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14. ABSTRACT We have proposed that an innate immune signaling axis formed by Toll-like receptor activation and maintained by the histone demethylase JMJD3 is deregulated in the bone marrow hematopoietic stem/ progenitor cells (HSPCs) of MDS. In this funding year, we have performed large scale expression and mutational analyses of key genes in this pathway in primary patient samples. We have achieved a systematic gene expression profiling of TLR1, 2, 6, JMJD3, IL8, and MYD88 in MDS. We have analyzed TLR2-F217S as a somatic mutation with biological gain-of-function property that occurs in 10% of 150 patients. Through clinical data analysis, we have defined associations of deregulation of TLR2-JMJD3 innate immunity genes with IPSS and survival of patients. At the biological level, we have characterized the impact of TLR2 signaling in primary HSPCs, which indicate that abnormal activation of TLR2 inhibits erythroid differentiation. Finally, we have demonstrated that interference of TLR2-JMJD3 innate immunity signaling through inhibition of TLR2 and JMJD3 rescues the differentiation of erythroid lineage in patients with lower-risk diseases (low-risk and intermediate-1). In summary, we have achieved a better understanding of the TLR2-JMJD3 innate immune pathway and its biology in MDS, including identification of potential biomarkers and novel therapeutic targets in this disease.						
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1. INTRODUCTION

MDS is a very heterogeneous group of bone marrow myeloid malignant disorders characterized by peripheral blood cytopenias and increased risk of transformation to acute myelogenous leukemia (AML). The molecular pathogenetic mechanism of MDS is still far from clear. Through preliminary studies we have identified that an innate immune signaling axis formed by Toll-like receptor activation of NF- κ B maintained by the histone demethylase JMJD3 is deregulated in the bone marrow hematopoietic stem/ progenitor cells (HSPCs) of patients and potentially contributes to disease pathogenesis. Based on this we propose a systematic analysis of the TLR2-JMJD3 pathway in MDS. In detail, we propose to perform a large scale expression profile of the key genes in this pathway in primary samples from patients with MDS; to study the molecular implications of deregulated TLR2/NF- κ B/JMJD3 signals in the pathogenesis of MDS; and to study the potential therapeutic effects of interfering with TLR2 function in MDS. The objective of the proposed studies is to achieve a better understanding of this innate immune pathway and its biology in MDS and, furthermore, to identify potential key biomarkers of prognosis and/ or novel therapeutic targets that eventually will improve the therapy of patients with MDS.

2. KEYWORDS

TLR2, lentivirus, CD34+ cells, colony formation, hematopoiesis, OPN305

3. OVERALL PROJECT SUMMARY

In year one, we completed the proposed systematic expression profiling of key component genes of the TLR2-JMJD3 innate immunity signaling pathway in the CD34+ enriched MDS bone marrow hematopoietic stem/ progenitor cells. We also demonstrated that interference of the TLR2-JMJD3 innate immunity signaling through shRNA inhibition of TLR2 and JMJD3 could rescue the differentiation of erythroid lineage in patient cells.

In the second year, we continued the studies and focused on the following two major directions: **1).** To characterize the effects of TLR2 WT and F217S-MUT overexpression in normal bone marrow hematopoietic stem/ progenitor cells; and **2).** To evaluate the potential therapeutic effects of the inhibition of TLR2 signaling via a TLR2 specific antibody in MDS.

In Aim 2 of this proposal, we proposed to study the molecular implications of TLR2 alterations in normal CD34+ cells via recombinant lenti viral transduction. To address this aim, we prepared and purified the recombinant lenti-virus expressing wild-type and mutant (F217S) TLR2. We then transduced four cases of normal bone marrow CD34+ cells that were isolated from healthy donors with wild-type, mutant TLR2, as well as control (GFP) virus. Overexpression of transduced TLR2 genes was confirmed by Q-PCR (**Figure 1A**). Following virus transductions, BM CD34+ cells were treated with TLR2 agonist PAM3CSK4. Proliferation and colony forming ability of cells were then analyzed. Results indicated that in this ex vivo culture condition, stimulation of TLR2 with PAM3CSK4 promotes the proliferation of CD34+ cells (**Figure 1B**). However, the overexpression of TLR2 WT or MUT cannot further alter the proliferation of CD34+ cells (**Figure 1B**). Similar results were observed in colony formation

assays: while PAM3CSK4 reduced the formation of erythroid colonies (BFU-E) (**Figure 1C and D**), there was no significant difference between GFP control with TLR2 WT or MUT transduction in the number of erythroid or myeloid colonies (**Figures 1C and D**).

These results implicate that TLR2 overexpression alone may not be sufficient to change the fate determination of bone marrow CD34⁺ stem/progenitor cells. An alternative interpretation may be that the ex vivo culture of bone marrow stem/progenitor cells may not be appropriate to analyze the biological effect of TLR2 overexpression on hematopoiesis. We therefore decided to generate the mouse model of altered TLR2/JMJD3 signaling and to analyze the effect on hematopoiesis in vivo. In an attempt to address this need, we are currently developing a hematopoietic-specific JMJD3 and TLR2 transgenic mouse model, in which the human JMJD3/TLR2 cDNA is overexpressed under the control of the murine hematopoietic Vav promoter. We have obtained preliminary results indicating that the Vav-JMJD3 mice have started demonstrating hematological phenotypes that are highly similar to critical clinical features in patients with MDS. This result implicates that the alteration of TLR2-JMJD3 signaling axis can impact hematopoiesis. Detailed in vivo assays of the mice will be performed.

In Aim 3 of this proposal, we plan to study the molecular effects of interfering with TLR2 function in MDS CD34⁺ cells. We initially applied the shRNA to inhibit the expression of TLR2 in MDS bone marrow CD34⁺ cells. Results of shRNA experiments were reported in the last annual report. Following that, we set up a collaboration with Opsona Therapeutics and acquired a fully humanised monoclonal antibody that specifically recognises TLR2, OPN305. The CD34⁺ BM cells from low risk MDS patients were obtained and were treated with OPN-305 ex vivo. TLR2 expression was also measured in these patients (**Figure 2A**). The efficacy of OPN-305 was investigated through ex vivo colony formation in methocult assays. Of key importance, results demonstrated that inhibition of TLR2 with OPN-305 elicits the same effect as TLR2-shRNA, which is the increase of CFU-E formation and no effect on CFU-G/M formation (**Figure 2B**). These results together indicate that TLR2 inhibition is a target specific event rather than an artifact in one experimental system. Based on these pre-clinical investigations, we have opened a pilot clinical trial in MDS using OPN-305, which as far as we know is the first clinical trial of anti-TLR antibody in MDS.

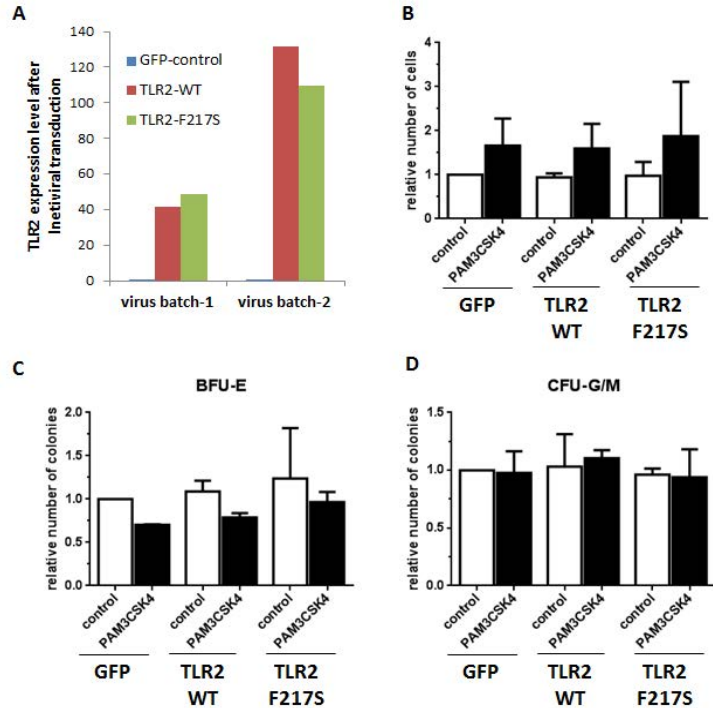
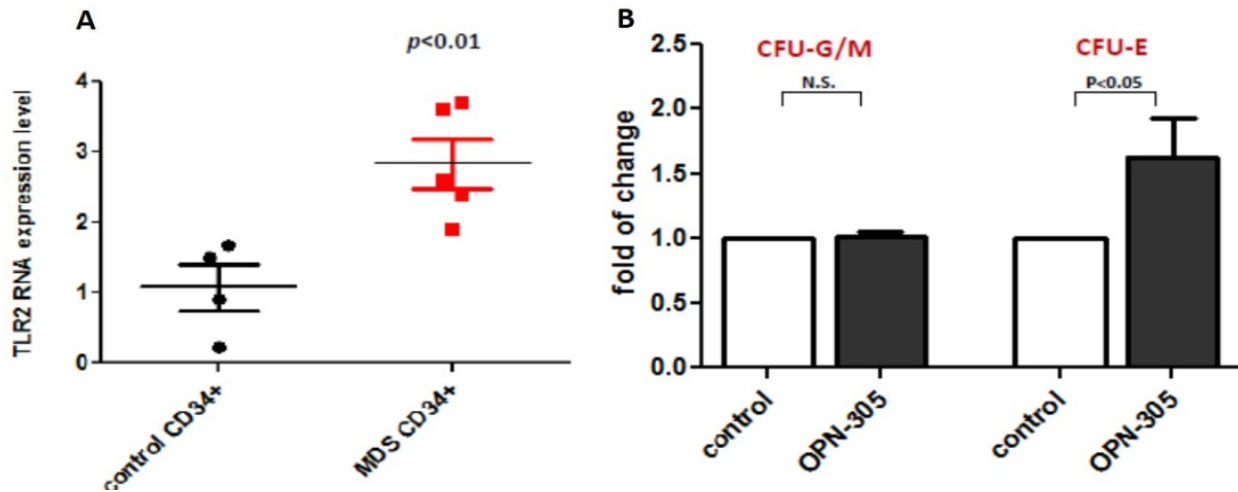


Figure 1. Lentiviral transduction of TLR2 WT and F217S MUT into normal BM CD34⁺ cells. (A) Expression of transduced TLR WT and MUT genes in CD34⁺ cells. (B) Proliferation of transduced BM CD34⁺ cells with and without PAM3CSK4. (C-D) Colony formation of transduced BM CD34⁺ cells with and without PAM3CSK4.



