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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 about 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 biological level, we have characterized the impact of TLR2 signaling in primary HSPCs, which indicated 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 better understandings of the TLR2-JMJD3 innate immune pathway and its biology in MDS, including identification of potential bio-marks and novel therapeutic targets in this disease.					
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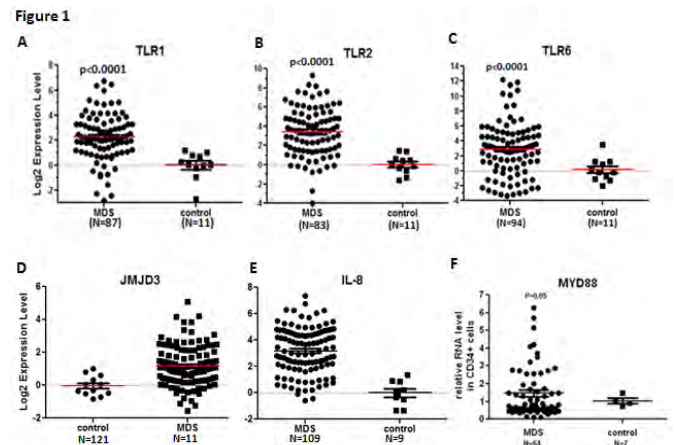
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Introduction

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. This potentially contributes to disease pathogenesis. Based on this, we propose a systematic analysis of the TLR2-JMJD3 pathway in MDS. Specifically, 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 will eventually improve the therapy of patients with MDS.

Progress Report

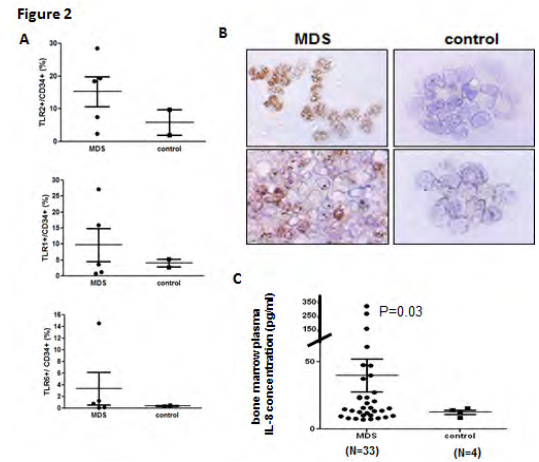
#1. In Aim 1 of this study we propose to perform gene expression analysis of TLR2 and its downstream signaling components in MDS CD34⁺ cells. We have performed QPCR analysis for the RNA expression of TLR 1, 2 and 6 in 103 cases of MDS BM CD34⁺ samples (Figure 1). Compared to controls, average expression levels in MDS samples were increased by 10-fold for TLR1 (74% over 2-fold increase, N=87, $p<0.0001$; Figure 1A), by 37-fold for TLR2 (82% over 2-fold increase, N=83, $p<0.0001$, Figure 1B), and by 168-fold for TLR6 (73% over 2-fold increase, N=94, $p=0.0001$, Figure 1C). RNA levels of JMJD3 were examined in 121 samples and the levels were found to be overexpressed in 54 percent of cases (> 2 fold increase; Figure 1D). The average JMJD3 RNA level in the whole cohort was 8-fold of control normal CD34⁺ cell samples ($p<0.0001$). Interleukin-8 (IL-8), a key transcriptional target of TLR2-JMJD3 pathway in HSPCs, has also been evaluated in 109 MDS BM CD34⁺ cells. Results indicated that IL-8 expression was significantly elevated in MDS (Figure 1E). We also compared their MYD88, which encodes a signal relay of the TLR2 pathway in 64 samples of MDS CD34⁺ samples. Results indicated that 27 percent of MDS patients (N=17) had a more than 2-fold increase of MYD88 RNA, and 14 percent (N=9) had a 1.3- to 1.9-fold increase. In total, 41 percent (N=26) of patients overexpressed MYD88 (Figure 1F).



