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- 1 Journal of Clinical Oncology
- 2 Review Article
- 3 Integrating the 'Immunome' in the stratification of myelodysplastic syndromes

4 and future clinical trial design

- 5 Running title: Systems immunology; a way forward in MDS
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Abstract 23

Myelodysplastic syndromes (MDS) are characterised by ineffective haematopoiesis and often 24 include a dysregulation and dysfunction of the immune system. In the context of population 25 26 ageing, MDS incidence is set to rise substantially, with exponential increases in health care 27 costs, given the limited and expensive treatment options for these patients. Treatment 28 selection is mainly based on calculated risk categories according to a Revised International 29 Prognostic Scoring System (IPSS-R). However, although IPSS-R is an excellent predictor of 30 disease progression, it is an ineffective predictor of response to disease-modifying therapies. 31 Redressing these unmet needs, the 'immunome' is a key, multifaceted component in the 32 initiation and overall response against malignant cells in MDS, and the current omission of 33 immune status monitoring may in part explain the insufficiencies of current prognostic 34 stratification methods. Nevertheless, integrating these and other recent molecular advances 35 into clinical practice proves difficult. This review highlights the complexity of immune dysregulation in MDS pathophysiology, and the fine balance between smouldering 36 37 inflammation, adaptive immunity, and somatic mutations in promoting or suppressing 38 malignant clones. We review the existing knowledge and discuss how state-of-the-art immune 39 monitoring strategies could potentially permit novel patient sub-stratification, thereby 40 empowering practical predictions of response to treatment in MDS. We propose novel 41 multicentre studies, which are needed to achieve this goal.

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Keywords: MDS, immune dysregulation, immune profile, patient stratification

43 Introduction

44 Myelodysplastic syndromes (MDS) represent a group of acquired clonal disorders of 45 haematopoietic stem and progenitor cells (HSPCs), characterised by ineffective 46 haematopoiesis, peripheral cytopenias, genetic instability, and an increased risk of 47 progression to acute myeloid leukaemia (AML)¹. Considering the higher prevalence in elderly 48 patients, the population ageing in developed countries as well as higher diagnostic awareness, 49 the incidence of MDS is set to rise substantially in coming decades².

50 Clinical outcomes can vary greatly, even between patients considered to have the same MDS 51 subtype. Thus, MDS display marked heterogeneity regarding prognosis and the risk of disease progression. To overcome this heterogeneity, the IPSS was introduced and then later revised 52 53 (IPSS-R) with the aim to provide discriminatory prognostic risk assessment regarding overall 54 survival and risk of progression to AML³. Whilst the IPSS-R reliably predicts the risk of disease 55 progression, it is not an effective tool to predict response to disease-modifying therapies⁴. 56 This is not surprising since the IPSS-R, like the original IPSS, was developed based on clinical data from untreated MDS patients. Recent advances in targeted and large-scale next 57 58 generation sequencing (NGS) have helped to illuminate the dynamic genomic landscape in MDS^{5–7}. Although none of the most common recurrent somatic mutations is disease-defining, 59 60 some have an independent impact on overall survival, such as in TP53⁸. Thus, addition of molecular data to the IPSS-R can improve its predictive power^{5, 8, 9}. 61

Recent advances have also highlighted the role of immune dysregulation in MDS pathogenesis but are currently omitted from IPSS-R. This includes both abnormal activation of innate immune pathways and associated inflammation as well as aberrant cellular immune responses of independent prognostic value, which dynamically evolve during disease

progression^{10–13}. The addition of comprehensive immunologic data to prognostic models 66 could, similar to mutational data, further help to refine risk stratification across the boundary 67 of lower- and higher-risk MDS. We envisage that continued clarification of the immune 68 69 pathways that are dysregulated in selected MDS subtypes will improve patient stratification, 70 the use and outcomes of existing treatments and novel immunotherapies, and drive the development of new targeted drugs. In this review, we highlight recent advances in the 71 understanding of immune dysregulation in MDS, discuss their clinical implications as well as 72 73 potential therapeutic applications, and outline how immune profiling could be implemented 74 in future clinical trials.

