137</sup> and a decreased osteogenic potential.<sup>91,138,139</sup> Perhaps most importantly, MDS MSC showed altered capacities to sustain HSPC maintenance both in MDS animal models<sup>140</sup> and in in vitro cocultures.<sup>91</sup> This could be partly attributed to the reduced expression of essential HSPC ligands, including CXCL12, ANGPT1, and KITL,<sup>141</sup> coupled with higher production of the proinflammatory cytokines IL-6 and TNF-α.<sup>59</sup>

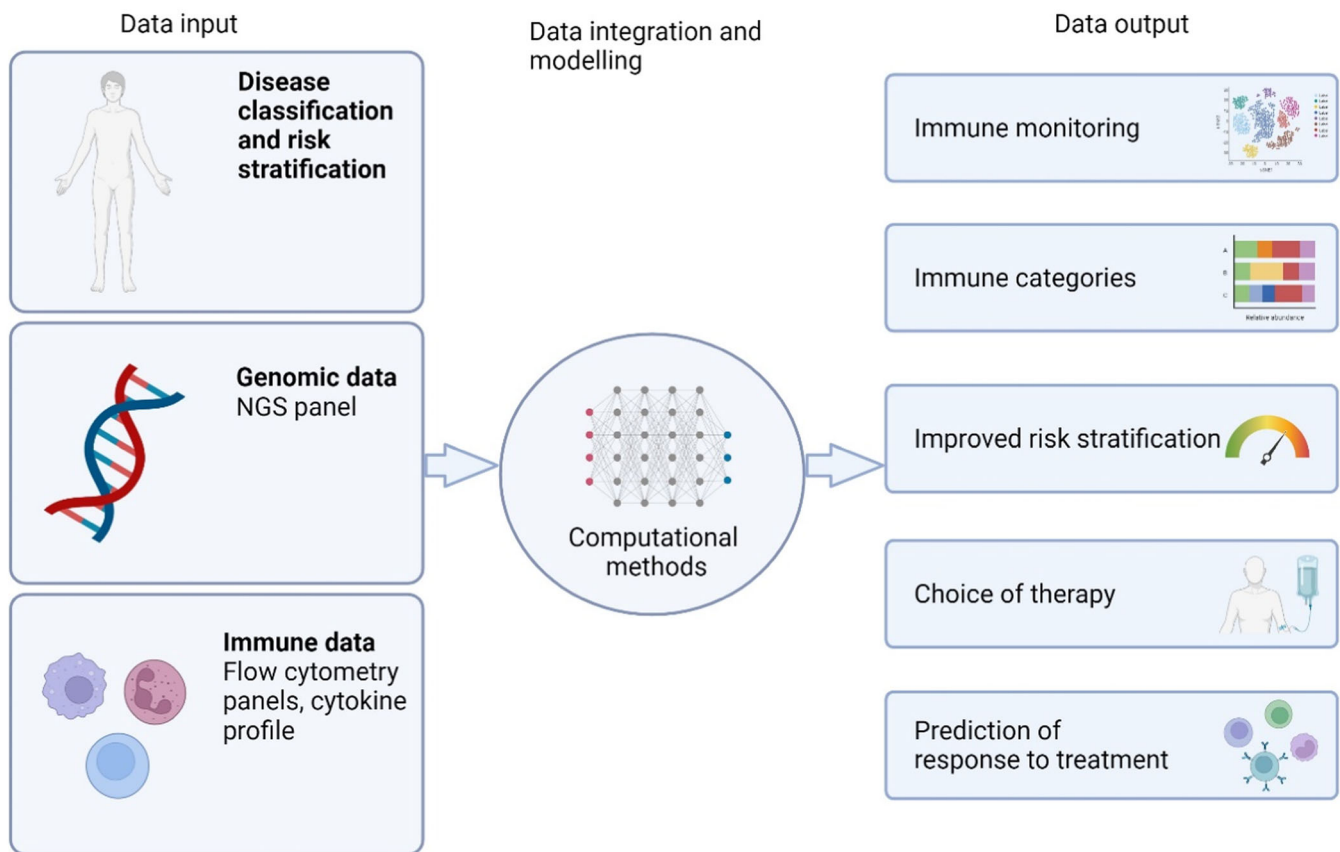
There is very little data regarding the dysregulation of other stromal and vascular niche cells in the context of MDS, however, BM stromal fibroblasts of MDS patients have also shown altered cytokine expression, including IL-6, TNF-α, IFN-γ, and TGF-β.<sup>142</sup>