To verify the overexpression of these innate immunity genes in MDS, we have also characterized their protein levels in initial cohorts of primary MDS bone marrow samples. We performed flow cytometry analysis of TLR1, 2, and 6 using primary MDS whole bone marrow samples. Data indicate that the average increase in BM CD34⁺ cells were 2.5-fold, 2.4-fold and 8.5-fold for TLR2, 1 and 6 respectively compared to controls (Figure 2A). Protein expression of JMJD3 was examined using immunohistochemical staining in primary BM CD34⁺ cytopins of patients with MDS (N=7) and healthy controls (N=2). Five of the seven MDS samples examined had strong JMJD3 signals in the cell nucleus, whereas both control CD34⁺ specimens had very weak nuclear JMJD3 signals (Figure 2B). Finally, the

IL-8 level in patient bone marrow plasma samples is evaluated using ELISA assays. IL-8 protein levels in patients with MDS (N=33) was also elevated in comparison to control counterparts (P=0.03; Figure 2C).

To further evaluate the prognostic value and clinical implication of the deregulation of these innate immunity genes, we correlate RNA expression data with other clinical annotations of the patients. First, our analysis has indicated that overexpression of two of the genes being characterized are associated with adverse clinical outcome, particularly overall patient survival: TLR6 expression had a tendency to be negatively correlated with OS (22.7 vs. 72.8 months, $p=0.18$; Figure 3A). Patients with higher MYD88 RNA expression in bone marrow CD34+ cells (split by median value) had a propensity for shorter survival ($p=.09$, HR 1.9, 95% CI 0.89-4.26; Figure 3B). Of interest, higher MYD88 expression correlated significantly with shorter overall survival in the multivariate model, adjusted for IPSS risk score and patient age ($p=.027$, HR 2.46, 95% CI 1.1-5.45). Furthermore, increased levels of TLR2 expression were associated with low-risk MDS by IPSS ($p=0.01$; Figure 3C), diploid cytogenetics ($p=0.04$), and a diagnosis of chronic myelomonocytic leukemia (CMML; $p=0.04$). In contrast, expression of TLR6 gradually increased with IPSS risk: high-risk patients had highest TLR6 expression, intermediate-1 and -2 patients had intermediate expression, and low-risk group had lowest TLR6 expression ($p=0.015$; Figure 3D). Patients that had lowest TLR6 expression were associated with diploid cytogenetics ($p=0.02$) and a diagnosis of CMML ($p=0.0004$). Furthermore, patients with higher TLR6 expression (above median) had increased percentage of bone marrow blasts (7.8% vs. 3.2%, $p<0.0001$).



#2. In Aim 1 we also propose to perform the mutational analysis of TLR2 (F217S) in bone marrow cells of MDS. We have analyzed a cohort of 149 MDS BM-MNC and identified that over 11 percent (N=17) of the cases bear the alteration of TLR2-F217S. In 15 of these 17 cases (88%) the TLR2-F217S alteration is somatic, whereas 2 cases carry the same mutation in control CD3+ T cells. No TLR2-F217S mutation was detected in normal control DNA samples (N=47) that were derived from human lymphoblastoid cells isolated from healthy donors. When correlating the presence of TLR2-F217S to other clinical outcomes, patients with TLR2-F217S had a significantly higher frequency of chromosome 7 deletion ($p=0.03$).

We have characterized the impact of TLR2-F217S on the activation of innate immunity signaling by expressing green fluorescent protein (GFP) fused wild-type or TLR2-F217S in 293T cells. In luciferase reporter assays, in the absence of TLR2 agonist, expression of wild-type or