75 Predisposing and potential driving immune factors

a) Smouldering inflammation and immunosenescence

Chronic inflammation due to long-lasting exposure to persistent infection or sterile 77 78 inflammation is a well-established predisposing factor for cancer^{14, 15}, and increasing evidence 79 implicates the activation of innate immune signalling in age-related haematopoietic senescence¹⁶, bone loss¹⁷, and MDS¹⁸. In fact, normal human ageing represents a state of 80 81 chronic low-grade sterile inflammation, similar to that originally described as 'parainflammation' by Medzhitov¹⁹, and commonly referred to as 'inflammaging'²⁰. Stressed, 82 83 damaged or otherwise malfunctioning, and/or dead cells release endogenous inducers of 84 sterile inflammation, including damage-associated molecular patterns (DAMPs) like high-85 mobility-group-protein B1 (HMGB1) and alarmin S100 proteins, which can be sensed through different receptors, such as Toll-like receptors (TLRs) and cytosolic nucleotide-binding domain 86 and leucine-rich repeat pattern recognition receptors (NLRs)^{19, 20}. The physiological purpose 87 of the ensuing inflammatory response early in life and adulthood is to restore functionality 88

89 and homeostasis in the tissue. However, in old age, a period in life largely not foreseen by evolution, the continuous exposure to inflammatory stimuli/stressors (the 'immune 90 biography') becomes detrimental, setting the biologic background favouring the susceptibility 91 92 to age-related inflammatory disorders, autoimmunity, and deterioration of haematopoiesis. 93 A reduced capacity to defend against pathogens and to initiate adaptive immunity is observed in ageing humans, together with enhanced pro-inflammatory reactions fuelled by 94 'endogenous/self-molecular garbage'^{20, 21}. The presence of 'smouldering' inflammation in the 95 96 elderly may aid the proliferation and survival of malignant MDS clones driven by genetic 97 alterations (including a recently described condition known as clonal haematopoiesis of indeterminate potential [CHIP]²²), subvert adaptive immunity, and alter cellular responses to 98 therapeutic intervention. 99

b) NLRP3 inflammasome: a driver of chronic inflammation in MDS

Increased levels of DAMPs (e.g. S100A8/9) and activated NLR family, pyrin domain-containing 101 protein 3 (NLRP3) inflammasomes are evident in MDS, particularly lower-risk disease^{18, 23–25}. 102 103 Notably, MDS HSPCs are specifically susceptible to DAMPs since they overexpress TLRs^{26, 27} along with signal transducers, such as IRAK1²⁸ and TRAF6²⁹. Ligation of S100A8/9 to TLR4 104 induces NF-kB-mediated transcription of pro-inflammatory cytokines, including pro-105 106 interleukin (IL)-1 β and IL-18, and transcriptional priming of inflammasome components³⁰. 107 Once activated, the NLRP3 inflammasome directs caspase-1-dependent conversion of pro-IL- 1β /IL-18 to their active forms and inflammatory pyroptotic cell death¹⁸. The consecutive 108 109 release of pro-inflammatory cytokines, reactive oxygen species (ROS), and other intracellular 110 contents into the extracellular milieu further activates the NLRP3 inflammasome, driving 111 pyroptosis of HSPCs, consequent cytopenias, and an inflammatory circuit (FIG. 1). This milieu 112 may support the propagation of the MDS clone through various pathways, including Wnt/ β - 113 catenin signalling³¹ or aberrant activation of the IL-1/p38MAPK pathway³². NLRP3 114 inflammasome activation appears to be licensed by S100A8/9 and MDS-related gene 115 mutations and is also evident in del(5q) MDS patients, featuring activation of the p53-116 S100A8/9-TLR4 axis ^{10, 18, 24}. However, whether inflammasome activation is a general feature 117 of lower-risk MDS or particular subgroups needs to be evaluated in larger cohorts in the 118 future.

119 TLR signalling pathway activation in MDS HSPCs makes the TLR axis a promising therapeutic 120 target (TABLE 1). In addition, novel NLRP3 inflammasome inhibitors or approved IL-1 β 121 inhibitors are in clinical development and may offer therapeutic promise in MDS¹⁰, which 122 highlights the importance of refined patient stratification to identify patients with prominent 123 'autoinflammatory' features, therefore most likely to benefit from inflammasome pathway 124 inhibition.

125 c) Somatic mutations and inflammatory status

A complex and dynamic landscape of genetic mutations and cytogenetic lesions is evident in 126 MDS^{5, 33}. Acquisition of serial mutations and clonal diversification not only reflect on disease 127 128 progression but also give an indication of the (in-)efficacy of the immune system to control outgrowth of malignant clones, as suggested in other malignancies^{34, 35}. Underlying 129 smouldering inflammation could contribute to the genomic instability and acquisition of 130 additional mutations, as shown in gastrointestinal malignancies^{36, 37}. In MDS, mutations 131 132 affecting epigenetic modifiers (e.g. TET2, ASXL1) and RNA splicing factors (e.g. SF3B1, SRSF2) appear to represent predominantly 'founder' events³³. Mutations in several of these genes 133 have been linked to activated NLRP3 inflammasomes and enhanced innate immune 134 signalling^{18, 38–40}. Such mutant gene licensing of innate signalling pathways in myeloid 135

progenitors may provide the selective immune pressure conducive to malignant progression in MDS/AML. On the other hand, the observation of 'founder' mutations in the lymphoid lineage raises questions about the potential effect of intrinsically aberrant lymphocytes on the adaptive immune response and MDS/AML pathogenesis^{33, 41}.