In summary, MDS is characterized by a multitude of intricate and interconnected dysregulations within cellular immunity and the BM microenvironment. These dysregulations exhibit heterogeneity across MDS subtypes, genetic mutations, and disease stages. This diversity underscores the importance of establishing standardized FCM panels and cytokine measurements for in-depth exploration of immunity in MDS and for risk stratification (Figure 3).

**TABLE 2** Summary of major cytokine alterations in MDS.

	Proinflammatory	Anti-inflammatory	Growth factors	Outcome
Cytokines	In all MDS: ↑ IL-1RA, IL-15, TNFα, IL-6, <sup>130,131</sup> IL-8, <sup>130,132</sup> CCL2, CXCL10 <sup>132</sup> ; ↓ CCL3, <sup>130</sup> CCL4 <sup>131</sup> (PB + BM) In LR-MDS: ↑ IL-7, IL-12, CCL5, INF-γ, <sup>8</sup> IL-17 <sup>8,133</sup> (PB + BM) In HR-MDS: ↓ IL-18 <sup>70</sup> (BM)	In all MDS: ↑ TGF-β, <sup>130</sup> IL-27 <sup>132</sup> ; ↓ IL-4 <sup>130</sup> (PB + BM) In HR-MDS: ↑ IL-10, soluble IL-2R <sup>8</sup> (PB)	In all MDS: ↑ G-CSF <sup>131</sup> (PB) ↑ VEGFA <sup>130</sup> (PB)	↑ TGF-β correlated with higher BM blasts % <sup>54</sup> (BM) ↑ TNF-α correlated with higher BM blasts %, adverse prognosis <sup>134</sup> (PB) ↑ IL-6, IL-7, and CXCL10 correlated with adverse prognosis <sup>135</sup> (PB)

Abbreviations: BM, bone marrow; PB, peripheral blood.



**FIGURE 3** Multiomics modeling pipeline to integrate the assessment of the immune signature in myelodysplastic neoplasms.

## RECOMMENDATIONS FOR IMMUNE PROFILING IN MDS PATIENTS

Guided by current knowledge and expert consensus, the i4MDS consortium presents the following recommendations as an initial step toward standardizing the assessment of immune parameters in patients with MDS.

All patients, whether they are under evaluation due to suspicion of MDS or have a confirmed diagnosis, should be requested to provide prescreening consent. This consent allows for the collection of the necessary samples for local or centralized immune profiling and the ongoing storage of research samples for future evaluations. Besides patients with suspected/diagnosed MDS, patients who are currently undergoing evaluation for persistent unexplained cytopenias (Hb <13 g/dL in men and <12 g/dL in women, ANC  $<1.8 \times 10^9$ , PLT  $<150 \times 10^9$ ) as well as those who have already received a diagnosis of immune aplastic anemia, clonal cytopenia of undetermined significance (CCUS), VEXAS syndrome, or idiopathic cytopenia of undetermined significance (ICUS) are considered eligible for participation (Figure 4). When possible, patients will be part of registries where central diagnosis screening is not prohibited.

Concomitant clinical data to be collected should include coexisting conditions (especially autoinflammatory and autoimmune disorders) and immunomodulatory drugs (especially systemic steroids >10 mg/day). Laboratory data should include CRP and autoimmunity-related tests if indicated and available. Molecular sequencing (standard MDS NGS panels) should also be performed in all patients, to complement both molecular and immune data.