4. KEY RESEARCH ACCOMPLISHMENT

The most important accomplishment derived from the proposed studies is that we are able to demonstrate that TLR2 innate immune signaling is excessively activated in MDS bone marrow stem/progenitor cells and that inhibiting this pathway (by shRNA or the antibody OPN-305) can improve hematopoietic differentiation. Based on these observations, the clinical trial, a collaboration between our group and Opsona Therapeutics, has been opened in patients with low risk MDS. This clinical trial, a prospective, open label phase I/II study to assess the safety and efficacy of cycles of intravenously infused doses of OPN-305 in second-line lower (low and intermediate-1) risk myelodysplastic syndrome (MDS), is under the direction of Dr Guillermo Garcia-Manero.

5. CONCLUSION

In summary, our work provides systematic evaluation of the TLR2-JMJD3 innate immune pathway in the HSPCs of MDS in a large patient cohort. We have achieved critical preclinical evidence that inhibition of this signaling can improve the hematopoietic differentiation of MDS HSPCs. This information has been applied toward development of the OPN305 TLR2 antibody clinical trial. Finally, ex vivo study of the overexpression of TLR2 does not demonstrate significant effect on the fate of normal bone marrow HSPCs, suggesting that in vivo studies are needed to better evaluate the impact of TLR2 signaling in hematopoiesis. We have started to address this by generating mouse models of TLR2 and JMJD3 using a hematopoietic tissue specific (Vav) system.

Our future plan is to evaluate the efficacy of OPN305 in low-risk MDS patients in the clinical trial. Furthermore, correlative molecular studies will be performed in the hematopoietic specimens, including BM HSPCs, that are collected in responding and non-responding patients to OPN305. These molecular studies will include the evaluation of innate immune signal activation, NF- κ B activity, as well as the levels of the inflammatory cytokines that are known to be regulated by TLR2-JMJD3 signals. The other direction of our future studies is to characterize

the Vav-JMJD3/ Vav-TLR2 mouse model in order to assess the in vivo effect of this pathway in hematopoiesis and the fate determination of hematopoietic stem/ progenitor cells.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Lay Press: Nothing to report

b. Peer-Reviewed Scientific Journal: Deregulation of Innate Immune and Inflammatory Signaling in Myelodysplastic Syndromes. Irene Gañán-Gómez, Yue Wei, Daniel T. Starczynowski, Simona Colla, Hui Yang, Mónica Cabrero-Calvo, Zachary S Bohannon, Amit Verma, Ulrich Steidl, Guillermo Garcia-Manero (Leukemia, submitted October, 2014)

c. Invited Articles: Nothing to report

d. Abstracts to be published in November, 2014 issue of Blood Journal /Poster Presentations:

1). Association Between Down-Regulation of EZH2 and Abnormal Karyotype, Response to Hypomethylation Treatment, and Patient Survival in Myelodysplastic Syndromes. **American Society of Hematology (ASH) Annual Meeting, Dec 2014, San Francisco**

2). Association Between Downregulation of POT1 Expression and Chromosome 7 Deletion, Response to Hypomethylation Agent Treatment, and Patient Survival in Myelodysplastic Syndromes. **American Society of Hematology (ASH) Annual Meeting, Dec 2014, San Francisco**

7. Inventions, Patents, and Licenses: Nothing to report

8. Reportable Outcomes: Nothing to report

1 **Deregulation of Innate Immune and Inflammatory Signaling in Myelodysplastic**
2 **Syndromes**

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22

1 **Abstract**

2 Myelodysplastic syndromes are a group of heterogeneous clonal hematologic malignancies that
3 are characterized by defective bone marrow hematopoiesis and by the occurrence of
4 intramedullary apoptosis. During the past decade, the identification of key genetic and
5 epigenetic alterations in patients has improved our understanding of the pathophysiology of this
6 disease. However, the specific molecular mechanisms leading to the pathogenesis of MDS
7 have largely remained obscure. Recently, essential evidence supporting the direct role of innate
8 immune abnormalities in MDS has been obtained, including the identification of multiple key
9 regulators that are overexpressed or constitutively activated in bone marrow hematopoietic stem
10 and progenitor cells. Mounting experimental results indicate that the dysregulation of these
11 molecules leads to abnormal hematopoiesis, unbalanced cell death and proliferation in patients'
12 bone marrow and plays an important role in the pathogenesis of MDS. Furthermore, there is
13 compelling evidence that the deregulation of innate immune and inflammatory signaling also
14 affects other cells from the immune system and the bone marrow microenvironment, which
15 establish aberrant associations with hematopoietic precursors and contribute to the MDS
16 phenotype. Therefore, the deregulation of innate immune and inflammatory signaling should be
17 considered one of the driving factors in the pathogenesis of MDS. In this article, we review and
18 update the advances in this field by summarizing the results from the most recent studies and
19 discussing their clinical implications.

20

21 **1. Introduction**

22 Myelodysplastic syndromes (MDS) are a heterogeneous group of hematologic stem cell
23 malignancies clinically characterized by cytopenias associated with defective hematopoiesis,
24 myeloid dysplasia and increased risk of transformation to acute myelogenous leukemia (AML).
25 One distinctive feature of this malignancy is the presence of increased apoptosis in bone
26 marrow (BM), which is, in contrast, generally hypercellular, although it can also be normal or
27 hypocellular. This heterogeneity of the disease characteristics complicates the diagnosis and
28 management.¹ Although significant efforts to understand the pathophysiology of MDS made
29 during the past decade have led to the identification of key genetic and epigenetic alterations in
30 patients,² the definite pathogenetic mechanisms of MDS are still not fully understood. Except for
31 a small subset that is eligible for stem-cell transplantation, most MDS patients have a poor
32 prognosis because frontline pharmacological therapies are not curative owing to the lack of well-
33 defined molecular targets.^{1, 2} Thus, there is an urgent need to characterize the molecular

1 mechanisms involved in the pathogenesis of MDS to allow the establishment of good diagnostic
2 protocols and specific and effective targeted therapies.

3 Remarkable breakthroughs have recently been achieved in this field. Clinical and molecular
4 studies of MDS have yielded accumulating evidence suggesting that abnormal activation of
5 innate immune signals and associated inflammation contribute to the pathogenesis of MDS.
6 New findings have improved our understanding of the molecular mechanisms triggering MDS
7 and have led to the development of promising therapeutic strategies. This article will review this
8 topic and present the most recent and significant studies, summarizing their results and
9 discussing their clinical implications and therapeutic applications.

10

11 **2. The Inflammatory and Autoimmune Nature of MDS**

12 **2.1. Abnormal Levels of Cytokines and Chemokines**

13 The presence of abnormal levels of cytokines, chemokines and growth factors in peripheral
14 blood (PB) and BM of MDS patients has been extensively documented (Table 1). In general,
15 levels of tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), transforming growth
16 factor beta (TGF- β), interleukin 6 (IL-6), IL-8 and the myeloid growth factors granulocyte
17 colony-stimulating factor (G-CSF) and macrophage CSF (M-CSF), among others, are increased
18 in MDS patients,³⁻¹³ which reflects dysregulation of both inflammatory signaling and myeloid
19 differentiation. Increased levels of some of these cytokines can affect the clinical outcomes of
20 patients. Higher levels of serum TNF- α are a potential adverse prognostic factor in AML and
21 high-risk MDS and are associated with higher leukocyte counts and higher levels of β 2-
22 microglobulin, creatinine, uric acid and alkaline phosphatase,¹⁴ Similarly, TNF- α , IL-6 and IL-1
23 receptor (IL-1R) levels have been related to ratings of fatigue in MDS.¹⁵

24 Several recent studies have utilized more comprehensive approaches, such as multiplex-based
25 analyses, to systematically determine the association between elevated cytokine levels and the
26 clinical characteristics and outcomes of MDS patients (Table 1). Kornblau et al. performed a
27 parallel profiling of 27 cytokines/chemokines in the peripheral plasma of 114 MDS patients and
28 found that the mean expression of several cytokines was significantly higher in MDS, whereas
29 the pleiotropic cytokines IL-10 and IL-4 were expressed at lower rates and directly correlated
30 with patient survival.¹⁶ Kornblau et al. further clustered the cytokines into 9 recurrent expression
31 patterns as “cytokine signatures” and studied their impact on clinical outcomes. Eight of the 9
32 signatures had prognostic implications, which included effects on remission, primary resistance,

1 relapse rates, and overall survival. However, there were some remarkable discrepancies
 2 between the results of this work and those of previous clinical studies. Indeed, some of the
 3 cytokines found to be highly produced in MDS before, such as IL-6 or IFN- γ , appeared to be
 4 downregulated in this study. In turn, two later large-scale studies in the PB of 57 MDS patients¹⁷
 5 and BM of 78 patients¹⁸ analyzed the expression of 32 and 30 cytokines, respectively, and both
 6 studies confirmed previous clinical data, in disagreement with the report by Kornblau et al.