The intricate relationship between mutagenesis and inflammatory processes is not limited to established MDS. Patients with CHIP²², a condition that likely precedes MDS and is characterised by the presence of MDS-related mutations in *DNMT3A*, *TET2*, *ASXL1*, or *JAK2*, were found to have an increased risk of inflammatory-related diseases, such as coronary heart disease^{42, 43}. Recent studies point to the existence of shared autoinflammatory NLRP3-related pathways in CHIP/MDS and associated co-morbidities⁴⁴, and suggest *NLRP3* as a shared genetic risk factor for MDS and para-neoplastic Sweet syndrome⁴⁵.

147 The other important and yet poorly investigated aspect of MDS pathophysiology is the 148 reciprocal effect of the (cellular) immune response on frequency and type of somatic 149 mutations, and whether these mutations induce immunogenic neoantigens, as shown in other malignancies³⁴. Due to the overall lower somatic mutation burden in both AML and MDS 150 151 compared to other types of tumours⁴⁶, the potential immunogenicity of these mutations is 152 largely unexplored. We previously adopted an algorithm to predict neoantigens and combined 153 this with mass cytometry to identify neoantigen-related immune signatures⁴⁷. This initial 154 investigation suggested that the presence of predicted neoantigens has a protective effect in patients with lower-risk disease. 155

d) The microbiome and its impact on inflammation and immunome

157 Profound changes in the microbiota and its interaction with the immune system are 158 increasingly recognised to contribute to chronic inflammatory diseases, including haematologic disorders^{48, 49}. Various factors can reduce microbial diversity and commensalism, including treatment with broad-spectrum antibiotics, poor dietary patterns, drugs, chemotherapy, and environmental factors. For example, depletion of intestinal microbial flora by broad-spectrum antibiotic treatment of mice has been shown to cause a decrease in HSPC numbers and concomitant anaemia, highlighting the intricate relationship between host-microbiome and haematopoiesis⁵⁰.

165 Although no detailed study exists concerning the microbiome composition in MDS, the role of microbial-dependent inflammation in the development of pre-leukaemic myeloproliferation 166 167 has been demonstrated recently in Tet2-deficient mice, in which intrinsic (Tet2 deficiency-168 induced IL-6R α overexpression) and extrinsic (microbial-induced IL-6) inflammatory cues cooperate and trigger proliferation of highly sensitive Tet2-deficient haematopoietic 169 170 progenitor cells³⁹. Clinically, overuse of antibiotics and/or a poor dietary pattern/nutritional 171 reserve is also common in MDS/AML, and could lead to decreases of microbial diversity and 172 commensalism in the gut, resulting in compromised immune responses and increased risk of 173 inflammation. One study concerning relapse after allogeneic haematopoietic stem cell 174 transplantation (HSCT) demonstrated that higher abundance of a bacterial group composed 175 mostly of *Eubacterium limosum* could decrease the risk of relapse and disease progression⁵¹. 176 Lack of commensal microbes like E. limosum or their immunomodulatory metabolites (e.g. 177 short-chain fatty acids) can increase the risk of gut permeability, and result in translocation of pathobionts and overexpression of inflammatory cytokines⁵². Thus, identifying microbiome 178 signatures that contribute to immune system deterioration in MDS may lead to novel 179 180 therapeutic strategies to control inflammation and potentially prevent disease progression.

e) Immune dysregulation in MDS: autoimmunity or autoinflammation?

Although there is evidence for the presence of both innate immune-related 182 'autoinflammation' as well as adaptive autoimmune responses in MDS^{10, 53, 54}, these two terms 183 are sometimes used interchangeably, which may cause some confusion. The term 184 'autoimmunity' coins a condition associated with the presence of autoreactive T cells and high 185 186 autoantibody titres, whereas 'autoinflammation' generally refers to a condition with 187 dysregulated myeloid-driven innate immune responses only. This view clearly separated autoinflammation and autoimmunity as distinct immunological diseases. However, and this 188 may be true for MDS, some chronic inflammatory diseases may lie on a spectrum from 189 190 autoinflammatory to autoimmune, sharing genetic associations, common inflammatory pathways (TLR, PI3K-Akt, and NF-KB signalling), and connecting by variable degrees of 191 interaction between innate and adaptive immune responses^{55, 56} (FIG. 2). 192

Autoimmune features were long considered as a coincidence rather than a predisposing factor 193 for MDS. Spurred from case reports and smaller studies, a large population-based study was 194 195 designed, which demonstrated an increased risk of MDS among patients with antecedent 196 autoimmune disease (AID) (OR 2.1; 95% CI 1.7-2.6) or infectious disease (OR 1.3; 95% CI 1.1-197 1.5), indicating that chronic immune stimulation (the 'immune biography') might act as a trigger for MDS development⁵⁷. On the other hand, AID can be a favourable prognostic factor 198 in patients with established MDS⁵⁴, but additional large prospective studies are necessary to 199 200 confirm these results.