We recommend the collection of PB and, if feasible, BM aspirates to monitor the immune signature. Notably, significant variations in the innate and adaptive cell composition and status between peripheral and BM samples can provide valuable insights into the mechanisms underlying defective immune surveillance. Aspirates should be performed at the time of diagnosis and before initiating any new therapies.

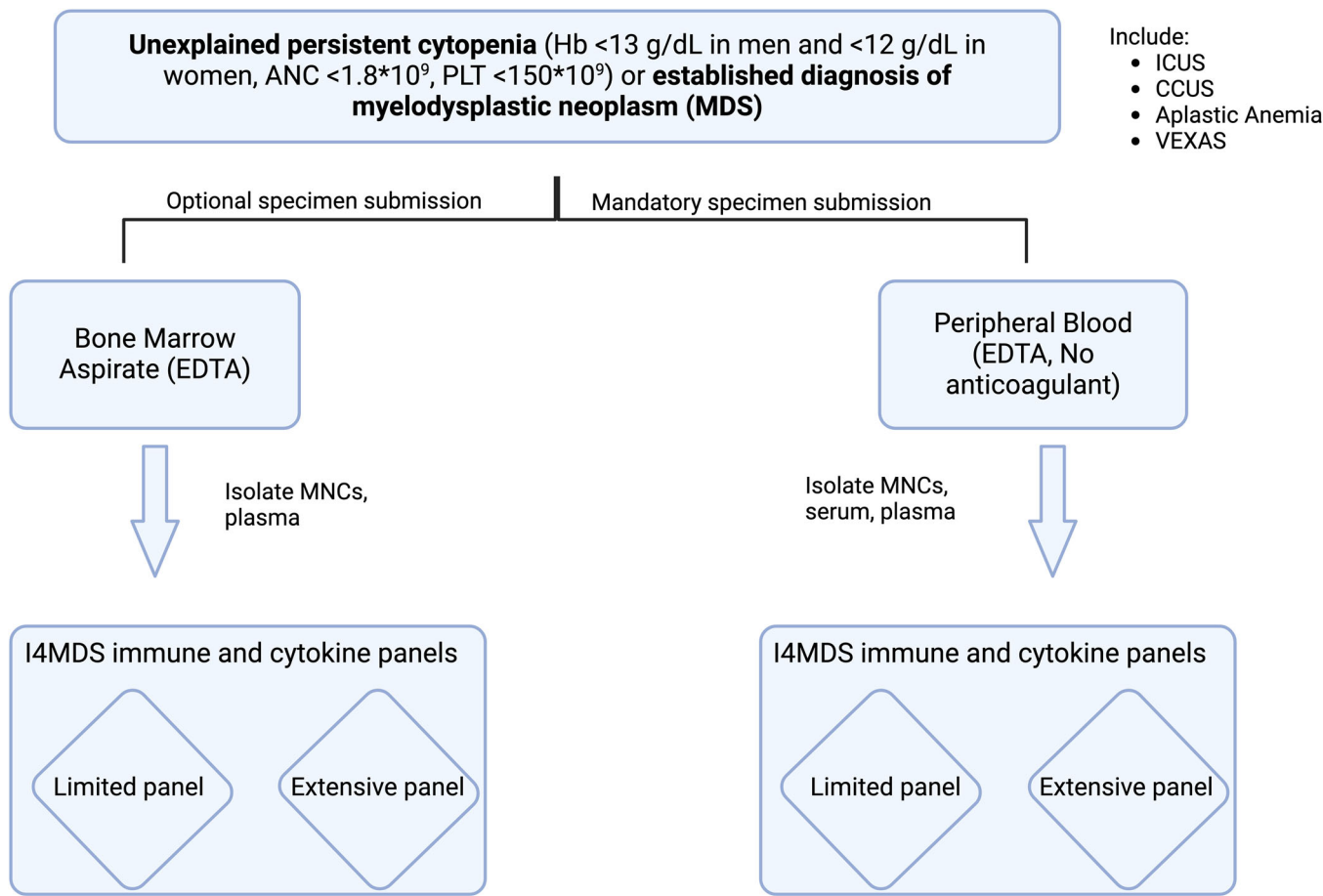
We strongly encourage sequential assessments in accordance with institutional guidelines to monitor the dynamic evolution of the immune response during treatment. This is particularly crucial for patients undergoing treatment with hypomethylating agents (HMA) for a minimum of three cycles, given the potential immunomodulatory properties of this therapy.