7 Discrepancies between studies need to be considered, especially with regard to the substantial
 8 differences that are found when comparing MDS subtypes with different rates of apoptosis.
 9 Whereas low-risk disease (or subtypes without excess blasts such as refractory anemia, RA) is
 10 characterized by an elevated apoptotic index, high-risk MDS and the subtypes with high counts
 11 of blasts (RA with excess blasts, RAEB) are associated with more aggressive clonal expansion,
 12 tolerance to self-immunity and poor response to immunosuppressive therapy. The occurrence of
 13 apoptosis in MDS BM is closely associated with TNF- α levels. Thus, the secretion of TNF- α and
 14 other related cytokines, such as IFN- γ or IL-6, is higher in low-risk MDS, whereas these and
 15 other cytokines are more likely to be downregulated in high-risk cases. Likewise,
 16 immunosuppressive cytokines, such as IL-10, are more intensely secreted in high-risk MDS, in
 17 which the survival of the malignant clone is vital for the progression of the disease.¹⁹ Thus,
 18 cytokine secretion profiles vary between types of MDS, and this fact might be the origin of the
 19 discrepancies among some of the cytokine profiling studies summarized above. For instance,
 20 patients with RAEB are particularly abundant in the cohort studied by Kornblau et al.,¹⁶ which
 21 might explain their discrepant results.

Table 1. Cytokines and growth factors differentially expressed in MDS					
Cytokine/factor	Levels	Tissue	Plasma/cell type	Associations	References
GM-CSF	Normal/ low	PB, BM	Plasma		3
EGF	Low	PB	Plasma		17
CXCL5	Low	PB	Plasma		17
IL-10	Low	PB	Plasma		16
CCL5	Low	PB	Plasma		18
M-CSF	High	BM	Mononuclears		4
G-CSF	High	PB	Plasma	Direct: BM cellularity	16-18
TNF- α	High	BM, PB	Whole BM aspirates, BM plasma, BMMC cultures, fibroblast cultures, macrophage cultures, PB plasma	Direct: macrophage count, rate of BM apoptosis, FAB subtypes*, WHO stratification, fatigue; Reverse: Hb levels, patient survival	3-9, 14, 15, 17, 18
IFN- γ	High*	BM, PB	Whole BM aspirates, mainly in		5, 18

			myeloid cells		
TGF-β	High*	BM	Whole BM aspirates		3, 11
IL-1α/1βR	High*	BM, PB	Mononuclears, parenchyma, PB plasma	Direct: fatigue	4, 15-18
IL-1RAP	High	BM	Stem and progenitor cells	IPSS risk (high)	20
IL-4	High*	PB	Plasma		18
IL-6	High*	BM, PB	Plasma, BMMC cultures, fibroblast cultures	Direct: fatigue; Reverse: survival	7, 10, 15, 17, 18
IL-7	High	PB	Plasma	Reverse: survival	18
IL-8	High*	BM, PB	Plasma	Direct: WHO stratification, IPSS risk	10, 16, 18
IL-12	High	PB	Plasma		16
IL-13	High	PB	Plasma		18
IL-15	High	PB	Plasma		16, 18
IL-17	High	PB	Plasma		19
VEGF	High	BM, PB	PB serum, whole BM aspirates		12, 13, 18
ANG	High	PB	Plasma		12
CXCL10	High*	PB	Plasma	Direct: circulating blasts, thrombocytopenia; Reverse: survival	16, 18
CCL3	High	PB	Plasma		17
CCL4	High	PB	Plasma		17
HGF	High	PB	Plasma		17, 18
MIP-1β	High	PB	Plasma		18
MIG	High	PB	Plasma		18
Eotaxin	High	PB	Plasma		18
MCP-1	High	PB	Plasma		18

Abbreviations not included in the text: vascular endothelium growth factor (VEGF), angiogenin (ANG), C-X-C motif-ligand (CXCL), C-C motif ligand (CCL), World Health Organization (WHO), French-American-British classification (FAB), macrophage inflammatory protein 1β (MIP-1β), monokine induced by IFN-γ (MIG), monocyte chemotactic protein 1 (MCP-1).

* Discrepancies exist among different studies: IFN-γ and IL-6 were reported to be downregulated by one work¹⁶; IL-1 was found to be expressed at normal levels¹⁶; direct association of TNF-α with FAB subtypes was not confirmed by some works^{7, 16}; IL-4 levels were low according to one report¹⁶; no differences were found in IL-8 and CXCL10 levels in one study¹⁷.

1

2 **2.2. Association with Inflammatory and Autoimmune Disorders**

3 In addition to the elevated levels of cytokines found in clinical studies, case-report studies have

4 traditionally associated MDS with the coexistence of other inflammatory disorders, which have

5 an incidence between 10-30% in MDS patients and even higher in chronic myelomonocytic

6 leukemia (CMML).²¹

7 Very early in the study of MDS, clinicians noticed its frequent association with rheumatic

8 manifestations, especially rheumatoid arthritis. Although this is a common disease in the elderly,

9 the observations suggested that those associations were not fortuitous.²² Interestingly, a recent

1 literature review reported that arthritis preceded MDS in 55% of cases, and both pathologies
2 were concomitantly diagnosed in 27% of cases,²² which suggests that the existence of
3 inflammation precedes the appearance of MDS. Similarly, MDS and inflammatory bowel
4 disease (IBD) are frequently diagnosed simultaneously. However, in this case, many patients
5 diagnosed with IBD frequently presented with clinical manifestations of MDS before diagnosis²³⁻
6 ²⁵. Other acute and chronic autoimmune disorders associated with MDS are diverse types of
7 vasculitis, autoimmune anemias, several rheumatic and skin disorders and certain thyroid
8 diseases.^{21, 26, 27}

9 Larger-scale epidemiologic studies have confirmed that patients with autoimmune disorders
10 have increased risk of developing MDS when compared to matched controls. A five-year case-
11 control study including 84 MDS patients was the first to report that these disorders actually
12 precede MDS instead of being part of its clinical manifestations.²⁸ Later, a large retrospective
13 analysis of the population-based case-controlled Surveillance Epidemiology and End Results
14 (SEER)-Medicare database, which included 2471 MDS patients, confirmed that the risk of
15 developing AML and MDS is associated with the preexistence of an autoimmune condition.²⁹
16 Particularly, the association between MDS and rheumatoid arthritis was further supported by a
17 long-term follow-up study that used a cohort of 91291 patients with rheumatoid arthritis or
18 osteoarthritis who had received a knee arthroplasty.³⁰ This study revealed an especially high
19 incidence of MDS in those patients, pointing to arthritis rather than the surgical procedure as the
20 responsible factor for the increase in MDS risk. In parallel, a population study of the central
21 registries in Sweden validated the significant associations between MDS and most of the other
22 disorders mentioned before.³¹

23 Interestingly, IBD is the only of the abovementioned conditions for which an association with risk
24 of MDS has not been confirmed by large-scale population studies.^{32, 33} Indeed, patients
25 frequently present with clinical manifestations of MDS before diagnosis of IBD.²³⁻²⁵ This has led
26 to the speculation that MDS and IBD could have a common pathogenesis. For instance, one
27 case study reported that the same abnormal BM karyotype is associated with the development
28 of both MDS and IBD.²⁵

29 Besides autoimmune diseases, more recent epidemiologic studies support the increased risk of
30 MDS in patients affected by acute and chronic infections. Two similar population studies carried
31 out with data from the Swedish registries³¹ and the American SEER-Medicare database³⁴
32 analyzed the occurrence of infectious diseases in 1662 and 3072 patients with MDS,
33 respectively. Both studies showed that history of infection was significantly associated with a

1 higher risk of MDS, particularly with several infections of the respiratory tract. Interestingly, risk
2 of MDS was consistent and, in some cases, even higher when longer periods of latency (of up
3 to 3-4 years) were considered. This may suggest that chronic infections make patients more
4 susceptible of developing MDS.

5 Taken together, these data strongly indicate that some inflammatory and autoimmune disorders
6 favor the development of MDS. This causal association may be a consequence of the
7 pharmacological treatment of such disorders, but the associations are not specific to treated
8 conditions. Other factors that deserve to be discussed and studied in depth are the common
9 genetic predisposition to autoimmune alterations and MDS and the possibility that the
10 underlying inflammatory/autoimmune conditions can directly damage BM precursors and drive
11 malignant transformation.^{29, 34} Provided that the acquisition of certain infections is also related to
12 a high MDS risk, the latter option seems more compelling, although the two situations are not
13 mutually exclusive.

14 Even though the idea of autoimmune disorders being a direct cause of MDS is very intriguing
15 and attractive, the existing literature reviews are not conclusive about the prognostic meaning
16 of these pathologies in MDS or about their association with the MDS subtypes or cytogenetics;
17 therefore, epidemiologic data need to be carefully interpreted and studied coordinately with
18 mechanistic data.

19

20 **3. Deregulation of Innate Immune and Inflammatory Signaling in MDS**

21 **3.1. Pro-Inflammatory Signaling and Death Receptor Pathways**

22 Probably the greatest difficulty for the understanding of the pathogenesis of MDS and also a
23 source of controversy in this field is the coexistence of increased cell proliferation and cell death
24 in BM³⁵.