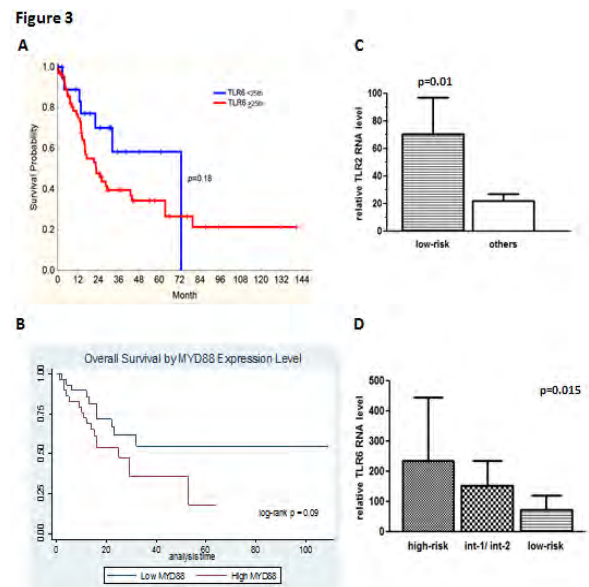
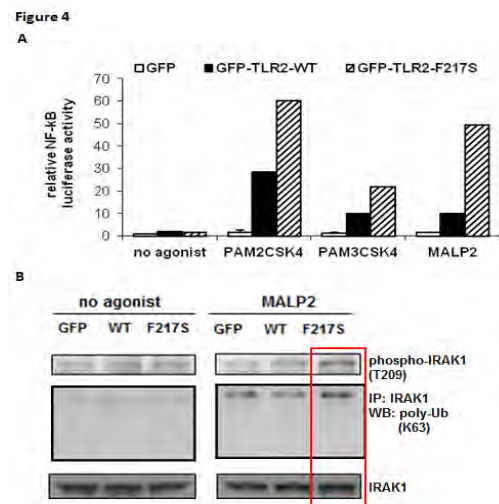


Fig 3. Clinical association of deregulated innate immunity genes. (A-B) Adverse association of TLR6 and MYD88 RNA level with patient survival (C-D) Association of TLR2 and TLR6 levels with IPSS scores.

F217S mutant TLR2 led to similar levels of NF- κ B activation. When a TLR2 agonist (PAM2CSK4, PAM3CSK4 or MALP2) was added, NF- κ B activation was increased in TLR2-F217S versus wild-type transfected cells (Figure 4A). Similar effects on AP1 activity and associated phospho-p38MAPK were also observed. We then examined the status of IRAK1 protein, a key downstream signal mediator of TLRs. In the absence of TLR agonist, a slight increase of a slowly migrating IRAK1 band (~180KD) was observed in both wild-type and F217S transfected 293T cells. After MALP2 and PAM2CSK4 treatment, the density of this high-molecular IRAK1 band was higher in TLR2 (F217S) than in wildtype-transfected cells. IRAK1 immuno-precipitation followed by immunoblotting indicates that this higher molecular form of IRAK1 contained phospho- and polyubiquitin- IRAK1, two active isoforms of this protein (Figure 4B). Taken together, these results indicate that TLR2-F217S is a gain-of-function SNP that augments TLR2 mediated down-stream signaling.



#3. In Aim 2 of the proposal, we plan to study the molecular implications of the activation of TLR2/NF- κ B/JMJD3 signaling in hematopoietic cells and to evaluate its potential impacts on MDS pathogenesis. We assessed the effects of several TLR2 agonists (MALP2 and PAM3CSK3). Results indicated that in the ex vivo cultured BM CD34⁺ cells, these agonists increase the expression of both JMJD3 and IL-8 expression. Stimulation of TLR2 has been shown to alter hematopoietic differentiation in cultured mouse HSPCs. Furthermore, flow cytometry assays indicated that MALP2 caused a significant decrease of an erythroid precursor cell population, which was defined by strong CD71 expression and absence of HLA-DR. PAM3CSK4 had similar but less significant effect (Figure 5A). The negative influence of TLR2 agonists on erythroid lineage was further confirmed by colony formation assays in methylcellulose medium (methocult). After 2 weeks in methocult culture, MALP2 treatment led to a 55 percent reduction in erythroid forming units (CFU-E; Figure 5B). Consistent with the results of flow cytometry, PAM3CSK4 had a similar but less significant effect on CFU-E formation. We also assessed the impact of IL-8