201 Immune surveillance, microenvironment, and MDS progression

a) Immune surveillance and MDS progression

203 The immune response to cancer requires a series of carefully regulated events that in principle should amplify and broaden cellular immune responses⁵⁸. Chronic inflammation affects 204 205 immune surveillance and has two overlapping effects in MDS. On the one hand, DAMPs and/or 206 founder gene mutations license the NLRP3 inflammasome to generate an inflammatory feed-207 forward process characterised by excess pro-inflammatory cytokines, such as IL-1 β , TNF- α , 208 and IFN- γ (FIG. 1). Pro-inflammatory cytokines may facilitate the selection of neoplastic clones 209 by simultaneously enhancing their growth and exhausting non-neoplastic clones, as demonstrated by the paradoxical effects of IL-1 β on AML versus normal progenitors³². 210 211 Moreover, cytokine-mediated induction of immunoinhibitory molecules like programmed cell death-ligand 1 (PD-L1) may contribute to T cell suppression and reduced immune 212 213 surveillance⁵⁹. On the other hand, excess DAMPs may expand myeloid-derived suppressor cells (MDSCs)⁶⁰, which overproduce suppressive cytokines, such as IL-10 and transforming 214 215 growth factor- β (TGF- β), contributing to immunosuppression and ineffective haematopoiesis^{60, 61}. 216

In general, low-risk disease is related to a more pro-inflammatory immune response and higher numbers of effector-type cells, such as IL-17⁺ CD4⁺ cells¹¹, while higher-risk disease is characterised by a predominantly suppressive milieu with significant expansion of immunosuppressive cells, such as Tregs^{62, 63} and MDSCs^{12, 60}, accompanied by a reduction in the number and function of bone marrow (BM) dendritic cells⁶⁴, peripheral CD8⁺ T⁶⁵, and NK cells⁶⁶ (FIG. 1). The proliferative capacity of Tregs appears compromised during earlier disease stages, but is restored during disease progression⁶⁷. A positive correlation between the

224 numbers of circulating MDSCs and Tregs has been observed, suggesting a role of MDSCs in the 225 expansion of Tregs and subsequent disease progression¹². Moreover, an independent 226 prognostic value of peripheral Treg and BM progenitor B cell frequencies in lower-risk MDS has been suggested^{13, 62}. Reduced NK function in higher-risk MDS likely supports immune 227 228 evasion and disease progression^{66, 68}. Hence, a novel strategy to restore NK cell function and overcome MDSC-mediated suppression in MDS patients has been proposed (TABLE 1)⁶⁹. In 229 230 addition, the presence of KIR haplotype A on NK cells may represent an independent risk 231 factor for the progression of MDS to AML⁷⁰.

Overall, similar to the role of inflammation in the initiation of MDS, the cellular immune response in established MDS is multifactorial and follows a stepwise transformation from an activated protective to a more immunosuppressive response as the disease progresses. Discrete patterns of cytokine expression may be evident throughout MDS progression and an integrative approach is required to study specific components of MDS pathogenesis in relation to cytokine network dynamics and immune cell states.

238 b) Microenvironment and MDS progression

239 Inflammatory cues from the surrounding microenvironment may actively contribute to the 240 formation and/or maintenance of a mutagenic environment in MDS and might suppress immune effector responses^{71–74}. Mesenchymal stromal cells (MSCs) and their progeny are 241 242 important components of the HSPC niche and regulate haematopoiesis by cell-to-cell contact or through paracrine signals⁷⁵. MSCs undergo functional decline with systemic ageing ⁷⁶. This 243 244 is further aggravated in MDS/AML MSCs, which have accumulated structural, epigenetic and functional alterations, chromosomal aberrations different from those found in HSPCs, and 245 display activation of key inflammatory pathways^{77–81}. Interestingly, MDS haematopoietic cells 246

can instruct healthy MSCs to acquire MDS-like features⁷⁸. In turn, MDS MSCs produce a variety
of cytokines and other factors (e.g. S100A8/9^{25, 81}), and exert immunomodulatory/suppressive functions that could further promote propagation of malignant HSCs^{25, 82}.
Mesenchymal S100A8/9 expression has been shown to be predictive of leukaemic evolution
and progression-free survival in a cohort of homogeneously treated low-risk MDS patients,
suggesting molecular characteristics of the mesenchymal niche as an important determinant
of disease outcome²⁵.