25 Increased rates of intramedullary apoptosis are considered the main cause of the PB cytopenias
26 that characterize MDS. This apoptosis is thought to be initiated by the death receptor Fas and
27 its specific ligand (Fas-L), which is overexpressed and correlates with the rate of apoptosis in
28 MDS^{6, 8, 36, 37} (Figure 1). Although BM CD34⁺ progenitors do not express Fas under physiologic
29 conditions, they can do so after exposure to cytokines such as TNF- α or IFN- γ .^{38, 39} Accordingly,
30 high levels of TNF- α are directly associated with apoptosis rates in MDS BM cells.³

1 The role of TNF- α in the pathogenesis of MDS is not limited to the induction of the expression of
2 Fas. TNF- α selectively binds two receptors, TNF receptor (TNFR) 1 and TNFR2 (Figure 1). The
3 primary role of TNFR1 is the induction of apoptosis through caspase-8 activation, whereas
4 TNFR2 has anti-apoptotic functions induced by the c-Jun N-terminal kinase (JNK) pathway.⁴⁰
5 Therefore, TNF- α secretion may also induce Fas-independent apoptosis in MDS via TNFR1.
6 Furthermore, it may modulate the progression of the disease because a switch in TNFR
7 expression associated with changes in apoptotic rates has been reported in MDS BM cells.^{9, 41}
8 Whereas TNFR1 is abundantly expressed in RA, TNFR2 is more highly expressed in RAEB,
9 indicating a correlation with the apoptotic activity in BM. Accordingly, RA patients also
10 overexpress TRADD, FADD and RIP compared to controls and RAEB,⁹ whereas levels of Fas
11 are lower in advanced stages of the disease and negatively correlated with higher counts of BM
12 blasts.³⁶ The blockade of TNF- α or Fas function by specific antibodies can partially restore
13 growth of MDS hematopoietic progenitors,^{8, 37} which shows a reverse association between the
14 functionality of TNF/Fas-dependent signaling and BM cellularity. Further evidence that pro-
15 apoptotic signaling is strongly associated with lower-risk MDS is that apoptosis has been found
16 to be one of the most significantly upregulated functional groups of genes in CD34⁺ cells from
17 RA versus controls and RAEB.⁴²

18 On the other hand, various cytokines, such as TGF- β , IFN- α and TNF- α itself, activate the p38
19 mitogen-activated protein kinase (MAPK) downstream signaling pathway in hematopoietic stem
20 and progenitor cells.⁴³ P38 MAPK is known to be hyperactivated in MDS bone marrows and to
21 increase apoptotic signaling in hematopoietic stem cells.⁴⁴

22

23 **3.2. The Role of Transcription Factor NF- κ B**

24 Transcription factors from the NF- κ B family are activated in response to a variety of stimuli,
25 such as inflammatory cytokines (like TNF- α), pathogenic antigens, oxidative stress, DNA
26 damage, and activation of Pattern Recognition Receptors (PRRs). NF- κ B activation triggers the
27 expression many target genes involved in the adaptive response to different types of stress and
28 in regulating the expression of a number of inflammatory cytokines and chemokines including
29 TNF- α , IL-1, IL-6 or IL-8, inducible enzymes, adhesion molecules and proteins regulating
30 immune responses. Importantly, this factor also regulates the expression of several anti-
31 apoptotic proteins and proliferative factors.^{45, 46}

1 There is a body of evidence that indicates that NF- κ B plays an important role in the survival of
2 MDS progenitors. NF- κ B activity is significantly elevated in MDS BM progenitors and cell lines
3 and has been correlated with the progression of the disease, with later stages of MDS
4 presenting with the highest activity levels.⁴¹ The blockade of NF- κ B activity has been shown to
5 induce apoptosis in normal and MDS BM precursors,⁴⁷⁻⁴⁹ suggesting that constitutive NF- κ B
6 signaling provides malignant cells, which overpopulate BM in late stages of MDS, with a survival
7 advantage.

8 There is also substantial evidence that the NF- κ B pathway participates in myeloid and lymphoid
9 differentiation from early progenitors and also at different levels of maturation of the
10 granulocytic/monocytic, erythroid and B/T-cell lineages.⁵⁰

11 These roles in differentiation suggest that the deregulation of the transcriptional activity of NF-
12 κ B may lead to or enhance the differentiation and proliferative abnormalities characteristic of
13 MDS. Although it has been postulated that canonical NF- κ B constitutive activation is not
14 sufficient to induce changes in CD34⁺ cell growth and differentiation,⁵¹ there are data showing
15 that mice deficient in the NF- κ B inhibitor I κ B α develop a premalignant dysregulation of
16 hematopoiesis.^{52, 53} Discrepancies among the few existing studies may be caused by the fact
17 that the effects of NF- κ B on hematopoiesis are not cell-autonomous. For instance, its activation
18 in myelopoietic cells alone is not sufficient for the induction of an MDS phenotype, but the
19 deregulation of NF- κ B in the non-hematopoietic compartment causes a myeloproliferative
20 disorder.⁵³ This paradoxical effect of NF- κ B activation should be analyzed in depth in the future.

21 Studies of some target genes of NF- κ B also provide indirect evidence of the potential relevance
22 of this factor in MDS. One example is the proinflammatory cytokine IL-6, which stimulates B and
23 T-cell differentiation and is also a macrophage and granulocyte inducer. IL-6-transgenic mice
24 develop a transplantable myeloproliferative disorder characterized by thrombocytosis, anemia
25 and transient neutropenia with progression to leukocytosis.⁵⁴

26 Overall, NF- κ B activation plays an important role in the pathogenesis of MDS by inducing the
27 expression of inflammatory cytokines and pro-survival factors and probably also by contributing
28 to dysregulated hematopoiesis. Furthermore, the activation of this factor is emerging as a hub in
29 which the multiple innate immune signaling pathways involved in this disease converge;
30 therefore, NF- κ B is a molecule of great interest in the study of MDS.

31 **3.3. The Deregulation of Toll-Like Receptor (TLR) Signaling**

1 The TLR family comprises some of the most important types of cell-associated mammalian
 2 PRRs, which play a major role in innate immunity.^{55, 56} Remarkably, TLRs participate in the
 3 pathogenesis of several non-infectious inflammatory and autoimmune diseases that are
 4 clinically associated with increased risk of MDS, such as chronic polyarthritis.⁵⁷

5 There are ten different TLRs in humans (Table 2), all of which recognize different microbial
 6 antigens and self-components released in response to stress, tissue damage and cell death.
 7 Most TLRs are localized on cell surfaces and are especially abundant in macrophages, dendritic
 8 cells (DCs) and neutrophils, whereas other TLRs are associated with intracellular membranes
 9 from organelles such as the endoplasmic reticulum, endosomes and endolysosomes.^{55, 56}

Table 2. Human TLRs and their ligands

TLR	Localization	Canonical ligand	Origin of the ligand
TLR1	Plasma membrane	Triacyl lipoproteins	Bacteria
TLR2	Plasma membrane	Lipoproteins (dimerizes with TLR1 or TLR6)	Bacteria, viruses, parasites, self
TLR3	Endolysosomes	dsRNA	Viruses
TLR4	Plasma membrane	Lipopolysaccharide (LPS)	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoproteins	Bacteria, viruses
TLR7	Endolysosomes	ssRNA and small purine analogs	Bacteria, viruses, self
TLR8	Endolysosomes	ssRNA	Bacteria, viruses, self
TLR9	Endolysosomes	CpG-DNA	Bacteria, viruses, protozoa, self
TLR11	Plasma membrane	Unknown	Unknown

10
 11 Engagement of TLRs by their specific ligands leads to the activation of transcription factors that
 12 cooperatively regulate the expression of IFNs and pro-inflammatory cytokines and chemokines
 13 (Figure 2). Moreover, in monocytes/macrophages, TLRs upregulate the expression of hundreds
 14 of other genes that might be involved in antimicrobial defense, metabolic changes, tissue repair
 15 and differentiation.^{55, 58} Interestingly, TLR signaling also induces the expression of several
 16 microRNAs (miRNAs) such as miR-146a/b, miR-147, miR-155, miR-181 and miR-21, which
 17 participate in the fine-tuning of the inflammatory response and some of which are likely involved
 18 in the pathogenesis of MDS.⁵⁹

19 Gene expression profiling assays have revealed that a number of TLRs, as well as many of the
 20 adaptor proteins and signal transducers in this pathway, are overexpressed in a high proportion
 21 (40-80%) of MDS patients. Maratheftis et al. were the first to postulate that TLR4 is
 22 overexpressed in BM mononuclear cells (BMMCs) and CD34⁺ cells of MDS patients, and found

1 its levels to be significantly correlated with apoptotic rates.⁶⁰ Another study by Kuninaka et al.
2 reported increased expression of TLR2 and TLR9 in all MDS subtypes, with levels of TLR9 also
3 correlated with those of TNF- α , and the expression of both decreased with disease progression
4 to AML.⁶¹ Notably, TLR4 levels in MDS cells, which did not significantly differ from those of
5 controls in that study, were also correlated with TNF- α expression. Results were further
6 confirmed by our group by gene expression profiling in a cohort of MDS CD34⁺ cells⁶².
7 Importantly, we found that not only was TLR2 highly expressed in a great majority of MDS
8 patients, but also that TLR1 and TLR6, its heterodimerization partners, were significantly
9 overexpressed when compared to healthy controls. Moreover, we showed that TLR2/TLR1 and
10 TLR2/TLR6 dimers were functional and associated with the inflammatory milieu observed in
11 MDS. Additionally, we identified an MDS-related somatic mutation of TLR2, TLR2-F217F, which
12 was present in 11% of patients and is associated with enhanced NF- κ B activation.