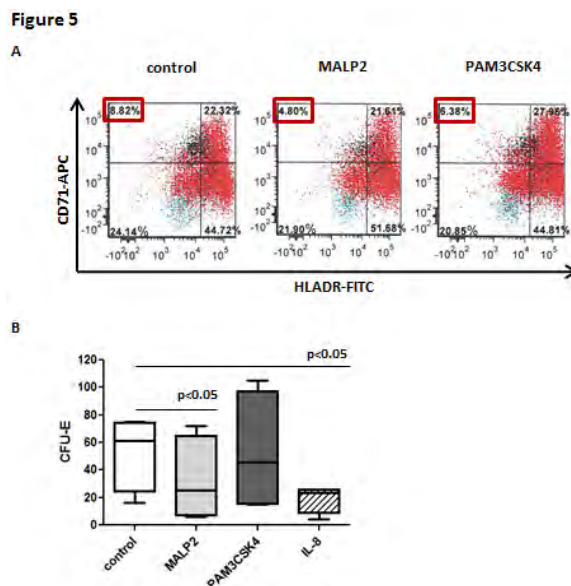
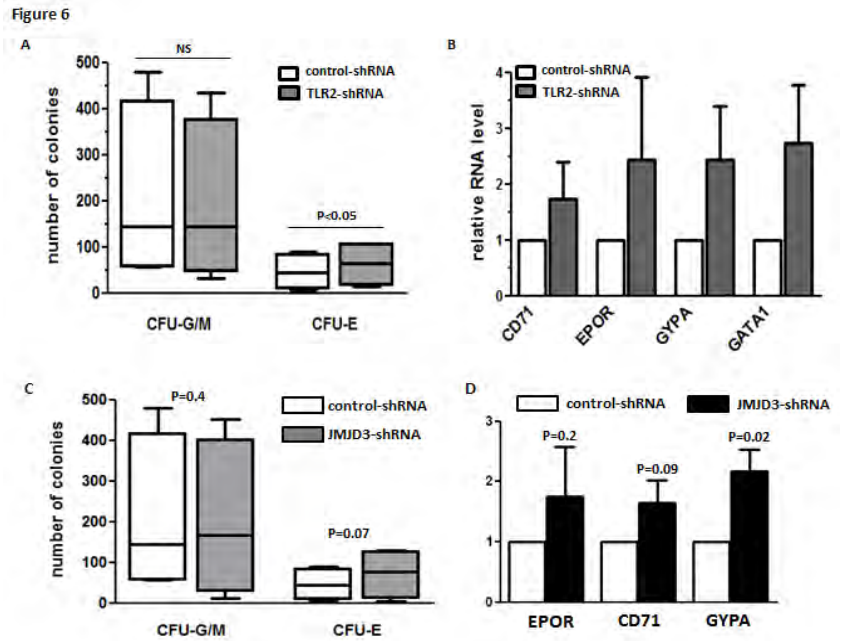


Fig 5. Biological impact of TLR2 activation in primary BM CD34⁺ cells cultured ex vivo. **(A)** Flow cytometry analysis detected decrease of CD71-high/ HLA-DR-low erythroid blast cells in TLR2 agonists MALP2 and PAM3CSK4 treated cells. **(B)** Decreased erythroid colonies (CFU-E) in methocult culture when CD34⁺ cells are treated with TLR2 agonists MALP2 and PAM3CSK4 or IL-8.

#4. In Aim 3 of the proposal, we plan to study the potential therapeutic effects of interfering with TLR2 function in MDS through recombinant retrovirus mediated shRNA transduction and also a TLR2 specific antibody. First, we transduced primary BM CD34⁺ cells isolated from patients with newly diagnosed lower-risk MDS (N=4) and higher-risk MDS

