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Deconstructing innate immune signaling in Myelodysplastic Syndromes

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Abstract

Overexpression of immune-related genes is widely reported in Myelodysplastic syndromes (MDS), and chronic immune stimulation increases the risk for developing MDS. Aberrant innate immune activation, such as due to increased Toll-like receptor (TLR) signaling, in MDS can contribute to systemic effects on hematopoiesis in addition to cell-intrinsic defects on hematopoietic stem/progenitor cell (HSPC) function. This review will deconstruct aberrant function of TLR signaling mediators within MDS HSPC that may contribute to cell intrinsic consequences on hematopoiesis and disease pathogenesis. We will discuss the contribution of chronic TLR signaling to the pathogenesis of MDS based on evidence from patients and mouse genetic models.

Introduction

Myelodysplastic syndromes (MDS) are heterogeneous clonal hematopoietic stem cell (HSC) disorders¹⁻⁶. MDS patients present with blood cytopenia, myeloid cell dysplasia, and ineffective hematopoiesis due to de novo mutations or prior chemotherapy^{7,8}. As patients progress towards bone marrow failure (BMF), ensuing hematologic complications are fatal if untreated. Moreover, approximately one third of MDS patients also develop AML due to acquisition of additional mutations in the defective hematopoietic stem/progenitor cells (HSPC)^{9,10}. MDS is comprised of distinct subtypes based on biological, genetic, and morphological features¹¹. Regardless of subtype, MDS arises from a HSC that has acquired genetic and/or epigenetic abnormalities³⁻⁵. MDS are genetically defined by somatic mutations and chromosomal abnormalities affecting epigenetic plasticity, ribosome function,

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spliceosome function, and immune signaling. Overexpression of immune-related genes is widely reported in MDS^{12,13}, and chronic inflammation and autoimmune disorders increase the risk of developing MDS¹⁴. Multiple independent observations suggest that hyperactivation of innate immune/Toll-like receptor (TLR) signaling is a feature of MDS: association with chronic inflammatory and autoimmune disorders, increased expression of inflammatory cytokines and chemokines, abnormal cellular immunity, and overexpression of TLR mediators, along with reduced expression of negative TLR regulators. The importance of innate immune signaling in primary MDS also is supported by the use of IRAK1/4 inhibitors in preclinical models as effective agents to suppress innate immune activation and the MDS clone¹⁵.

Aberrant innate immune activation in MDS can contribute to non-cell autonomous effects on hematopoiesis (Figure 1) in addition to cell-intrinsic defects within hematopoietic stem/progenitor cells (HSPC) (Figure 2). Several recent comprehensive reviews have described the systemic and non-cell autonomous consequences of chronic innate immune activation in MDS, including the effects of increased inflammation and abnormal adaptive and innate immunity on BM HSC^{16,17}. Herein, we will systematically deconstruct aberrant function of TLR signaling mediators within MDS HSC that may contribute to the cell intrinsic consequences associated with ineffective hematopoiesis and disease pathogenesis (Figure 2).

Mediators of Innate Immune Signaling in MDS

Toll-Like Receptors

The innate immune system recognizes foreign pathogens by cell surface pattern recognition receptors (PRRs). These receptors recognize foreign pathogen components, termed pathogen-associated molecular patterns (PAMPs), as well as host cellular by-products, referred to as damage-associated molecular patterns (DAMPs). Among the first PRRs to be identified were TLRs. Together with the Interleukin-1 receptor (IL1R), TLRs form the TLR/IL1R (TIR) superfamily, which share a common a TIR domain. TLRs consist of a single-pass transmembrane protein with a leucine-rich ectodomain. There are 10 human and 12 murine TLRs, which can be divided into two main groups based on subcellular location: extracellular (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) and intracellular, or endosomal (TLR3, TLR7, TLR8, and TLR9). The location of the receptors, in turn, relates to their specific ligands. The intracellular receptors bind to features of pathogen nucleic acids, such as dsRNA, and unmethylated CpG DNA; whereas the extracellular receptors bind to pathogen membrane components, the best characterized being TLR4 binding to lipopolysaccharide (LPS) of gram-negative bacteria.

Binding of a TLR to its ligand results in recruitment of a TIR domain-containing adaptor protein. There are two main TLR adaptor proteins, Myeloid differentiation primary response (MyD88) and TIR-domain-containing adapter-inducing interferon- β (TRIF), which induce the activation of separate innate immune signaling pathways. All of the TLRs, with the exception of TLR3, use MyD88 (Figure 1). Through a series of mediators, signaling through this adaptor results in activation of MAPK and NF- κ B pathways, leading to transcription of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF α), interleukin-1 (IL-1), IL-8, and IL-6. Instead of MyD88, TLR3 recruits TRIF. Signaling through TRIF

results in activation of IRF3 and MyD88-independent activation of NF- κ B, leading to transcription of similar pro-inflammatory cytokines as the MyD88 pathway with the addition of type 1 interferons. TLR4 is the only receptor that utilizes both MyD88 and TRIF¹⁸⁻²⁰.