13 In line with these findings, multiple TLR downstream signaling mediators have been shown to
14 be also overexpressed in MDS. Velegraki et al. demonstrated increased expression of a wide
15 panel of genes involved in TLR4 signaling in MDS BMMCs.⁶³ A gene expression microarray
16 showed that TRAF6 is overexpressed in MDS CD34⁺ cells when compared to healthy controls.⁶⁴
17 Furthermore, DNA arrays revealed the amplification of the TRAF6 locus (chromosome 11p12)
18 and the TIRAP locus (chromosome 11q24.2) in MDS.^{65, 66} Our group recently reported that
19 MyD88 is also overexpressed in BM progenitors of MDS and is associated with risk stratification
20 and patient survival.⁶⁷ Lastly, Rhyasen et al. demonstrated IRAK1 upregulation in MDS
21 BMMCs.⁶⁸

22 Several functional studies in MDS patient cells have confirmed increased TLR signaling, leading
23 to NF- κ B hyperactivation and to the elevated secretion of cytokines. Maratheftis et al. showed
24 that increased TLR4 signaling contributes to the elevated BM levels of TNF- α in MDS BMMCs
25 and that TNF- α , in turn, induces TLR4 expression in a positive feedback loop.⁶⁰ Velegraki et al.
26 later demonstrated that MDS BM plasma can induce TLR4-dependent cytokine secretion in
27 BMMCs from both healthy and MDS subjects.⁶³ Authors ascribed this effect to HMGB1, which
28 they found to be upregulated in MDS BM plasma, and further showed that this protein is
29 released by apoptotic BMMCs. In turn, we showed that specific ligands of the two TLR2
30 heterodimers induce IRAK1 phosphorylation, NF- κ B activation and IL-8 secretion in BM
31 precursors.⁶² Notably, TLR2 stimulation also induced the expression of the histone demethylase
32 JMJD3,⁶² which we reported to be significantly overexpressed in MDS and to form a positive
33 feedback loop with NF- κ B activation, leading to the expression of IL-8.⁶⁹ Taken together, these

1 results suggest that TLR4 and TLR2 signaling is not only activated in MDS but also has the
2 ability to self-maintain. Additional evidence was recently provided by Rhyasen et al, who
3 showed that IRAK1 is constitutively active in MDS BM cells and that its inhibition significantly
4 downregulates genes involved in the inflammatory response, including TLR6 and IL-8.^{68, 70}
5 Importantly, these results point to the existence of additional feedback loops sustaining TLR2
6 signaling. Another hint of the existence of feedback loops is the elevated expression of IL-1R
7 accessory protein (IL-1RAP), an IL-1-dependent alternative activator of the MyD88/IRAK-
8 1/TRAF6 signaling axis, in CD34⁺ cells of high-risk MDS.²⁰

9 Although the precise role of TLR-mediated signaling in MDS has not yet been elucidated, *in*
10 *vitro* and *in vivo* assays suggest that the deregulation of this pathway might be involved in the
11 loss of progenitor cell function and impaired differentiation in BM cells. By studying gene
12 expression in the 5q deletion (5q-) syndrome, Starczynowski et al. reported the loss of miR-145
13 and miR-146a, which are encoded in 5q and respectively target the downstream TLR
14 transducers TIRAP and TRAF6.⁷¹ Both the functional knock-down of miR-145 and miR-146a
15 and the enforced overexpression of TRAF6 in mouse BM lead to multiple hematopoietic
16 abnormalities that recapitulated features of 5q- syndrome and were associated with NF-κB
17 activation and increased production of IL-6. Moreover, mice transplanted with TRAF6-
18 expressing cells presented relevant hematological phenotypes as well as progress to AML.
19 More recently, our group showed that the blockade of TLR2-mediated signaling in primary
20 CD34⁺ cells with a specific inhibitor of MyD88 increased the number of erythroid colonies and
21 the expression of erythroid marker genes.⁶⁷ We obtained similar effects by inhibiting the IL-8
22 receptor⁶⁷ and knocking down TLR2.⁶² Blockade of IRAK1 activity in MDS by Rhyasen et al, in
23 turn, decreased overall cell growth and colony formation of MDS BM cells.^{68, 70} On the contrary,
24 Velegraki et al. reported a TLR4-dependent decrease in the clonogenic potential of both normal
25 and MDS CD34⁺ cells in the presence of apoptotic BMMCs or recombinant HMBG1.⁶³ Taken
26 together, these results indicate that TLR-dependent signaling deregulates hematopoiesis and
27 HSC growth in MDS, although its specific effects are still not clear. Interestingly, IRAK1
28 inhibition efficiently suppressed MDS xenografts in immunodeficient mice, significantly
29 improving survival rates of the recipients.⁶⁸

30 These findings suggest that TLR signaling exerts direct effects on HSC function and
31 hematopoietic differentiation. For instance, the main function of TLR-mediated signaling in BM
32 hematopoietic precursors is the replenishment of the cellular components of the innate immune
33 system.⁷² Upon stimulation, both *in vivo* and *in vitro*, these receptors initiate a transcriptional

1 response that mediates MyD88-dependent and growth factor-independent differentiation of
2 common myeloid and lymphoid progenitors into monocytes/macrophages and DCs, at the
3 expense of lymphopoiesis.^{58, 73-75} This process requires quiescent HSCs to reenter the cell cycle
4 and is accompanied by the secretion of inflammatory cytokines/chemokines frequently
5 overexpressed in MDS.^{58, 73} Interestingly, activation of different TLRs may favor the
6 differentiation of CD34⁺ cells into different mature myeloid cells. TLR1/2 stimulation seems to be
7 more effective in inducing monocytic differentiation, whereas TLR7/8 activation is more effective
8 at inducing the DC subset.⁷³ Translated into TLR-related pathological conditions, the continuous
9 signaling could cause abnormalities in myeloid/lymphoid differentiation and eventually affect the
10 outcomes of hematopoiesis. In line with this hypothesis, a study has shown that chronic TLR
11 stimulation induces durable changes in mouse BM physiology that are very similar to the MDS
12 phenotype, including increased cycling rates and limited self-renewal of HSCs and loss of
13 lymphopoietic potential. Remarkably, the authors found a correlation between these changes in
14 chronic TLR stimulation and aging.⁷⁶

15 Although many TLR-induced cytokines are transcriptionally activated by NF- κ B, the effects of
16 TLRs on differentiation do not necessarily have to be ascribed to this factor. A global expression
17 profiling and hierarchical clustering analysis carried out in the CD34⁺ cells of 183 patients⁴²
18 showed that the most significantly deregulated pathway for upregulated genes in MDS was the
19 IFN signaling pathway (Figure 1). IFN- γ , which is secreted in response to the activation of
20 several TLRs and also secreted in high levels in MDS patients, appears to have a strong
21 inhibitory effect on hematopoietic progenitors and stem cells that includes impairing
22 erythropoiesis⁷⁷ and reducing the long-term repopulation potential of HSCs.⁷⁸ Thus, the effects
23 of TLR signaling on hematopoiesis could be mediated by different effectors other than NF- κ B.

24 Furthermore, the stimulation of TLR4 and TLR2 *in vitro* induces apoptosis in primary BM cells.^{60,}
25 ^{79, 80} In the case of TLR2, this apoptosis is NF- κ B-independent,^{79, 80} which is consistent with the
26 pro-survival role of NF- κ B. Notably, a p38 MAPK inhibitor significantly decreased TLR2-
27 dependent cell death, suggesting that p38 is involved in the induction of NF- κ B-independent
28 apoptosis by TLRs.⁷⁹

29 Recent evidence has also shown that the role of TLR signaling in MDS is not limited to its
30 effects on HSCs and early progenitors. DIAPH1 is encoded in the 5q region and downregulated
31 in patients with 5q- syndrome.⁸¹ The gene product of DIAPH1, mDia1, participates in actin
32 polymerization. Keerthivasan et al. recently reported that young mDia1 heterozygous or knock-

1 out mice have granulocytopenias originating from defects in differentiated granulocytes and that,
2 upon aging, they acquire prominent myeloid dysplasia with neutropenia, which is characteristic
3 of MDS.⁸² This mDia1 deficiency induced upregulation of the TLR4 adaptor protein CD14, which
4 was dramatically overexpressed in committed granulocytic progenitors and, especially, in
5 mature granulocytes. Chronic TLR4 stimulation in CD14-overexpressing mice mimicked the
6 hematologic phenotype of MDS, which suggests that the deletion of DIAPH1 in 5q- syndrome
7 could contribute to the pathogenesis of MDS by inducing the overexpression of CD14. In
8 agreement, the overexpression of CD14 was confirmed in granulocytes of 5q- patients.