When feasible, a portion of the PB and BM mononuclear cells and serum samples should be biobanked to enable more advanced multiparameter technologies, such as cytometry by time of flight or spectral flow cytometry, which can facilitate more in-depth immune profiling. Thus, the collection of these important immunological and clinical/demographic data will enable advanced analyses to answer outstanding research questions to improve the experience and outcomes of patients with MDS.

## IMMUNE CELL MONITORING USING FLOW CYTOMETRY

Table 3 summarizes the primary antigens the i4MDS panel recommends for the identification, characterization, and functional assessment of immune cells during clinical care. For each type of





**FIGURE 4** Patients screening for immune signature evaluation. ANC, absolute neutrophil count; CCUS, clonal cytopenia of unknown significance; Hb, hemoglobin; ICUS, idiopathic cytopenia of unknown significance; MNC, mononuclear cells; PLT, platelets.

immune cell lineage, we have devised a suggested panel along with optional markers. The selection of these additional markers should be guided by local protocols, equipment availability, available resources, and the source of aspirates.

This approach has been designed to facilitate effective and routine immune cell assessment in clinical laboratories, particularly in secondary and tertiary centers.

In the case of BM, hemodilution should be prevented, if possible, by using the first aspirate pull. Samples can be collected in ethylenediaminetetraacetic acid tubes and processed up to 72 h after collection. Nevertheless, if high dimensional flow cytometry is to be performed, we would generally recommend citrate collection tubes as they allow both genomic and flow cytometry analysis from the same sample. We recommend ammonium chloride (homemade or commercially available) lysis of mature RBC before antibody staining and suspension in paraformaldehyde for data acquisition.<sup>143</sup>

The panels of marker combinations in Table 3 have been constructed based on 10 colors FCM, CD45 hematopoietic marker, and a viability stain.<sup>132</sup> Optional markers are proposed for exploring cell type-specific functions (immune-related) or those known or suspected to be dysregulated in the context of MDS (malignancy-related).

In the case of BM aspirates, we also recommend the addition of a combination that includes CD34 and other appropriate

markers to distinguish hematopoietic stem and progenitor cells (HSPC). Based on the current level of evidence regarding alterations in cell types in MDS (as outlined in Table 3), we strongly recommend performing, at a minimum, the T- and B-cell panels. The choice to implement additional panels may be influenced by the intended treatment strategy.