Overactivation of TLRs have been associated with autoinflammatory disorders. More recently, TLR signaling has been implicated in HSC development^{21,22}, suggesting that changes in TLR expression and function could lead to dysregulated hematopoiesis. Several mouse studies have shown that chronic administration of low levels of LPS in vivo, meant to model chronic infection, result in loss of HSC quiescence, increased HSCs, myeloid skewing, and decreased progenitor function²³⁻²⁵. Taken together, chronic TLR signaling impairs HSC function and alters normal hematopoiesis, which suggests a causal role in MDS.

Indeed, overexpression or gain-of-function mutations in a subset of TLR has been described in MDS. TLR4 is overexpressed at both the mRNA and protein levels in CD34+ cells isolated from the bone marrow (BM) of MDS patients²⁶. MDS CD34+ cells with overexpressed TLR4 exhibited normal functional capacity as assessed by ICAM-1 expression after LPS stimulation. Interestingly, TNF α was shown to increase TLR4 expression in a dose-dependent manner, while depletion of TNF α with anti-TNF antibody treatment of MDS BM cells resulted in decreased TLR4 expression, suggesting that the TLR4 expression is TNF α dependent. Furthermore, Maratheftis et al. found a significant correlation between TLR4 expression and apoptosis in CD34+ MDS BM cells, providing correlative evidence for increased TLR4 expression and cell intrinsic defects associated with MDS HSPC. However, TLR4 expression within the total BM population from MDS patients did not differ from normal controls, nor was there a correlation between TNF α levels and TLR4 expression²⁷. Kuninaka et al. described TLR9 overexpression in MDS BM cells, which positively correlated with TNF α levels. Interestingly, TLR9 expression decreased once the MDS transformed to leukemia in these patients, further supporting the link between TLR expression and balance of cell survival signals in MDS. Both of these studies also describe TLR2 overexpression in MDS HSPC, and through deep sequencing analysis, a more recent study identified a TLR2 gain-of-function variant (F217S) in the CD34+ BM cells of 11% of MDS patients examined²⁸. Treatment with the TLR2 agonist MALP2 inhibited erythroid differentiation in healthy CD34+ BM cells²⁸. Furthermore shRNA knockdown of TLR2 in MDS CD34+ BM cells increased erythroid progenitors in colony formation assays²⁸.

Taken together, these studies provide evidence for the role of HSPC-expressed TLRs in the pathogenesis of MDS. There is some discrepancy in which specific TLRs are most important in the context of MDS since they regulate many of the same pathways. The specific TLR, however, may not be as important to identify as the hematopoietic lineage exhibiting overexpression of the TLR and the downstream effects that result from TLR dysregulation. Inhibition of the induction of these receptors, particularly through TNF α , which was implicated in both TLR4 and TLR9 expression, may be an attractive therapeutic strategy in the treatment MDS.

MyD88

MyD88, one of two intracellular TLR adaptor proteins, functions as a central node for innate immune signaling. It contains three domains: a death domain (DD), an intermediate domain, and a TIR domain^{29,30}. Upon TLR activation, TIR-TIR domain interaction recruits MyD88 to activated TLRs³¹. Following this interaction, IL-1 receptor associated kinase 4 (IRAK4), a serine-threonine kinase, complexes with MyD88 through the association of their respective DDs (Figure 2). IRAK family members (such as IRAK1 and IRAK2) are subsequently recruited and phosphorylated, forming a multiprotein complex referred to as the Myddosome^{32,33}. Phosphorylated IRAK1 and IRAK2 can then interact with TNF receptor associated factor 6 (TRAF6), an E3 ubiquitin (Ub) ligase, which ubiquitinates and activates TAK1^{34,35}. Activated TAK1 then signals to the MAPK and NF- κ B pathways³⁵. Moreover, MyD88 knockout mice exhibit hematopoietic system, immune signaling, and cell survival defects. The immune system abnormalities in these animals result in increased susceptibility to bacterial and viral infections³⁶.