9 10 **4. Inflammation and Innate Immunity in Other Cell Types**

11 **4.1. Involvement of the BM Microenvironment in MDS**

12 The BM niche is comprised of different types of stromal cells, including adipocytes, fibroblasts
13 and osteoblasts, and of mesenchymal stem cells (MSCs), which are primitive, non-
14 hematopoietic stem cells that give rise to all the above lineages.⁸³ MSCs can carry out
15 immunosuppressive functions through the impairment of DC maturation.⁸⁴ Because BM HSCs
16 and MSCs may have a common multipotent progenitor, malignant HSCs could coexist with a
17 malignant MSC clone with altered immunosuppressive properties in MDS. Many efforts have
18 been made to detect abnormal MDS MSC clones, but various groups have failed to show
19 significant morphological and functional differences between patients and healthy individuals.^{85,}

20 ⁸⁶ Although BM MSCs from many patients have chromosomal alterations, these seem to have
21 no correlation with the karyotypic/cytogenetic abnormalities of their HSC counterparts,^{85, 86}
22 which indicates that MDS-derived MSCs and their progeny have a different clonal origin.
23 Nevertheless, it was recently demonstrated that the immunosuppressive capacity of MSCs is
24 decreased in MDS and that these cells fail to efficiently inhibit DC maturation. Remarkably, this
25 effect was only observed in cells from low-risk MDS, whereas immunosuppressive functions of
26 high-risk MDS-derived MSCs were similar to those of controls.⁸⁷ These results indicate that,
27 despite not belonging to the malignant clone, the functionality of MSCs is altered in MDS and
28 may favor the expansion of cytotoxic T-cells in the early stages of the disease.

29 The notion of the active participation of MSCs in the pathogenesis of MDS has been reinforced
30 by a recent publication showing that MDS HSCs can “reprogram” MSCs by inducing changes in
31 their gene expression profiles. “Reprogrammed” MDS-derived MSCs showed increased ability
32 to allow the *in vivo* engraftment of MDS CD34⁺ cells, which exhibited long-term renewal and

1 myeloid skewing of differentiation.⁸⁸ Interestingly, “response to inflammation” and “cytokine-
2 cytokine receptor interaction” were two of the functional groups of genes upregulated in MSCs,
3 which suggests that MDS HSCs induce adaptation of their neighboring cells to the inflammatory
4 microenvironment.

5 Another line of evidence of the abnormal behavior of the BM niche in MDS is the fact that Fas-L
6 is more prominently expressed in stromal cells and macrophages than in hematopoietic cells,
7 which in turn widely express Fas and TNFR.^{9, 89} This distribution suggests that non-
8 hematopoietic cells in the BM niche could be responsible for the induction of apoptosis in
9 hematopoietic precursors. In agreement with that hypothesis, Stirewalt et al. demonstrated that
10 the apoptotic effects of TNF- α on hematopoietic cells depend on their direct contact with stromal
11 cells, in which TNF- α induces significant changes in gene expression, particularly in apoptosis-
12 related genes and cytokines/chemokines such as IL-6 and IL-8.⁹⁰

13 Taken together, these results suggest that MDS-derived MSCs and BM stromal cells are
14 determinants of the fate of hematopoietic progenitors and play an important role in the
15 pathogenesis of MDS.

16 **4.2. The Role of Cellular Immunity**

17 Myeloid-derived suppressor cells (MDSCs) are inflammatory and immunosuppressive effectors
18 localized to the BM that express the immune-receptor CD33.⁹¹ Chen et al. found that MDS
19 patients have increased MDSCs count and that they induce defects in myeloid and erythroid
20 differentiation. Furthermore, MDSCs appear to reduce T-cell proliferation and functionality in
21 MDS patients. These effects are mediated by CD33, for which the inflammatory signaling
22 molecule S100A9 is a specific ligand.⁹² S100 molecules, including S100A8 and S100A9, are
23 also the ligands of other innate immune receptors, such as TLR4,⁹³ and are known to be
24 overexpressed in MDS BM CD34⁺ cells.⁶⁹ Moreover, S100A9 also appears to be upregulated in
25 the hematopoietic cell compartment of telomere-dysfunctional mice, an animal model of
26 premature aging with perturbed BM hematopoiesis.⁹⁴ Chen et al. found that the levels of
27 S100A9 are also elevated in MDS BMDCs, supporting the increased counts of MDSCs and the
28 secretion of immunosuppressive cytokines. Furthermore, S100A9-transgenic mice developed
29 an MDS-like phenotype with multilineage cytopenias and cytological dysplasia.⁹² Forced
30 maturation of MDSCs restored hematopoiesis, suggesting that these cells are deeply involved in
31 the pathogenesis of MDS. Of interest, and potentially linked to the switch to immunosuppression

1 during MDS progression, the development of MDSCs relies on inflammatory cytokines, and GM-
2 CSF and IL-6 generate the most suppressive MDSCs.⁹⁵

3 Macrophages are also potentially involved in MDS. It was recently shown that there is a
4 recurrent and specific loss of granulocyte/monocyte progenitor (GMP) populations in the BM of
5 low-risk MDS, which is likely due to the increased phagocytosis of GMPs by macrophages. This
6 deregulated phagocytosis is proposed to be regulated by the interaction between cell surface
7 calreticulin on target cells and the low-density lipoprotein receptor-related protein (LRP1)
8 receptor on macrophages.⁹⁶ Macrophages also mediate angiogenesis, which is elevated in
9 high-risk MDS.^{12, 13}

10 Innate immunity is not the only dysregulated immune mechanism in MDS. Although MDS
11 patients generally present with lymphopenias, cellular immunity may be upregulated in low-risk
12 MDS. These patients have higher counts of cytotoxic (CD8⁺) and helper (Th17) T-cells and NK
13 cells and lower counts of T-regulatory lymphocytes (Treg).^{19, 97-99} The expansion of CD8⁺ cells is
14 particularly detrimental in patients with chromosome 8 trisomy because CD8⁺ cells specifically
15 target WT1, which is overexpressed in the CD34⁺ progenitors of these patients. Probably for this
16 reason, patients with trisomy 8 are more responsive to immunosuppressive therapy.¹⁰⁰ Overall,
17 these changes in cell number and functionality cooperate with the release of inflammatory
18 cytokines and trigger an autoimmune response against hematopoietic cells that may contribute
19 to intramedullary apoptosis.^{99, 101-103} In agreement with this hypothesis, the depletion of the CD8⁺
20 cells allows colony formation in primary BMMCs from MDS patients; however, it remains unclear
21 if the proliferating cells belong to the normal or the malignant clone.¹⁰³

22 On the other hand, in high-risk cases, impaired cellular responses with lower levels of CD8⁺,
23 Th17 and NK cell function and increased numbers of Tregs are more common.^{98, 99, 104, 105} The
24 number of these cells is also associated with higher levels of IL-10⁹⁹ and a poorer prognosis.¹⁰⁶
25 Similarly, the decreased cytolytic function of NK cells correlates with MDS progression.¹⁰⁵
26 Overall, this dysregulation leads to the acquisition of immune tolerance by the proliferating clone
27 and enhances the risk of progression.

28 The events triggering the clonal expansion of CD8⁺ cells in low-risk MDS, as well as the switch
29 in the CD8⁺:Treg ratio during progression, are poorly understood. The expansion of CD8⁺ cells
30 could be induced to fight the malignant clone or contribute to the annihilation of normal
31 hematopoietic progenitors. Despite numerous efforts to identify putative antigenic sequences in
32 MDS T-cell receptors, the causal antigens eliciting the CD8⁺ cell response, other than WT1,

1 have not yet been characterized.^{97, 107, 108} Similarly, little is known about the decline in the
2 proportion of CD8⁺ cells in later stages of MDS. However, data from our group suggest that this
3 phenomenon could be related to the expression of the negative co-stimulatory T-cell receptor
4 programmed death-1 (PD-1) and its ligand, PDL-1. These molecules are upregulated in a
5 subset of MDS patients, with PD-1 being highly expressed in PB mononuclear cells and PDL-1
6 being preferentially overexpressed in BM CD34⁺ cells.¹⁰⁹ These results suggest that MDS BM
7 cells may actively participate in the repression of the CD8⁺ T-cell response. Indeed, we found
8 that higher levels of PD-1/PDL-1 in BM cells are associated with resistance to therapy and with
9 a poorer prognosis. More research in this field is necessary to shed light on the role of T-cell
10 immunity in the pathogenesis of this disease. Other frequent alterations that are common to all
11 stages of MDS are the deficiency of B cells¹¹⁰ and $\gamma\delta$ T cells,¹¹¹ important regulators of T-cell
12 responses.

13

14 **5. Progress of Anti-Innate Immune Therapies**

15 There is wide clinical experience on the treatment of MDS with immunosuppressive therapy
16 (IST), which was used before molecular evidence of innate immune involvement in MDS arose.
17 Some of the first approaches used cyclosporine, but the risk of renal failure made other
18 treatments, such as antithymocyte globulin (ATG) or lenalidomide, the ISTs of choice.¹¹²
19 Another immunosuppressive agent is alemtuzumab, an antibody directed to the abundant
20 lymphocyte antigen CD52¹¹³. About 30% of patients treated with IST become transfusion-
21 independent and improve cell counts, although these do not revert to normal.¹¹²

22 Encouragingly, recent findings regarding innate immune and inflammatory signals in MDS have
23 provided a strong biological rationale for the development of novel therapeutic strategies.
24 Preclinical studies have demonstrated that this interfering strategy may lead to promising
25 therapeutic effects. As explained above, the specific inhibition of the activity or expression of
26 TLR2, JMJD3, MyD88, IL-8 and IRAK1 in primary BM cells of MDS significantly improved
27 differentiation, induced apoptosis and impaired their clonal generation potential, particularly in
28 cells from patients with lower-risk disease. Interestingly, the effects of an IRAK1 inhibitor can be
29 further improved when combining it with a Bcl-2 specific inhibitor.^{62, 67-69} Furthermore,
30 interference with TRAF6 sensitized MDS/AML cells to bortezomib-induced cytotoxicity.¹¹⁴
31 Similarly, inhibition of the p38 MAPK pathway by the specific inhibitor SCIO-469 stimulates
32 hematopoietic activity *in vitro* while simultaneously decreasing the expression of TNF- α or IL-1 β -