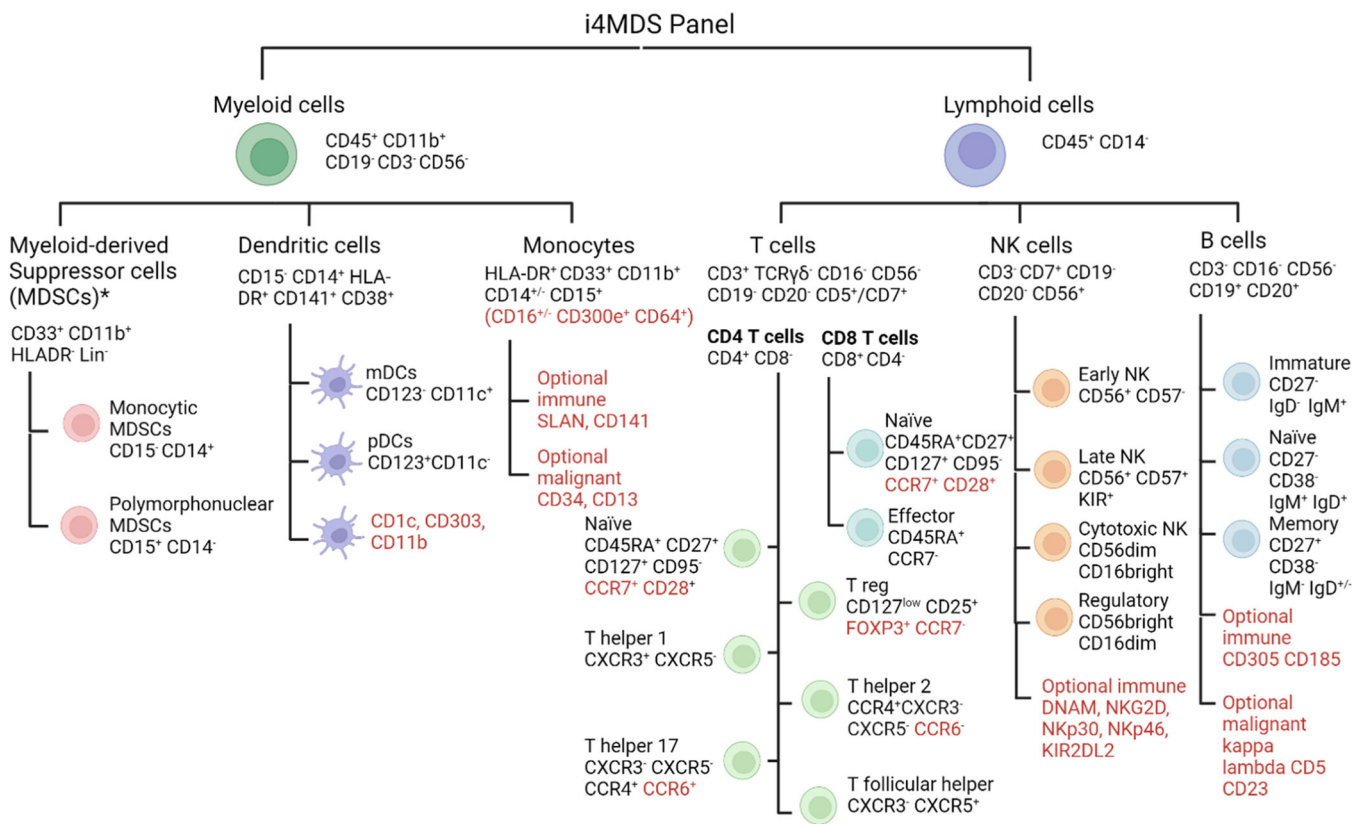
We designed specific tubes for T-cell monitoring (Panel A) and set a core panel of markers to distinguish cytotoxic, Th, and Tregs, as well as their naïve/memory phenotype (Panels E and F). For both Th (CXCR3, CCR4, CCR6, and CXCR5) subsets and Treg (CD25, CD127), cell-surface identifying markers were preferred given their good correlation with intracellular staining and to facilitate routine implementation.<sup>144</sup> Panel B explores B cell isotypes, activation status, and repartition. NK cell panel D discriminates CD56<sup>bright/dim</sup> NK subsets and major activating/inhibitory/licensing NK receptors known to be dysregulated in MDS. Myeloid panel C investigates monocyte (classical, nonclassical, and intermediate) subsets along with M-MDSC and PMN-MDSC based on CD14/15 differential expression, while panel G analyzes plasmacytoid and myeloid DC subsets (Figure 5).

Neutrophils can be individualized based on FSC/SSC scales and assessed for SSC-based granularity. Given the lack of data regarding the immune impact of red cell nucleated progenitors and megakaryocytes in MDS, we do not recommend their monitoring in routine workup of MDS immune profiling.