Aberrant MyD88 expression and function is widely implicated in hematopoietic disease. Somatic mutation of Leucine-265 to Proline (L265P) within the TLR domain are observed in lymphoid malignancies and Waldenstrom macroglobulinemia, as well as in del(5q) MDS following gammopathy of undetermined significance^{37,38}. L265P results in a gain of function MyD88 protein, as the mutated TIR domain of MyD88 displays an intrinsic propensity for augmented oligomerization and spontaneous formation of cytosolic Myddosome aggregates³⁹. Although the level of MyD88-L265P expression in malignant lymphoid cells is comparable to wildtype MyD88 in normal tissues, its molecular consequences resemble that of MyD88 overexpression, indicating that L265P or overexpression of wildtype results in increased MyD88 signaling. Although MyD88 is not commonly mutated in myeloid malignancies, overexpression of wildtype MyD88 mRNA and protein is reported in MDS HSPC. Dimicoli et al. found that MyD88 mRNA is increased in the CD34+ BM cells of 40% of MDS patients examined. Furthermore, MDS patients with increased MyD88 expression in CD34+ BM cells were found to have shorter survival than patients with lower MyD88 expression, indicating that MyD88 expression has prognostic value⁴⁰. Blocking MyD88 signaling with a 26-amino acid inhibitory peptide in CD34+ cells from lower risk MDS patients results in increased differentiation of the erythroid lineage, coinciding with upregulation of erythroid differentiation genes. Additionally, hematopoietic differentiation genes, including GATA1 and GATA2, were upregulated in these cells, with an increased GATA1/GATA2 ratio, supporting the positive effect of a MyD88 blockade on erythroid differentiation of MDS CD34+ cells. Similarly, inhibition of IL-8, a downstream inflammatory cytokine dependent on MyD88, results in increased erythroid differentiation of CD34+ cells from lower risk MDS patients^{40,41}. Taken together, these findings implicate MyD88 activation and suggest a potential therapeutic role for inhibiting MyD88 and downstream effector signaling in MDS.

TIRAP

Myddosome assembly is also catalyzed by TIR domain containing adaptor protein (TIRAP) (Figure 2). TIRAP-deficient mice respond normally to TLR5, TLR7, and TLR9 ligands and to IL-1 and IL-18; however they exhibit defects in cytokine production and NF- κ B and

MAPK activation in response to LPS and bacterial lipoproteins⁴². TIRAP is composed of 3 domains: a TIR domain, a PEST domain, and an N-terminal PIP2 binding domain. TIRAP primarily functions as a molecular bridge between MyD88 and receptor complexes for TLR-2 and TLR-4 signaling. TIRAP recruits cytosolic MyD88 to interact with activated TIR domains of TLR dimers at the cell membrane, thus catalyzing formation of the Myddosome⁴³⁻⁴⁵.

TIRAP has MyD88-independent functions, such as activation of Rac1-PI3K-Akt signaling⁴⁶. Additionally, TIRAP binds TRAF6 and recruits it to the plasma membrane, which is required for the serine phosphorylation of p65/RelA⁴⁷. TLR2- and TLR4-induced NF- κ B activation requires Bruton's tyrosine kinase (Btk) phosphorylation of TIRAP. Also relevant to hematopoietic malignancies, TIRAP enhances cAMP responsive element protein (CREB) activation by signaling through TRAF6, Pellino3, p38 MAPK, and MK2⁴⁸.

The relevance of TIRAP to MDS was based on findings related to miR-145 expression, which targets TIRAP mRNA and is located within the commonly deleted region (CDR) on chromosome 5 (q33.1) in MDS⁴⁹. Since TIRAP is a direct target of miR-145, MDS patients with deletion of miR-145 exhibit increase TIRAP mRNA and protein expression⁴⁹. Using retroviral approaches, Ibrahim et al. demonstrated that TIRAP overexpression in HSPC results in BMF, independent of TRAF6⁵⁰. The contribution of TIRAP to MDS is supported by the genetic loss of miR-145 in mice as knockout of miR-145 results in phenotypically similar hematologic disease as TIRAP-overexpressing mice. Both mouse models exhibit defects in short-term repopulating HSC and progenitors of the myeloid lineage⁵¹.