1 induced proinflammatory chemokines in BM stromal cells.^{44, 115} Finally, SB-332235, a specific
 2 inhibitor of the IL-8 receptor, also significantly reduces growth and colony formation in primary
 3 MDS BM CD34⁺ cells.¹¹⁶

4 Consistent with these preclinical findings, clinical trials of novel innate immune-targeted
 5 interventions are starting to emerge. ARRY614 is a potent dual inhibitor of p38 MAPK/Tie2, key
 6 downstream effectors of innate immune signaling. In an open-label phase I study in patients
 7 with low-risk MDS, nearly 30% of patients achieved hematological improvement with this drug,
 8 almost all of which had previously failed treatment with azanucleosides. Based on these results,
 9 a new formulation of ARRY614 has entered a phase I/II study in patients with low-risk MDS.¹¹⁷
 10 Another drug that targets immune signaling and has entered clinical studies is OPN-305, a
 11 TLR2-directed antibody with promising utility in MDS. OPN-305 has completed phase I clinical
 12 studies¹¹⁸ and is about to start phase II studies in MDS patients. Lastly, an oral small molecule
 13 inhibitor of TGF-beta receptor I kinase, LY-2157299, is being tested in a phase II trial in low-
 14 and intermediate-risk MDS.¹¹⁹ Ongoing preclinical and clinical trials of targeted innate immune
 15 interventions are summarized in Table 3.

16

Table 3. Summary of recent anti-innate immune pre-clinical and clinical trials in MDS			
Targeted molecule	Reagent	Potential therapeutic effect	References
TLR2/JMJD3	shRNA (preclinical)	Improvement of erythroid differentiation of MDS BM CD34 ⁺ cells	64, 70
MyD88	Inhibitory peptide (preclinical)	Improvement of erythroid differentiation of MDS BM CD34 ⁺ cells	67
IL-8	Neutralizing antibody (preclinical)	Improvement of erythroid differentiation of MDS BM CD34 ⁺ cells	67
IL-8 receptor	SB-332235 (preclinical)	Reduction of growth and colony formation in MDS BM CD34 ⁺ cells	116
IRAK 1	RNAi and specific inhibitor molecule (preclinical)	Induction of apoptosis and impairment of clonal generation in in MDS BM cells	67, 68
p38 MAPK	SCIO-469 (preclinical)	Enhancement of hematopoiesis and reduction of apoptosis in MDS BM CD34 ⁺ cells, anti-inflammatory effects in BM stromal cells	44, 115
p38 MAPK	ARRY614 (clinical)	Hematological improvement in patients who previously failed azanucleoside treatment	117
TLR2	OPN-305 (clinical)	Improvement of erythroid differentiation of MDS BM CD34 ⁺ cells	118
TGF-β Receptor I Kinase	LY-2157299	Improvement of MDS BM progenitor colony formation <i>in vitro</i> and <i>in vivo</i> , stimulation of hematopoiesis	119

17

1 **6. Concluding Remarks**

2 With the recent development of new technologies and the consequent experimental
3 optimization, understanding of the role of innate immune deregulation in the MDS pathogenesis
4 has been greatly improved. It is now commonly recognized that constitutively activated innate
5 immune and inflammatory pathways can directly affect hematopoiesis, lead to altered cytokine
6 secretion and impact T-cell immunity. All these biological effects contribute to the development
7 and progression of MDS (Figure 3). Furthermore, innate immune deregulation seems to be
8 chronic rather than transient and affects all stages of the disease. This deregulation could arise
9 from cellular stresses associated with senescent changes, genomic instability, and other genetic
10 and epigenetic abnormalities that occur in hematopoietic cells with aging, but it could also be
11 initiated by abnormal cellular interactions in the BM microenvironment. To better evaluate the
12 biological and clinical implications of innate immune signaling in MDS, deep investigations of
13 innate immune alterations are necessary, especially in purified specific BM hematopoietic
14 populations. Special attention should be drawn to the key aspects that remain unknown, such
15 as the signaling pathways activated by innate immunity that determine disease evolution and/or
16 define distinct MDS subtypes. Significant efforts are also needed to identify the endogenous
17 ligands responsible for TLR activation and the conditions that contribute to their release or make
18 MDS patients more vulnerable to the deleterious effects of TLR signaling. This information could
19 eventually be applied to develop effective therapeutic regimens. Because about 50% of deaths
20 in MDS patients are related to cytopenias rather than to progression to AML, the development of
21 immunomodulatory therapies potentially improving hematopoiesis is of great interest for the
22 management of MDS.

23

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4 **Conflict of Interest**

5 Authors declare no conflict of interest.

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21

22 **Figure 1. Signaling pathways frequently deregulated in MDS.**

23 The transmembrane receptors Fas (CD95), TNFR1, TNFR2, Toll-like receptors (TLRs) and IFN-
24 γ receptor (IFNGR) and their associated signal transducers are frequently overexpressed and/or
25 constitutively activated in MDS. Fas/CD95 is specifically engaged by Fas-L/CD95L, which
26 induces caspase-dependent cell death by activating the initiator caspase-8 via its FAS-
27 associated death domain (FADD). TNFR1 and TNFR2 activate the adaptor protein TNFR-
28 associated death domain (TRADD), which in turn activates TNFR-associated factors (TRAFs) to
29 ultimately induce the phosphorylation of the MAPKs JUN N-terminal kinase (JNK) and/or p38
30 MAPK, the latter via the receptor-interacting protein (RIP). JNK induces the transcriptional
31 activity of AP-1 and p38 MAPK, in turn, activates other transcription factors (TF) that carry out
32 various functions. TNFR1/2 can also activate the transcription factor NF- κ B via I κ B kinase
33 (IKK). In addition, TNFR1 can directly induce apoptosis through the death receptor pathway by
34 activating FADD via TRADD, initiating caspase cleavage. TNFR2 lacks a death domain, so its
35 functions are predominately pro-survival. After recognition of pathogen- or damage-associated
36 molecular patterns (PAMPs and DAMPs, respectively), TLRs signal through several specific
37 adaptor molecules that ultimately lead to the activation of AP-1, p38 MAPK and NF- κ B. Lastly,
38 IFN- γ initiates a transcriptional response mediated by the activation of the Janus kinase
39 (JAK)/signal transducer and activator of transcription (STAT) pathway. The transcriptional
40 programs activated by these receptors generally lead to the expression of genes involved in the

1 innate immune response, survival and differentiation but may also lead to the transcription of
2 pro-apoptotic genes.

3
4 **Figure 2. TLR signaling and its activation in MDS.**

5 TLRs transduce their signals through two different adaptor molecules, myeloid differentiation
6 primary response gene 88 (MyD88) and TIR domain-containing adaptor inducing IFN- β (TRIF).
7 Virtually all TLRs signal via MyD88, except for TLR3 (not depicted here), which is an
8 intracellular receptor signaling via TRIF. In addition, TLR4 is the only TLR that can use both
9 mediators. MyD88-driven signaling mediates a rapid and acute pro-inflammatory response
10 through the activation of NF- κ B, AP-1 and p38 MAPK-dependent transcription factors. The
11 intracellular receptors TLR7/8 and TLR9 additionally activate interferon-regulatory factor (IRF)-
12 7, which induces the expression of type I IFN. In contrast, TRIF triggers a delayed pro-
13 inflammatory response mediated by NF- κ B and IRF-3-dependent type I IFN expression. Herein,
14 TLR2 and TLR4 are depicted as examples of cytoplasmic membrane-bound TLRs, and TLR9 is
15 shown as an example of intracellular TLRs. Receptors and mediators colored in different
16 shades of red represent molecules found to be overexpressed or constitutively activated in
17 MDS.

18 Abbreviations not defined in the text: toll-interleukin 1 receptor domain-containing adaptor
19 protein (TIRAP), TRIF-related adaptor molecule (TRAM), TGF- β activated kinase (TAB), TANK-
20 binding kinase 1 (TBK1), NF- κ B-inducing kinase (NIK), ubiquitin (Ub).

21
22 **Figure 3. Proposed model for the central role of inflammation/innate immunity in the**
23 **pathogenesis of MDS.**

24 (1) The malignant clone or MDS HSC originates in the BM of patients with the characteristic
25 phenotype of aging. The main characteristics of the BM in old individuals are summarized in the
26 box. In this context, the MDS HSC might originate from genetic/epigenetic changes occurring in
27 susceptible individuals during aging; be generated by exposure to various types of stress,
28 including DNA damage; or could develop after a sustained exposure to inflammatory molecules
29 derived from an existing or past inflammatory condition. (2) Either the changes in gene
30 expression or the pre-exposure to inflammatory molecules trigger the activation of innate
31 immune signaling pathways and the subsequent secretion of cytokines, chemokines and growth
32 factors, which create an inflammatory microenvironment. (3) As a consequence, and maybe