254 Clinical experience with immune interventions

255 Immunomodulatory therapies have long been employed for MDS, with benefits for selected 256 patient subgroups. Immunosuppressive therapy (IST) with antithymocyte globulin (ATG), and 257 in combination with prednisone or cyclosporine, provides a therapeutic option for selected 258 lower-risk patients, particularly those with hypoplastic MDS, a still poorly defined subgroup⁸³⁻ 259 ⁸⁶. The immunomodulatory drug lenalidomide has shown a high rate of activity in lower-risk 260 del(5q) MDS⁸⁷, but also yields sustained responses in 26.9% lower-risk non-del(5q) MDS, while 261 predictive immunological biomarkers associated with this response are lacking⁸⁸. Allogeneic 262 HSCT is another type of immunotherapy which has long been used in MDS and could lead to 263 a beneficial graft-versus-leukaemia (GvL) effect. The success of this therapeutic approach may 264 also be based on its capacity to reprogram the niche-driven immune dysregulation in MDS.

While recent progress in cancer immunology and the emergence of novel cancer immunotherapies brought new hope for many cancer patients, including those with MDS and AML^{69, 89–92} (TABLE 1), the overall response rates to these therapies are variable and less than 50% in the majority of malignancies, including MDS. So far, single-agent application of PD-1/PD-L1 as well as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) checkpoint inhibitors 270 (CIs) has shown limited efficacy in advanced disease after hypomethylating agent (HMA) 271 failure, with variable overall response rates as low as 0% for nivolumab (0/15)⁹³, 4% for 272 pembrolizumab (1/27)⁹⁴, and 3.4 (1/29)-22% (2/9) for ipilimumab^{93, 95}. Hence, combination strategies with CIs both in the upfront as well as HMA-refractory setting to counteract HMA-273 induced checkpoint upregulation are currently under intensive investigation^{89, 92, 96}. 274 Nonetheless, single-agent therapy might display disease-modifying activity in selected 275 patients, including elderly AML patients⁹⁷. Recent studies have also indicated the potential of 276 targeting the innate immune checkpoint CD47-SIRP α in cancer, including haematologic 277 cancers^{98, 99}. So far, blocking the interaction between the 'don't-eat me' signal CD47 and the 278 phagocyte inhibitory immunoreceptor SIRP α has shown low activity in a small AML/MDS 279 cohort (1/10), but initial results from the combination therapy with 5-Aza are promising¹⁰⁰. 280 Altogether, there is growing evidence that the combination of drugs with different mechanism 281 282 of action might offer clinical benefit in MDS/AML, while the search for reliable biomarkers for response continues. This will require innovative and multicentre clinical trial designs to obtain 283 284 meaningful results in larger patient cohorts¹⁰¹. It is worth mentioning that reliable predictors 285 are also lacking for routine monotherapies. For instance, recent studies have evaluated how mutations correlate with clinical benefit from HMA therapy. While earlier studies reported a 286 favourable effect of TET2 mutations on response rates^{102, 103}, this association was not 287 confirmed in a different cohort¹⁰⁴. 288

Finding predictive biomarker(s) for response to therapy is of particular relevance for the elderly population, which often displays lower response and higher toxicity rates. However, finding a magic 'fits all' predictive biomarker in MDS is an unlikely scenario, considering the complexity of the disease and the role of several genetic, immunological, and environmental factors in its pathophysiology. Technological advances in recent years, thanks to affordable 294 omics experiments, led to a so-called 'big data revolution'. The challenge, however, is to 295 integrate the massive amount of data and create computational models to build knowledge 296 and identify signatures that are important in patients' stratification for immunotherapy¹⁰⁵. To 297 overcome this challenge, a more comprehensive and combinatorial approach is necessary, 298 which utilises individual biomarkers as part of the bigger picture rather than the whole story.