(N=3) with a recombinant retroviral mediated shRNA against TLR2. After two weeks of culture in methocult medium, all four lower-risk samples had an increased number of erythroid colonies (CFU-E) after TLR2 shRNA transduction. On average, 64 CFU-E were formed per 10^4 MDS BM CD34+ cells plated in response to the transduction of TLR2-shRNA, which was a 35 percent increase compared to the number of CFU-E formed after control-shRNA transduction (Figure 6A). No significant effect on the formation of myeloid colonies (CFU-G/M) was observed after TLR2 inhibition. In contrast to lower-risk MDS, we did not observe a positive effect on the formation of erythroid or myeloid colonies in any of the three BM CD34+ cells isolated from patients with higher-risk MDS. To further verify the effect of TLR2 inhibition in lower-risk samples, we measured



transcripts of several genes known to be positively associated with erythroid differentiation, including Glycophorin-A (GYPA), CD71, EPOR and GATA1 (Figure 6B). In the cells collected from methocult colonies, expression of all four genes was increased after TLR2 inhibition. The effect on gene expression further confirmed the positive impact of TLR2 inhibition on the differentiation of erythroid lineage in MDS BM CD34+ cells of lower-risk type of MDS. Besides TLR2, we also evaluate the effect of JMJD3 inhibition by applying a shRNA that targets JMJD3 in primary MDS CD34+ cells of patients with newly diagnosed lower-risk MDS (N=4) and higher-risk MDS (N=4) cultured ex vivo with a recombinant retroviral-mediated shRNA against JMJD3. Three out of four lower-risk samples had an increased number of CFU-E after JMJD3 shRNA transduction (Figure 6C). Similar to TLR2-shRNA, no significant effect on the formation of myeloid colonies (CFU-G/M) was observed after JMJD3 inhibition. In contrast to the observations in lower-risk MDS, we did not observe positive effect on the formation of erythroid or myeloid colonies in any of the four BM CD34+ cells isolated from patients with higher-risk MDS. In the cells collected from methocult colonies, expression of Glycophorin-A (GYPA), CD71 and EPOR were all increased after JMJD3 inhibition (Figure 6D).

Key Research Accomplishments

1. Achieved a systematic gene expression profiling of key components of the TLR2-JMJD3 mediated innate immunity signaling pathway, including TLR1, 2, 6, JMJD3, IL8, MYD88, in the CD34+ enriched MDS bone marrow hematopoietic stem/ progenitor cells;
2. Established preliminary associations between the deregulation of TLR2-JMJD3 innate immunity genes and key prognostic results of patients with MDS, including their IPSS score and overall survival time;

3. Analyzed the TLR2-F217S as a potential somatic mutation with biological gain-of-function property and occurs in over 10% of patients with MDS;
4. Characterized the biological impact of the activation of TLR2 signaling on cultured primary bone marrow CD34+ HSPCs, which indicated that abnormal activation of this signal may inhibit erythroid differentiation from HSPCs. This observation is relevant to one of the important clinical features of patients with MDS, which is persistent anemia; and
5. Demonstrated that interference of the TLR2-JMJD3 innate immunity signaling through inhibition of TLR2 and JMJD3 could rescue the differentiation of erythroid lineage in patients with lower-risk diseases (low risk and intermediate-1 by IPSS score).

Reportable Outcomes

A. Publications:

11. **Wei Y**, Chen R, Dimicoli S, Bueso-Ramos C, Neuberg D, Pierce S, Wang H, Yang H, Jia Y, Zheng H, Fang Z, Nguyen M, Ganan-Gomez I, Ebert B, Levine R, Kantarjian H, Garcia-Manero G. Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells. *Leukemia*. e-Pub 3/2013. PMID: 23538751.
14. Dimicoli S, **Wei Y**, Bueso-Ramos C, Yang H, Dinardo C, Jia Y, Zheng H, Fang Z, Nguyen M, Pierce S, Chen R, Wang H, Wu C, Garcia-Manero G. Overexpression of the Toll-Like Receptor (TLR) Signaling Adaptor MYD88, but Lack of Genetic Mutation, in Myelodysplastic Syndromes. *PLoS One* 8(8):e71120, 2013. e-Pub 8/2013. PMCID: PMC3744562.
15. **Wei Y**, Dimicoli S, Bueso-Ramos C, Chen R, Yang H, Neuberg D, Pierce S, Jia Y, Zheng H, Wang H, Wang X, Nguyen M, Wang SA, Ebert B, Bejar R, Levine R, Abdel-Wahab O, Kleppe M, Ganan-Gomez I, Kantarjian H, Garcia-Manero G. Toll-like receptor alterations in myelodysplastic syndrome. *Leukemia* 27(9):1832-40, 9/2013. e-Pub 6/2013. PMID: 23765228.