IL-1 RAP

Another member of the TIR superfamily, interleukin receptor accessory protein (IL1RAP) is evolutionarily conserved among species and required for appropriate host responses to injury and infection. IL1RAP is expressed on cells required for the innate and adaptive immune response, including mast cells, monocytes, macrophages, neutrophils, basophils, NK cells, B cells, T helper type 2 cells (TH2), T helper 17 (TH17), and certain subsets of basal epithelial cells⁵²⁻⁵⁵. In humans, alternative splicing of the gene produces soluble (sIL1RAP) and membrane bound (mIL1RAP) isoforms. The membrane-bound isoform contains three extracellular immunoglobulin domains and a TIR domain in the cytoplasmic portion, and it was originally described as a necessary partner of the interleukin 1 receptor 1 (IL1R1)⁵⁶⁻⁵⁹ to initiate IL1 signaling. Binding of IL1 α or IL1 β to the IL1R1 recruits mIL1RAP, which increases the avidity of the ligand to IL1R1. The juxtaposition of the cytoplasmic TIR domains of mIL1RAP and IL1R1 anchors MYD88, IRAK4, TRAF6 and other signaling intermediates⁶⁰⁻⁶², leading to the induction of NF- κ B and MAPK pathways⁶³⁻⁶⁶. As a result, the expression of pro-inflammatory genes, IL6, TNF α , Interferon α/β , Tumor Growth Factor, and IL8, is upregulated. Active cytokines are then released from the cell. mIL1RAP interacts with other receptors including the interleukin-1 receptor 2 (IL1R2)^{67,68}, the interleukin 33 receptor (ST2)⁶⁹⁻⁷¹, the receptor tyrosine kinase KIT⁷² and the receptor for interleukin 36 family (IL1RL2)⁷³. Through its cytoplasmic domain, mIL1RAP transiently associates with the p85 subunit of phosphatidylinositol 3-kinase (PI3K) upon IL1 stimulation and mediates IL1 β -dependent activation of NF- κ B p65/RelA

subunit via Akt⁷⁴. In murine cells, Ruhul Amin et al. reported that mIL1RAP complexes with the glycoprotein SIRP α 1 to activate Akt and Erk in response to IL1 β stimulation⁷⁵. The soluble form of IL1RAP, lacking the transmembrane and cytoplasmic domains, counteracts IL-1 signaling by binding to the decoy receptor IL1R2 and increasing its affinity for IL1 α or IL1 β ⁷⁶. Similarly, in mast cells, sIL1RAP inhibits IL33 signaling⁷¹.

Dysregulated expression of IL1RAP has been observed in MDS⁷⁷, AML^{77,78}, and chronic Myeloid leukemia (CML)⁷⁹⁻⁸¹ as well as during inflammation⁸²⁻⁸⁵. In MDS, mRNA and protein levels of mIL1RAP are increased in CD34+ or phenotypically defined HSC from high-risk MDS patients while protein levels in patients with low risk MDS are lower⁷⁷, suggesting a role in disease progression. In AML patients with monosomy 7 and complex karyotype, IL1RAP is overexpressed in phenotypically defined hematopoietic stem and progenitor cells^{77,78}. AML patients with normal karyotype present variable levels of mRNA expression of IL1RAP in HSC. In this subtype of AML, patients with higher levels of IL1RAP have lower overall survival than patients expressing lower levels of IL1RAP in BM cells⁷⁷, indicating a prognostic value for IL1RAP. Inhibition of IL1RAP with short hairpin RNAs in AML cell lines and primary high-risk MDS cells leads to reduction of clonogenic capacity and cell death^{77,86}. IL1RAP is also proposed as a stem cell marker in CML, where its expression is restricted to CD34+ cells that express BCR-ABL⁸⁰. Further studies are warranted to determine the mechanistic role and therapeutic potential of IL1RAP in the pathogenesis of MDS.

IRAK

The IRAK family of proteins consists of four members: IRAK1, IRAK2, IRAK4, and IRAK-M. These proteins are serine/threonine kinases involved in signaling downstream of TLR/IL1-R activation. As mentioned above, upon TLR activation and recruitment of MyD88, MyD88 forms a multi-unit complex with IRAK4 via DD interactions (Figure 2)³³. Following its activation, IRAK4 phosphorylates IRAK1 at Threonine-209 followed by Threonine-387, which permits IRAK1 to form a complex with the Ub ligase TRAF6. Lysine 63 ubiquitination of IRAK1 by TRAF6 assembles a scaffold for recruitment of additional downstream proteins⁸⁷. Ultimately, the IRAK-mediated signal results in the activation of NF- κ B and MAPK pathways. In addition to activating IRAK1, phosphorylation of IRAK1 by IRAK4 has been shown to induce degradation of IRAK1, providing a negative feedback mechanism for TLR signaling⁸⁸. Originally it was thought that both IRAK2 and IRAK-M lacked kinase activity; however recent studies have shown that IRAK2 can act as a kinase and plays a major role in TLR-induced NF- κ B activation, particularly from TLR3, TLR4 and TLR8⁸⁹. Additionally, it has been proposed that IRAK-M may have kinase-independent activity regulating alternative NF- κ B activation⁹⁰.