**TABLE 3** Flow cytometry panel recommendations.

Panel	Immune subsets	Recommended markers	Optional (immune)	Optional (malignant)
A	T cell	CD45, CD45RA, CD3, CD4, CD8, CD5, CD7, CD16/56, CD27, TCRγδ	HLADR, CD38, CD95, CD62L, CD45RO, PD1/CTLA4/TIM3	TRBC1, CD26
B	B cell	CD45, CD20, CD19, CD10, CD27, CD38, IgD, IgM, CD25, CD22	CD305, CD185	kappa, lambda, CD5, CD23
C	Monocyte/MDSC	CD45, CD14, CD16, CD64, CD300e, CD56, HLADR, CD11b, CD33, CD15, Lin	SLAN, CD141, CD45RO	CD34, CD13
D	NK cell	CD7, CD56, CD8, CD94, CD57, CD161, CD16, CD3, KIR3DL1/DL2, CD45RO	DNAM1, NKG2D, NKp30, NKp46, KIR2DL2	
E	T cell subset – 1	CD3, CD4, CD8, CD19, CD25, CD127, CXCR5, CXCR3, CCR4, CCR6	CD95, CD28, CCR7	
F	T cell subset – 2	CD45RA, CD3, CD4, CD25, CD127, CD194 (CCR4), CD95, CD28, CCR7, CD8	FOXP3, CXCR5, CXCR3, CCR6, CD45RO	
G	Dendritic cells	CD45, Lin, CD123, CD88, HLADR, CD5, CD11c, CD141, CD163, CD11B, CD14	CD1c, CD303, CD11b, CD45RO	

Note: Each panel explores a specific immune subset with a set of core markers and additional extra-markers to explore their status and function (immune) or known to be dysregulated in the context of cancer (malignant).



**FIGURE 5** Cell markers to identify cell subtypes from the International Integrative Immunology for MDS panel (optional markers in red). \*Functional assay is necessary for definite identification of myeloid-derived suppressor cells.

### CYTOKINE MONITORING IN MDS PATIENTS

Cytokine detection has classically used enzyme-linked immunosorbent assays (ELISA), whose routine implementation has been limited by the analysis of one analyte at a time, and a relatively large sample volume to

ensure accurate detection.<sup>145</sup> Multiplex immunoassays overcome the limitations of single-analyte ELISA allowing simultaneous analysis of multiple cytokines with a reduced sample volume. While routine implementation of cytokine analysis is not yet widespread, we feel these techniques will play an important role in the therapeutic management of many inflammatory diseases, including MDS.

**TABLE 4** Cytokines monitoring panel recommendations.

Cytokine panel	Recommended Markers (ranked in priority order)	Optional markers
Cytokines	IFN- $\gamma$ , IL-6, IL-8, IL-1RA, IL-7, IL-12, CCL5, IL-10, IL-2, IL-2R, TNF- $\alpha$ , IL-17, IL-1 $\beta$ , TGF- $\beta$ , VEGF, GM-CSF, CXCL10, IL-4, CCL2, CCL3, CCL4, CXCR4	IL-5, IL-15, CXCL9, CXCL12, CXCR1, CXCR2

Serum cytokine levels have been shown to robustly reflect BM cytokine concentration<sup>8</sup> and there is high agreement between serum and plasma concentrations.<sup>146</sup> Peripheral blood serum (5 mL dry tube) should be the preferred source for cytokine analysis, although we also encourage BM supernatant biobanking. Lastly, it is crucial to exercise diligence regarding the patient's clinical condition to prevent sample collection during concurrent inflammatory or infectious episodes that could confound results. Additionally, any ongoing treatments, particularly those involving growth factors, immunomodulators, or novel inflammasome inhibitors, should be documented and reported, as they have the potential to impact result interpretation.

Table 4 summarizes the recommended cytokine panel, ranked in priority order established based on their potential role in disease genesis and their reported altered expression in MDS patients. Multiplex assays may allow simultaneous analysis of both proinflammatory (IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-2, IL-2R, IL-6, IL-7, IL-8, IL-12, VEGF, GM-CSF, CCL2, CCL3, CCL4, CCL5, CXCL10) and anti-inflammatory (IL-4, IL-10, TGF- $\beta$ ) molecules known to be dysregulated in the context of MDS. We also suggest additional markers: IL-5 and IL-15 for their broad proinflammatory roles in granulocyte stimulation, NK and T cell activation and proliferation, respectively; CXCL9 and CXCL12 for their important function in immune cell migration toward inflamed and BM tissue, respectively.