299 Systems Immunology; a way forward

a) Framework for comprehensive immune monitoring in clinical trials

301 Overall, sufficient evidence exists to support the role of the 'immunome' as an important and 302 independent factor in MDS/AML patients` stratification. Nonetheless, immune responses 303 against malignant clones require coordination between cell types and across tissues, and a 304 systems immunity screening approach is necessary to evaluate the overall 'immune fitness' in cancer, as previously shown¹⁰⁶. Data from recent cancer studies highlighting the power of 305 306 integrative approaches are encouraging^{105, 107}. Nevertheless, there is still no standard or 307 widely accepted method for monitoring the overall immune response in haemato-oncology in 308 general or MDS in particular. Data from state-of-the-art immune monitoring strategies need 309 to be merged with clinical data and other omics data for multiomics-driven analysis to identify robust and predictive immune-signatures, and map the interaction between disease-310 311 associated inflammation and potentially host-beneficial cellular immune responses (FIG. 3). 312 Multiomics-driven analysis has shown the power to identify key molecular pathways in cancer 313 progression and could identify pathway-enriched cancer driver modules based on DNA, RNA, and protein data¹⁰⁸. For instance, web tools like LinkedOmics provide a user-friendly platform 314 to explore, analyse, and compare cancer multiomics data within and across tumour types¹⁰⁹. 315 316 The widespread use of NGS technologies and the maturation of cutting-edge technologies,

such as single-cell RNA-seq¹¹⁰, CITE-seq¹¹¹/Ab-seq¹¹², and mass cytometry by time of flight
(CyTOF)¹¹³, generate large datasets that can be mined for immunologically relevant
parameters and serve as input for integrative data analysis.

Over the last years, NGS technologies are increasingly used in the clinical setting for 320 mutational profiling in MDS, utilising comprehensive myeloid NGS panels¹¹⁴. In many clinics, 321 multiparameter flow cytometry (MFC) is increasingly used to reinforce MDS diagnosis^{115, 116}. 322 MFC has also been extensively applied to characterise the immune landscape in MDS^{11, 12, 60,} 323 324 ^{62–67, 117} and has demonstrated utility for monitoring immune-modifying agents in high-risk 325 MDS/AML¹¹⁸ or minimal residual disease monitoring, as has been shown in multiple myeloma¹¹⁹. CyTOF, which achieves an even higher resolution of the single-cell proteome, has 326 been broadly applied in the solid cancer field to profile the tumour immune landscape^{120, 121}, 327 328 to monitor checkpoint-blockade-induced immune responses, and predict response to PD-1 329 immunotherapy^{122, 123}. CyTOF has also been already successfully adopted for immunophenotypic analysis of clinical samples in MDS¹²⁴, prospective immune monitoring of 330 331 patients with chronic myeloid leukaemia (CML)¹²⁵, and to further characterise the immune signature in a wider range of T cell subsets in MDS¹²⁶. 332

There are, however, two important questions to be addressed: 1) Which immunological 333 334 markers to use? 2) How will we define an immunoscore? We are still in the early days but 335 resources are already available, which could be used and customised for MDS/AML. In an 336 attempt to identify and characterise all major human immune cell lineages in a single assay, 337 Hartmann et al. have designed and validated a CyTOF panel that can be incorporated into cancer immunotherapy trials¹²⁷. This framework provides a set of markers also relevant for 338 future clinical trials in MDS and may be extended by markers relevant for further 339 340 immunophenotyping of immune cell subsets and HSPCs (supplementary TABLE S1).

341 In solid tumours, infiltrating T cells have been generally associated with a positive prognosis, 342 which led to the development of the Immunoscore, a scoring system based on the 343 quantification of cytotoxic and memory T cells in the tumour centre and invasive margin^{128,} ¹²⁹. While this immunohistochemical tool has demonstrated prognostic value for solid 344 tumours¹³⁰, it cannot be directly applied to the MDS/AML BM microenvironment, which lacks 345 346 a clear invasive margin and a tumour core. However, automated image analysis of BM tissues 347 in combination with flow cytometry and clinical parameters has been shown useful for 348 predicting treatment responses in CML¹³¹. A comprehensive immunoscore for MDS will likely 349 be based on multivariate features derived from genomic, transcriptomic, and proteomic data (FIG. 3 and supplementary FIG. S1). The solid tumour field provides examples of how such 350 351 immune profiling can be used to train predictive models and generate immunoscores^{132–134}. 352 Overall, this will require an expanding computational toolbox to process, analyse and visualise 353 the highly complex and heterogeneous datasets being generated on bulk tissue and at single-354 cell level (reviewed by Finotello et al.¹³⁵) as well as validation of predictive biomarkers in 355 independent cohorts and across MDS subtypes.