IRAK4 deficiency has been reported in patients that show recurrent infections and poor inflammatory response⁸⁹. Interestingly, in these patients, the rate of infection decreases with age⁹¹, presumably due to intact adaptive memory responses, though further investigation is needed to determine an exact mechanism. IRAK knockout mice for each of the four IRAK family members have been generated. Mice deficient for IRAK1, 2, and 4 exhibit poor response to both viral and bacterial infections, recapitulating the human IRAK4 deficiency

syndrome⁹²⁻⁹⁵. Alternatively, IRAK-M knockout mice exhibit increased TLR-induced NF- κ B and MAPK activity, highlighting the regulatory role of IRAK-M⁹⁶.

Gene expression profiling of CD34+ BM cells from MDS patients show that IRAK mRNA is overexpressed in 10-30% of patient samples compared with normal CD34+ BM cells^{12,13,15,97}. IRAK1 protein levels correlated with its mRNA overexpression in primary MDS patient BM samples and several MDS/AML cell lines. Not only was IRAK1 protein level elevated, but phosphorylation of IRAK1 at Threonine-209 was also increased. IRAK1 inhibition, either by RNAi-mediated knockdown or through the use of an IRAK1/4 inhibitor, suppressed an MDS-like disease and delayed mortality in a xenograft model utilizing an MDS patient-derived cell line (MDSL)¹⁵. Alternatively, IRAK4 knockdown caused a less dramatic reduction of MDSL progenitor function and did not suppress IRAK1 phosphorylation, suggesting that IRAK4 is not as essential to MDS pathogenesis as IRAK1⁹⁷. These results suggest that IRAK inhibition could present a useful therapeutic target in the treatment of MDS. Selective IRAK4 small molecule inhibitors are in development for the treatment of lymphoid-derived hematologic malignancies, particularly those with MyD88 gain-of-function mutations⁹⁸, and thus present an exciting prospect for treatment of MDS. A comprehensive review of IRAK signaling in hematologic malignancies was recently published, emphasizing the potential importance of IRAK1 signaling and its inhibition in MDS⁹⁹.

TRAF6

During normal TLR/IL-1R activation, interaction of IRAK family members with TRAF6 is a rate-limiting step for efficient innate immune activation. TRAF6 contains a RING domain, which functions as an Ub ligase by exclusively forming K63 linkages on protein substrate lysines (Figure 2). The RING domain of TRAF6 binds a specific Ub-conjugating enzyme, UBE2N (Ubc13), which catalyzes the synthesis of Ub chains^{100,101}. Importantly, Ubc13 (and its non-enzymatic factor Uev1A) is essential for TRAF6-dependent IKK function^{101,102}. Inhibition of Ubc13 activity with a small molecule inhibitor abolishes TRAF6 Ub ligase function and transfer of Ub¹⁰³. Following formation of the IRAK1-IRAK4-MyD88 complex, TRAF6 ubiquitinates downstream signaling molecules necessary for of TLRs/IL-1R signaling¹⁰⁴. In parallel, TRAF6 induces its polyautoubiquitination and subsequent oligomerization¹⁰⁵, which further promotes its interaction with additional molecules, such as NEMO, TAB2/3, TAK1¹⁰⁶, and Akt¹⁰⁷. One of the key functions of TRAF6 is to activate NF- κ B in response to proinflammatory cytokines. IRAK1 undergoes TRAF6-mediated K63 polyubiquitination, which initiates activation of IKK and the subsequent induction of pro-inflammatory cytokines¹⁰⁸. In response to stimulation of TLR4, TRAF6 also links innate immune response to autophagy through the ubiquitination of Beclin-1¹⁰⁹. While significant progress has been made in identifying pathway-versatile regulatory roles for TRAF6, its full potential in inflammatory disease pathogenesis remains to be discovered.

Given the critical role of TRAF6 in immune signaling, it is not surprising that TRAF6 function and expression is tightly regulated. A major regulatory node for intracellular inflammatory signaling occurs at the TRAF6 mRNA level, via miR-146a. As discussed

below in more detail, miR-146a is an immune responsive target that is a feedback inhibitor by directly regulating TRAF6 (and also IRAK1). Moreover the dual ubiquitin-editing enzyme A20 antagonizes TRAF6 autoubiquitination by deubiquitinating the K63 linkages. TRAF6 function is also modulated by its other protein interactors, as a recent report suggests that protein phosphatase 1 (PP1- γ) directly associates with TRAF6, enhances its E3 ligase function, and promotes NF- κ B signaling¹¹⁰. Meanwhile, Jiao et al. found that MST4 directly interacts with and phosphorylates TRAF6 to prevent its oligomerization and autoubiquitination, which in turn suppresses its activity¹¹¹. Taken together, the balance of inhibitory and stimulatory effectors regulating TRAF6 plays a key role in fine-tuning cell-intrinsic response to inflammatory stimuli. Despite our understanding of how TRAF6 protein function is regulated, much less is known about transcriptional control of TRAF6.