## DISCUSSION AND FUTURE DIRECTIONS

In this work, we offer recommendations to establish a structured framework for evaluating immune cells and monitoring cytokine changes at the time of diagnosis and throughout the course of the disease to standardize the immune profiling of MDS patients. Currently, manual gating and expert analysis remain the conventional practices, but we anticipate that automated gating and cell identification algorithms, currently in development or used in research studies,<sup>147</sup> will likely find their place in clinical practice in the near future.

Looking ahead, more sophisticated technologies, such as mass cytometry or spectral flow cytometry, could become readily available. These advancements have the potential to streamline the monitoring process, potentially reducing the need for multiple tubes and enhancing the efficiency of immune profiling.

We envision the integration of comprehensive immunological data with existing genomic analyses through the setup of a big data consortium. Through advanced statistical analysis, this could lead to improved risk stratification, identification of new prognostic biomarkers and patient subsets most likely to benefit from immunomodulatory treatments, including allogeneic hematopoietic stem cell transplantation and emerging immunotherapies.

## AUTHOR CONTRIBUTIONS

Shahram Kordasti, Uwe Platzbecker, Catherine Cargo, Matteo G. Della Porta, and Lionel Adès designed the study and immune panels and supervised the project. Cristina A. Tentori and Lin P. Zhao performed the research, contributed to the panel design, and wrote the manuscript. Benedetta Tinterri, Kathryn E. Strange, Katharina Zoldan,

Konstantinos Dimopoulos, Xingmin Feng, Elena Riva, Benjamin Lim, Yannick Simoni, Vidhya Murthy, Antonella Poloni, Eric Padron, Bruno A. Cardoso, Michael Cross, Susann Winter, Aida Santaolalla, Bhavisha A. Patel, Emma M. Groarke, Daniel H. Wiseman, Katy Jones, Lauren Jamieson, Charles Manogaran, Naval Daver, Laura Gallur, Wendy Ingram, P. Brent Ferrell, Katja Sockel, Nicolas Dulphy, Nicolas Chapuis, Anne S. Kubasch, Astrid M. Olsnes, Austin Kulasekararaj, Hugues De Lavellade, Wolfgang Kern, Mieke Van Hemelrijck, Dominique Bonnet, Theresia M. Westers, Sylvie Freeman, Uta Oelschlaegel, David Valcarcel, Marco G. Raddi, Kirsten Grønbaek, Michaela Fontenay, Sanam Loghavi, Valeria Santini, Antonio M. Almeida, Jonathan M. Irish, David A. Sallman, Neal S. Young, AH, and Arjan A. van de Loosdrecht contributed to panel design and critically reviewed the manuscript.

## CONFLICT OF INTEREST STATEMENT

Shahram Kordasti: Novartis: Advisory Board, Speakers bureau, Alexion: Speakers bureau, Beckman Coulter: Speakers bureau, MorphoSys: Research Support (none is related to this publication). Wolfgang Kern declares part-ownership of MLL Munich Leukemia Laboratory. Austin Kulasekararaj: Research support (to institution): Celgene/BMS and Novartis. Speaker's fees: Alexion/AstraZeneca, Akari, Apellis, Celgene/BMS, Novartis, Pfizer, Ra Pharma/UCB, Roche, SOBI. Scientific advisory board: Alexion/Astra Zeneca, Apellis, Amgen, Agios, Biocryst, Celgene/BMS, Novartis, Pfizer, Regeneron, Roche, SOBI, Janssen, Samsung and Novo Nordisk (none is related to this publication).

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Pubmed at <https://pubmed.ncbi.nlm.nih.gov/>.

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