Moreover, comprehensive interrogation of cancer immunity in MDS requires longitudinal as well as paired sampling to evaluate the impact of a given therapy on peripheral blood immune cells and the BM immune microenvironment. Combinatorial agents, such as 5-Aza and lenalidomide, can exert direct immunomodulatory effects on immune cells and BM MSCs^{79,} 1^{36, 137}. Thus, careful dissection of the net immunomodulatory effects of combination therapy through serial assessment can provide adequate information regarding activation of alternative pathways and inform subsequent clinical trials.

b) Dissecting good and not so good immune responses

364 While it is, for instance, possible that autoinflammatory and autoimmune features are present 365 in a single patient, a dominant clinical representation of one of these conditions is more likely. 366 An important aspect of immune profiling in MDS would therefore be to identify MDS patients 367 with an underlying autoimmune response that could benefit from immunosuppressive 368 therapy (IST) or potentially Treg-based therapies to reinstate immune regulation (FIG. 3). 369 Immune profiling may also help to identify lower-risk MDS patients who harbour a signature 370 characteristic of smouldering innate inflammation in the absence of autoimmune disease. 371 These patients may benefit from novel therapies targeting S100A8/9-related inflammasome 372 activation or TLR pathways. Patients with potentially immunogenic somatic mutations may 373 benefit from novel vaccination therapies with or without immune CIs to reinstate the 374 beneficial immune response against dysplastic clones. On the other hand, it is equally 375 important to identify patients without dominant inflammatory/autoimmune features or 376 immunogenic somatic mutations who are less likely to respond to novel immunotherapies and 377 may benefit from other forms of therapies, such as early HSCT.

378 Conclusion

In conclusion, collection of comprehensive omics datasets will leverage the development of a computational pipeline specific to MDS that will help to identify key features at various biological levels, their interconnectivity, and to better predict patient outcomes. To achieve this, well-coordinated studies on large cohorts of patients are crucial to combine known as well as potentially relevant predictive immunological biomarkers with clinical data. We expect that applying validated immune signatures to routine clinical investigations will improve patients' stratification for therapeutic intervention, and ultimately improve patient outcomes.

386 Figure legends

387 Fig. 1: The immune contexture in MDS. Certain conditions associated with chronic immune 388 stimulation, such as ageing, chronic infection, and autoimmune disease, may contribute to set 389 the biologic background for MDS development (left). Chronic immune stimulation leads to 390 sustained TLR activation that may drive haematopoietic skewing and loss of stem cell 391 quiescence. Initial events may induce a 'myeloid bias' of HSCs and multipotent progenitors, 392 and such a bias could skew the accumulation of somatic mutations conferring clonal 393 advantage and/or differentiation defects towards the myeloid lineage. Elevated levels of pro-394 inflammatory cytokines, reactive oxygen/nitrogen species, and DAMPs induce activation of 395 the NLRP3 inflammasome, resulting in pyroptosis of HSPCs, consequent cytopenias, an 396 inflammasome-driven inflammatory circuit, and an increasing dysfunction of the 397 haematopoietic stem cell niche including mesenchymal alterations (middle). Subsequently, 398 the presence of smouldering inflammation may support the propagation of pre-malignant 399 clones (e.g. via ROS-dependent Wnt/ β -catenin pathway) and subvert adaptive immunity 400 (right). The immune contexture dynamically changes with disease progression. In higher-risk 401 MDS, an expansion of MDSCs and Tregs contributes to the suppression of antitumour 402 responses and immune evasion of malignant clones. Regarding CD4⁺ T cell subsets, which 403 display significant plasticity in response to changing environmental cues, different CD4⁺ T cell 404 signatures are to be expected in MDS subtypes with predictive value for disease progression 405 and response to therapy, as shown in other diseases like aplastic anaemia¹³⁸. Abbreviations: ASXL1, additional sex combs-like 1, transcriptional regulator; DAMP, damage-associated 406 molecular pattern; DC, dendritic cell; DNMT3A, DNA methyltransferase 3 alpha; HIF-1 α , 407 408 hypoxia-inducible factor 1, alpha subunit; HSPC, haematopoietic stem and precursor cell; IL-409 1R1, interleukin-1 receptor, type 1; IL-1RAP, interleukin-1 receptor accessory protein; M,

410 macrophage; MDSC, myeloid-derived suppressor cell; MSC; mesenchymal stromal cell; NK, 411 natural killer cell; NLRP3; nucleotide-binding domain and leucine-rich repeat pattern 412 recognition receptor (NLR) family, pyrin domain-containing protein 3; ROS, reactive oxygen 413 species; RNS, reactive nitrogen species; SF3B1, RNA splicing factor 3B, subunit 1; SRSF2, 414 serine/arginine-rich splicing factor 2; STAT3-P, signal transducer and activator of transcription 3, phosphorylated; TET2, tet methylcytosine dioxygenase 2; TLR, Toll-like receptor; TNFR, 415 416 tumour necrosis factor receptor; Treg, regulatory T cell; U2AF1, U2 small nuclear RNA auxiliary 417 factor 1.