1 also owing to the possible genetic/epigenetic abnormalities acquired, BM HSCs enter the cell
2 cycle and increase their cycling rates. Also driven by the release of cytokines, HSCs express
3 Fas and other immune receptors on their surface, and CD8⁺, Th17 and NK cells are recruited.
4 (4) The expression of death receptors and the continuous inflammatory signaling induce
5 apoptosis in some HSCs in addition to the T-cell mediated cytotoxicity. However, it is not clear if
6 the dying HSCs belong to the normal or MDS clone, or to both. (5) Regardless of HSC origin,
7 intramedullary apoptosis decreases the number of functional BM progenitors, which results in a
8 reduced number of fully differentiated cells. In addition, intrinsic defects on the differentiation
9 potential of the MDS clone, and the sustained inflammatory signaling, cause differentiation to be
10 dysregulated and skewed toward the myeloid lineage. (6) The released cytokines and
11 chemokines, and probably also certain cell-to-cell contact proteins, eventually trigger the
12 recruitment of MDSCs to the tumor site and induce profound gene expression changes in the
13 surrounding MSCs. MDSCs exacerbate the defects of differentiation by inducing myeloid
14 skewing and killing erythroid precursors, and they suppress the autoimmune response by CD8⁺
15 T-cells as well as probably participating in the switch to an immunotolerant microenvironment.
16 Likewise, “reprogrammed” MSCs express genes involved in the adaptation to inflammation. (7)
17 The high proliferation rates make MDS HSCs more prone to the accumulation of additional
18 genetic/epigenetic aberrations. In addition, unknown mechanisms lead to a switch in the
19 expression of TNFRs and probably also in the expression of other molecules, which makes
20 malignant cells resistant to apoptosis. (8) Altogether, these alterations confer the MDS clone a
21 survival advantage and contribute to the aberrant proliferation of the clone, which at this point
22 overpopulates the BM. (9) This switch in the cellular processes that prevail in the BM is
23 accompanied by the recruitment of immunomodulatory cells, which are probably triggered by
24 changes in the cytokine/chemokine milieu. Treg cells confer immune resistance to the MDS
25 clone and allow abnormally proliferating cells to escape the immune system. Along with step 8,
26 this event increases the risk of progression to AML.

27

Abstract #75644

Association Between Down-Regulation of EZH2 and Abnormal Karyotype, Response to Hypomethylation Treatment, and Patient Survival in Myelodysplastic Syndromes

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EZH2 encodes a key histone methylation regulatory molecule. Genetic mutations of EZH2 occur in ~10% patients with MDS and are associated with poor prognosis. However, the expression patterns of EZH2 are less well studied in MDS. To characterize these expression patterns, we assessed EZH2 mRNA expression in primary patient bone marrow CD34+ cells (n=78) (age: 33-91; IPSS: low 24%, Int-1 31%, Int-2 27%, High 14%; Karyotype: diploid 62%, 5q-/7q- 23%, others 15%). QRT-PCR assays indicated that 47% (n=37) of patients had reduced EZH2 mRNA expression (less than 50% of controls), but this finding was not statistically significant (p= 0.159). Subtype analyses based on various karyotypes revealed that EZH2 is significantly underexpressed in patients bearing chromosome 7 or 7q deletions (mean=0.4 fold, p=0.006). Seventy-five percent of patients with 7 or 7q deletions have EZH2 expression less than 50% of that of controls. Chromosome 7 deletions were also associated with lower EZH2 expression than that seen in diploidy and other cytogenetic abnormalities (p=0.041). We previously found that the overexpression of a group of innate immune genes contributes to MDS pathogenesis and is related to deregulation of histone methylation. Because EZH2 is a key regulator of histone methylation, we assessed the relationship between deregulation of these genes and EZH2 under-expression. To exclude the effects of cytogenetic defects and EZH2 mutations, we studied only the subset of patients with normal karyotypes and wildtype EZH2. We surveyed capture deep sequencing results of 32 of the diploid patients from the cohort that had sequencing data available. Three patients carry EZH2 mutations, including missense (nt148511133), nonsense (nt148524257), and splicing mutations (nt148524257). In the remaining 29 patients with normal karyotypes and wildtype EZH2, 14 (48%) had EZH2 under-expression. We then compared mRNA expression of 11 innate immune genes known to overexpress in MDS between the patients with EZH2 underexpression and others. We observed that mRNA levels of all 11 immune genes tested were higher in the EZH2 underexpression group and statistically significant (p<0.05) for the genes JMJD3, IL-8, IL-1B, TLR-2, and S100-A9.

We then performed survival analysis for EZH2 expression in MDS. Surprisingly, multivariate analysis in the whole cohort indicated that EZH2 underexpression is associated with better overall survival (OS) (HR 0.23, 95% CI (0.07-0.72); p=0.013). We also performed analysis in the subset without chromosome 7 deletion and observed a similar association (HR 0.18 (0.06-0.55) p=0.012). To investigate whether this result was related to responses to therapy, we

reviewed treatment records and found that 61% of patients in the cohort (n=53) received hypomethylating agents (HMA). In this HMA treatment subset, non-responders (n=27) tended to have lower EZH2 expression than responders (n=26) (mean EZH2 of 0.497 vs 0.944, p=0.12). However, we noticed that in the subset of HMA responders, EZH2 expression was significantly lower (p=0.02) in patients who achieved longer responses (more than 12 months, n=15) than in those who progressed or relapsed within 12 months following treatment. We are currently investigating whether this impact of EZH2 underexpression on HMA responses contributes to its effect on OS. Taken together, the results of this study indicate that underexpression of EZH2 in the bone marrow hematopoietic progenitor cell compartment may have unique effects on the molecular pathogenesis, prognosis, and treatment of MDS and may do so through a unique mechanism that differs from that of previously characterized EZH2 mutations. Further investigations are also required to determine the relationships between EZH2, HMA-based treatments, and patient survival.

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Association Between Down-Regulation of EZH2 and Abnormal Karyotype, Response to Hypomethylation Treatment, and Patient Survival in Myelodysplastic Syndromes

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Abstract #75898

Association Between Downregulation of POT1 Expression and Chromosome 7 Deletion, Response to Hypomethylation Agent Treatment, and Patient Survival in Myelodysplastic Syndromes

Yue Wei¹, Amit Verma, MD², Monica Cabrero, MD^{3*}, Yu Jia^{1*}, Hong Zheng^{1*}, Zhihong Fang^{1*}, Yiting Yu, PhD^{4*}, Simona Colla^{1*}, Zach Bohannan^{1*}, Teresa V. Bowman, PhD⁴, Jacqueline Boulton, PhD⁵ and Guillermo Garcia-Manero, MD¹

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The POT1 gene is located in chromosome 7 and encodes a key component of the shelterin complex, which is essential for the maintenance of telomere and chromosome integrity. Somatic mutations of POT1 have been identified in chronic lymphocytic leukemia, which indicates that POT1 dysfunction is involved in the pathogenesis of hematological neoplasms. At the same time, abnormal telomere shortening has been observed in MDS/AML and a spectrum of bone marrow failure syndromes. We therefore sought to study the potential role of POT1 in MDS by sequencing the gene and characterizing its expression in primary bone marrow specimens of patients with MDS.

We first sequenced all POT1 coding regions that are known to have mutations in CLL. PCR-Sanger sequencing was performed in bone marrow mononuclear cells (BM-MNMC) of a cohort of 30 patients with MDS (15 with RAEB/RAEBT, 11 with RA/RARS/RCMD/MDS-U, 2 with CMML, and 2 with 5q- syndromes). No genetic mutations in the POT1 gene were detected. This result suggests that genetic alteration of POT1 is rare in MDS.

We then evaluated the expression of POT1 using cDNA arrays (n=183) or RT-PCR (n=58) in a cohort of 241 patients with MDS from two centers. The median age of our patients was 71 years (32-95). Diagnoses included RAEB in 108 (45%), 5q- syndrome in 18 (8%), and other syndromes (RA, RCMD, and MDS-U) in 115 (47%) cases. In this cohort, 140 (58%) patients were diploid, 22 (9%) had chromosome 7 alterations, 21 (9%) had 5q deletion, and 58 (24%) had other cytogenetic abnormalities.

Results indicate that POT1 was underexpressed (less than 50% of the POT1 level in normal controls) in the bone marrow CD34+ hematopoietic progenitor cell population in 138 patients (57%). However, no significant difference was observed between the whole MDS cohort and control BM CD34+ cells from healthy donors (n=25). Further subset analysis based on karyotypes revealed that 81% of patients with chromosome 7 alterations (7- and 7q-) had lower expression of POT1 versus 38% of diploid patients, 35% of 5q patients, and 42% of

patients with other cytogenetic alterations ($p=0.001$). ANOVA testing indicated that expression of POT1 was significantly downregulated (less than 50% of control) only in patients with chromosome 7 alteration ($p<0.000$) but not in other cytogenetic subsets.

When we compared the survival of patients with POT1 downregulation to other groups, we observed a strong tendency toward shorter overall survival in patients with POT1 downregulation (median OS of 37 months [95% CI: 21-52] vs 53 months [95%CI: 30-75]; $p=0.139$). This tendency toward poorer OS was also observed when we excluded cases with chromosome 7 alterations (37 months [95% CI: 17-57] vs 53 months [95%CI: 25-80]; $p=0.186$).

Next, we evaluated the potential impact of POT1 expression on responses to therapies. In the subgroup of patients with available treatment records for analysis ($n=58$), a total of 42 patients received hypomethylating agents (HMA), and 47% of them achieved responses. When comparing POT1 expression levels to HMA response, we observed significantly lower POT1 expression in HMA non-responders than in responders (U Mann-Whitney test $p=0.028$). In a regression model for response to HMA, we also observed that downregulation of POT1 was associated with a poorer response to HMA (OR 4.96 [1.01-24.37]; $p=0.049$). However, when we introduced chromosome 7 alterations into the model, POT1 expression lost its effect, which suggests that the impact of POT1 on response to HMA is due to its interaction with chromosome 7 alterations.

Taken together, the results of this study indicate that the downregulation of POT1 gene expression, which is related to chromosome 7 deletions, may play a role in the pathogenesis and prognosis of MDS, including response to HMA-based therapies.

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