While it is generally accepted that inflammation plays a key part in tumorigenesis, many intracellular mechanisms underlying this process remain to be elucidated. Significant progress has been made in ascribing TRAF6 as a key molecule for cell-intrinsic inflammatory signaling and as a result, corroborative data generated from patient samples, in vitro studies, and in vivo mouse model characterizations have appreciably implicated increased TRAF6 signaling in the pathogenesis of MDS. Evidence of increased TRAF6 function was first described by Hofmann et al¹². Gene expression profiling of CD34+ BM cells from MDS patients revealed a 10-fold increase in TRAF6 mRNA as compared to normal CD34+ controls. This observation was further supported by derepression of TRAF6 mRNA and protein in del(5q) MDS as a consequence of miR-146a haploinsufficiency⁴⁹. Conversely, knockdown of TRAF6 in MDS/AML cell lines or patient samples resulted in rapid apoptosis and impaired malignant hematopoietic stem/progenitor function¹¹². Importantly in the context of elevated TRAF6 expression in MDS, overexpression of TRAF6 in human or mouse cells is sufficient to induce its oligomerization, autoubiquitination, and downstream pathway activation. Collectively, TRAF6 overexpression may explain immune pathway overactivation in the MDS-initiating HSPC.

Experimental mouse data supports the hypothesis that increased TRAF6 function has a causal role in the pathogenesis of MDS. Overexpression of TRAF6 in mouse HSPC using a retroviral approach resulted in elevated platelets, neutropenia, dysplasia, and myeloid leukemia in a subset of mice⁴⁹. Some of the effects are mediated by IL-6 as overexpression of TRAF6 in IL-6 deficient HSPC restored platelets and neutrophil counts. However, the IL-6 deficiency did not delay BMF and AML. Consistent with these observations, Zhao et al. similarly reported a fatal BMF syndrome and associated cytopenias in TRAF6-overexpressing mice. Despite similarities between miR-146a-deficient and TRAF6-transduced HSPC (see comparison below), the level of TRAF6 expression in transduced HSPC is at least 10-fold higher than observed in miR-146a-deficient HSPC. Therefore, to better decipher the contribution of TRAF6 to the miR-146a-deficient HSPC phenotype, enforced expression levels of TRAF6 in transgenic mice need to mimic what is observed in miR-146a-deficient HSPC and in MDS patients. Despite our understanding of TRAF6 Ub-dependent activation of IKK, very few additional TRAF6 substrates have been identified. More importantly, constitutive NF- κ B-dependent activation is not sufficient to induce MDS in mice^{113,114}, suggesting additional pathways downstream of TRAF6 contribute to initiation of MDS while NF- κ B activation may contribute to the maintenance of the diseased

phenotype¹¹⁵. The consequences of TRAF6 activation and relevant substrates have not been evaluated in HSC. Given its potential role in MDS, an extensive investigation is necessary to elucidate the function of TRAF6 in HSC and MDS.

Negative Regulators of Innate Immune Signaling in MDS

miR-146a

miR-146a belongs to a family of microRNAs, which also includes miR-146b. Both miRNAs have an identical seed region and putative mRNA targets. The miR-146 family was initially described as TLR4/NF- κ B target genes through a microarray study to identify miRNAs that were regulated upon LPS stimulation in THP1 cells¹¹⁶. Furthermore, a promoter analysis identified two functional and conserved NF- κ B binding sites upstream of the miR-146a gene. Follow up studies have confirmed that miR-146a is a rheostat for innate immunity by targeting several components of the TLR signaling pathway, including TLR4, TRAF6, and IRAK1¹¹⁷.

miR-146a lies within chromosome 5q33.3 and its deletion occurs in 80% of all del(5q) MDS patients. Consistent with deletion of a single miR-146a allele, expression in BM HSPC from del(5q) MDS patients is reduced by greater than half^{49,118}. miR-146a is also downregulated in non-del(5q) MDS patients suggesting that other mechanisms may contribute to its reduced expression in MDS^{119,120}. In this non-del(5q) MDS patient cohort, miR-146a is part of a miRNA diagnostic signature that distinguishes MDS patients from age-matched controls¹¹⁹. Although miR-146a is consistently downregulated in del(5q) MDS, additional miRNAs residing on chr 5q (i.e., miR-145) may also contribute to aspects of MDS pathogenesis. Concomitant knockdown of miR-145 and miR-146a using a miRNA decoy approach in mouse HSPC resulted in elevated platelets, neutropenia, megakaryocytic dysplasia, and myeloid leukemia. The distinction between miR-145 and miR-146a's contribution to the hematopoietic defects has been revealed by examination of the miR-146a-deficient mice.