Fig. 2: MDS across the autoinflammatory/autoimmune disease continuum. The clinical 418 419 heterogeneity of MDS may reflect the variable contribution of autoinflammatory and 420 autoimmune processes to disease pathogenesis. The classic autoinflammatory syndromes are 421 usually related to monogenic (e.g. cryopyrin-associated periodic syndromes [CAPS], TNF 422 receptor-associated periodic syndrome [TRAPS]) or polygenic mutations (e.g. Crohn's disease) in genes important in the regulation of the innate immune response. Several 423 424 autoinflammatory disorders, including CAPS¹³⁹ and Crohn's disease¹⁴⁰, have been linked to 425 mutations/genetic variants in NLRP3 and overproduction of IL-1 β . The adaptive immune 426 response plays the predominant role in the clinical expression of monogenic (e.g. immune 427 dysregulation polyendocrinopathy enteropathy X-linked syndrome [IPEX]) and polygenic (e.g. 428 rheumatoid arthritis, systemic lupus erythematosus [SLE]) autoimmune diseases. However, 429 innate immune mechanisms, in particular the NLRP3 inflammasome, are also emerging as important players in various autoimmune diseases, including SLE¹⁴¹. Some diseases, referred 430 to as mixed-pattern diseases, are on the borderline between autoimmune and 431 432 autoinflammatory diseases, and may share genetic associations, treatment responses and clinical manifestations¹⁴². Abbreviations: DAMPs, danger-associated molecular patterns; Mø, 433

macrophage; MHC class II, major histocompatibility complex class II; Mo, monocyte; Neu,
neutrophil; NLRP3; nucleotide-binding domain and leucine-rich repeat pattern recognition
receptor (NLR) family, pyrin domain-containing protein 3; PAMPs, pathogen-associated
molecular patterns.

438 Fig. 3: Multiomics pipeline for MDS. Implementing systems biology approaches in MDS is an 439 unmet and urgent clinical need to not only understand the pathophysiology of this complex 440 disease but also to create a more personalised approach to therapy. Multiple types of highly complex and rich omics data are being generated in large scale and are particularly helpful in 441 442 MDS patients' risk stratification and for identifying novel therapeutic targets. Different data 443 types, including clinical, genomic (multigene NGS-based sequencing panels), transcriptomic (single-cell RNA-seq), targeted transcriptomic (NanoString¹⁴³), proteomic/immunophenotypic 444 445 (CyTOF, flow cytometry), and metagenomic (16S ribosomal rRNA sequencing, high-446 throughput shotgun sequencing) datasets, will be combined with the development of a 447 bioinformatics pipeline, allowing an integrative view of the immunome in MDS patients. The 448 advent of new technologies like TARGET-seq¹⁴⁴, which combines high-sensitivity single-cell 449 mutational analysis and parallel RNA-seq, will further help to resolve inflammatory signatures 450 of MDS genetic subclones and non-mutant cells. The analytical pipeline will employ 451 customized computational methods to incorporate single-cell and bulk multiomics data, 452 leveraging on mathematical models to provide a holistic view of all components and modelling 453 of biological networks to identify disease signatures. This provides an unprecedented 454 opportunity to identify immune profiles, examine the association between common driver 455 mutations and immune subtype, and to better understand how somatic mutations and 456 immune cell activation states impact the disease course, response to treatment, and outcome. Abbreviations: ASXL1, additional sex combs-like 1, transcriptional regulator; BM, bone 457

- 458 marrow; HMA, hypomethylating agent; HR, higher-risk; HSCT, haematopoietic stem cell
- 459 transplantation; IS, immunosuppressive; IST, immunosuppressive therapy; PB, peripheral
- 460 blood; QOL/PRO, quality of life/patient reported outcome; SF3B1, RNA splicing factor 3B,
- 461 subunit 1; *TET2*, tet methylcytosine dioxygenase 2; TLR, Toll-like receptor.

462 Table legends

- 463 **Table 1: Novel therapeutic agents evaluating immune targets in MDS**
- 464 Aza, 5-azacytidine; BTK, Bruton's tyrosine kinase; CAR, chimeric antigen receptor; CCUS, clonal
- 465 cytopenia of undetermined significance; HMA, hypomethylating agent; int-1, intermediate-1;
- 466 int-2, intermediate-2; Len, lenalidomide; MM, multiple myeloma; RAEB, refractory anemia
- 467 with excess blasts; R/R, refractory/relapsed; TRIKE; trispecific killer engager.

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475

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481 Author contributions

482 S.W., S.K., and U.P. designed the review. All authors contributed to the writing and editing of483 the manuscript.

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FIG.2



FIG. 3



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Supplementary Fig. S1: Integrative immunoscore for MDS. Online version only. Integration of data from different omic platforms with clinical data could identify a biomarker panel to improve stratification of MDS patients.

Abbreviations: BM, bone marrow; HLA, human leukocyte antigen; QOL/PRO, quality of life/patient reported outcome.