In normal mouse and human hematopoietic cells, miR-146a expression is not restricted to particular hematopoietic lineages. Evidence suggests a role for miR-146a throughout development of blood and lymphoid cells due to a significant increase in its expression as HSCs mature. A germ-line knock out mouse model of miR-146a suggests minimal requirement for miR-146a during steady state hematopoiesis. Conversely, a model of enforced miR-146a expression in mouse HSPC has marginal effects on hematopoiesis. However, miR-146a is essential during stressed and age-associated hematopoiesis. Knockout of miR-146a results in an early onset of myeloid expansion in the BM, immune dysfunction, and progression to more aggressive diseases such as lymphomas, BMF, and myeloid leukemia¹²¹⁻¹²³. Furthermore, miR-146a-deficient HSPC produce higher levels of pro-inflammatory cytokines, such as IL-6, TNF α , GM-CSF and IL-1 β , in response to TLR activation. Additional concepts and description of miR-146a-deficient mice pertaining to the pathogenesis of MDS has been recently summarized¹¹⁷.

TNFAIP3

TNFAIP3(A20) regulates TLR/NF- κ B signaling through its effects on TRAF6. A20 is an anti-inflammatory signaling molecule that regulates several intracellular signaling cascades by modifying Ub chains on upstream adaptor proteins. Under normal cellular conditions, A20 is expressed at low levels. However, stimulation of PAMPs and pro-inflammatory cytokines acutely induce the expression of A20 in an NF- κ B-dependent manner¹²⁴. In response to several immune pathways including TNF receptor, IL-1R, and TLR signaling, A20 negatively regulates excessive NF- κ B signaling by modifying Ub chains on the key intermediates TRAF6 and Receptor Interacting Protein (RIP1)¹²⁵.

The most striking data to demonstrate the involvement of A20 in the innate immune system is that A20-deficient mice prematurely die of severe multiorgan inflammation¹²⁶. A20^{-/-} RAG1^{-/-} mice, which lack T and B lymphocytes, present with a similar phenotype, suggesting A20 regulates innate immune homeostasis independent of the adaptive immune system¹²⁷. Given the limitation of the perinatal lethality of A20-deficient mice, conditionally targeted mice have been generated for lineage specific deletions of A20, which uniquely mimic human autoimmune and inflammatory diseases that are associated with single nucleotide polymorphisms (SNPs) in the A20 genomic region^{125,128-133}. Cell-specific deletions highlight A20's role in maintaining tissue homeostasis and the prevention of inflammatory diseases. Mice with hematopoietic-specific deletions of A20 exhibited severe cytokine-mediated inflammation leading to premature death^{134,135}. Myeloid cells from these mice proliferated excessively and B cells underwent apoptosis accompanied by anemia and lymphopenia¹³⁴. Constitutive NF- κ B activity was observed in A20-deficient HSCs, leading to increased cell cycle entry. Loss of A20 in HSCs diminishes quiescence and depletes the HSC pool¹³⁵. Furthermore, transplants of these cells into recipient mice revealed impaired long-term HSC function¹³⁴.

A20 is a central regulator of multiple signaling cascades activating NF- κ B. As an NF- κ B target gene, A20 negatively feeds back to dampen NF- κ B signaling by several mechanisms: removal of polyubiquitin chains that participate in signaling activation, and attachment of polyubiquitin chains that facilitate the degradation of proteins involved in signaling complexes. Its deubiquitinase activity promotes the depolymerization of K63-linked polyubiquitin chains on RIP1. Subsequently, its E3 ligase activity covalently attaches K48-linked polyubiquitin chains on RIP1, which results in proteasomal degradation of RIP1, thereby regulating TNF α -induced NF- κ B signaling¹³⁶. K63-linked polyubiquitin chains are also deubiquitinated on TRAF6, while K48-linked polyubiquitin chains are ligated on the E2 enzyme Ubc13, resulting in the degradation of Ubc13 and the disruption of the E2 and E3 ligase interaction, and consequently terminating TLR4/ IL-1R1 signaling to NF- κ B¹³⁶.

The dual ubiquitin-editing functions of A20 are accomplished by two functional domains. The deubiquitination activity is mediated by the catalytic cysteine in the N-terminal ovarian tumor (OTU) domain while the C-terminal zinc fingers 4 and 7 mediate E3 ligase activity. Several studies have investigated the specific function of each domain in the molecular and cellular context. In Tnfaip3^{OTU/OTU} cells, which are mutant for A20 DUB function, RIP1 immunoprecipitation experiments showed increased polyubiquitin chains, suggesting that the OTU domain is responsible for the removal of K48 and K63-linked polyubiquitin chains

from RIP1¹³⁷. Bosanac et al. found that A20 zinc finger 4 appears to be necessary for the modification of NF- κ B signaling¹³⁸. Zinc finger 4 recognizes K63-linked Ub chain and exhibits binding capabilities. By overexpressing zinc finger A20 mutants, Tokunaga and colleagues determined that zinc finger 7 regulates NF- κ B activity through the recognition of linear polyubiquitin chains and also weakly interacts with K63-linked polyubiquitin chain¹³⁹. Therefore, there is evidence that both the DUB activity in the OTU domain and E3 ligase activity in zinc fingers 4 and 7 participate in the modulation of Ub chains to control intracellular immune signaling cascades that limit excessive inflammation.

Expression of A20 mRNA is extremely high in lymphoid organs and regulation of A20 in lymphocytes differs from other cell types¹⁴⁰. A20 has recently been identified as a recurrently mutated gene in lymphoid leukemias¹⁴¹. Since dysregulation of the NF- κ B signaling pathway has been found to be involved in leukemogenesis and deletions and mutations of A20 have been observed in lymphomas, A20 is considered a tumor suppressor¹⁴². Certain autoimmune and inflammatory diseases linked with SNPs in A20 are associated with increased risk of lymphomas¹⁴³.

Increasing evidence also suggests a connection between A20 expression and MDS pathogenesis. Consistent with the hypothesis that reduced A20 expression and increased innate immune function is associated with MDS¹⁴⁴, empirical data revealed A20 mRNA is significantly reduced in CD34+ cells isolated from the BM of MDS patients¹⁴⁵. Moreover, miR-125a, which targets A20, is overexpressed in MDS CD34+ cells^{16,146}. As mentioned above, population-based study revealed that autoimmune disorders are a potential trigger for MDS development¹⁴. Interestingly, the MDS-associated autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, and Sjogren's syndrome are also genetically linked to A20 SNPs and observed in A20-deficient mice. Taken together, this suggests an impelling case for A20 as a relevant immune-related gene in MDS.

Conclusions

In summary, chronic innate immune signaling and activation is widely reported in MDS. The consequences of increased TLR signaling in MDS can result in systemic effects on hematopoiesis, such as due to inflammation and abnormal adaptive and innate immunity on BM HSPC. In addition to these non-cell autonomous consequences of chronic innate immune activation, increased TLR signaling within HSPC from MDS is also widely reported, and thought to contribute to cell-intrinsic defects in HSC. Although mounting evidence implicates chronic innate immune signaling in the pathogenesis of MDS, there are many unresolved questions. How best to measure chronic innate immune signaling and activation in MDS? In addition to the evidence above, how else is the innate immune pathway activated in MDS, and in which hematopoietic cell lineage? Which MDS subtypes exhibit activation of innate immune signaling? Lastly, it is prudent to distinguish between therapies targeting the systemic effects of chronic innate immunity versus cell-intrinsic TLR signaling in MDS, as these treatments will likely have distinct consequences on disease outcome.

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Figure 1. Overview of non-cell autonomous effects of chronic innate immune activation in MDS
 The MDS bone marrow (BM) consists of MDS-derived hematopoietic cells and residual normal cells. The non-cell autonomous consequences of the MDS-derived BM include increased expression of cytokines, expansion of myeloid-derived suppressor cells (MDSC), and lymphocyte-mediated attack on hematopoietic cells. In addition, MDS HSC affect mesenchymal stromal cells (MSC), therefore altering the HSC niche and hematopoietic differentiation.

Figure 2. Overview of cell-intrinsic TLR activation in MDS HSPC cells

Toll-like receptor (TLR) and IL-1 receptor (IL1R) on HSPC recruit MyD88 and IRAK4, which initiate the assembly of the Myddosome complex. TIRAP can also increase the efficiency of Myddosome assembly by binding MyD88. IRAK4, a serine/threonine kinase, activates IRAK2 and/or IRAK1 through IRAK4-dependent phosphorylation. IRAK1 associates with an E3 Ub ligase, TRAF6, which mediates activation of NF- κ B (IKK α / β / γ complex) and MAPK (TAB2/TAB3/TAK1) through K63-linked Ub chains. TRAF6 can also regulate other proteins (indicated by “?”) that may contribute to immune signaling and

MDS. miR-146a suppresses IRAK1 and TRAF6 protein expression by direct binding at 3'UTR sites within IRAK1 and TRAF6 mRNAs. A20 suppresses TRAF6 by de-ubiquitinating TRAF6 K63 linkages. Reduced levels of miR-146a or A20 result in increased IRAK1/TRAF6-mediated signaling, impaired HSPC function, and altered myeloid differentiation. The red boxes represent molecules that are overexpressed/activated in MDS. The green boxes represent molecules that are deleted/downregulated in MDS.