B. Oral Presentations:

1. **Toll-Like Receptor (TLR) Signaling Adaptor Protein MYD88 in Myelodysplastic Syndromes (MDS)** Sophie Dimicoli, Yue Wei, Rui Chen, Carlos E. Bueso-Ramos, Sherry A. Pierce, Hui Yang, Yu Jia, Hong Zheng, Zhihong Fang, Irene Ganan-Gomez, Martin Nguyen, Michael Fernandez, Hagop M. Kantarjian, and Guillermo Garcia-Manero. **American Society of Hematology (ASH) Annual Meeting, Nov 2012, Atlanta**
2. **Targeting Innate Immunity Signaling in Myelodysplastic Syndrome (MDS).** Yue Wei, Sophie Dimicoli, Rui Chen, Carlos E. Bueso-Ramos, Sherry A. Pierce, Guillermo Garcia-Manero. **2012 National conference of Hematologic Malignancies, Oct 2012, Houston**

C. Poster Presentations:

1. **Serum Amyloid Protein A 1 (hSAA1) Is Overexpressed in Myelodysplastic Syndromes and Potentially Mediates Toll-Like Receptor 2 Innate Immunity Signaling in CD34+ Hematopoietic Stem Cells.** Yue Wei, Carlos E. Bueso-Ramos, Hui Yang, Yu Jia, Hong Zheng, Simona Colla, Martin Nguyen, Michael Fernandez, Hagop M. Kantarjian, and Guillermo Garcia-Manero. **American Society of Hematology (ASH) Annual Meeting, Nov 2012, Atlanta**

2. Deregulation of TLR2-JMJD3 Innate Immunity Signaling, Including a Rare TLR2 SNP As a Potential Somatic Mutation, in Myelodysplastic Syndromes (MDS). Yue Wei, Rui Chen, Sophie Dimicoli, Carlos E. Bueso-Ramos, Donna S. Neuberg, Sherry A. Pierce, Hui Yang, Yu Jia, Hong Zheng, Zhihong Fang, Martin Nguyen, Michael Fernandez, Sa A. Wang, Hagop M. Kantarjian, and Guillermo Garcia-Manero. **American Society of Hematology (ASH) Annual Meeting, Nov 2012, Atlanta**

Conclusion

In the first year of this grant, we have successfully provide multiple lines of evidence that abnormal activation of innate immunity signaling is involved in the pathogenesis of MDS. We have reported that an innate immune/ inflammatory signal network centered by Toll-like Receptor (TLR) and inflammation-associated histone demethylase JMJD3 (KDM6B) is deregulated in hematopoietic stem cells (HSCs) of MDS and contributes to disease pathology. We have demonstrated that inhibition of TLR2, JMJD3 and the inflammatory cytokines that are regulated by this signaling pathway can improve hematopoietic differentiation in the HSCs of MDS. In the next year, we will expand the gene profiling effort and focus on the characterization of the potential association of the overexpression of innate immunity genes and the occurrence of other known molecular lesions such as frequent genetic mutations in MDS. We will investigate the impact of co-occurrence of innate immunity signal deregulation and other molecular lesions on the progress and prognosis of the disease. Based on the effect of inhibition of TLR2 and JMJD3, we will characterize several inhibitors of this pathway, including TLR2 antibody and JMJD3 inhibitory compound, and their potential therapeutic effects in MDS. Finally, we have also performed pilot analysis using peripheral blood and bone marrow HSC samples collected from patients on hypomethylation agent (HMA)-based clinical trials and observed that multiple innate immune genes are actively induced by HMA, specifically in patients who failed treatment. These results suggest that activation of innate immunity signaling is not only involved in MDS pathogenesis but also in the mechanism of HMA resistance/ failure in MDS and potentially also in AML. We will further investigate this hypothesis and try to develop novel therapeutic strategies of combining anti-innate immunity agents with HMA drugs to improve treatment of patients with MDS.

ORIGINAL ARTICLE

Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34 + cells

Y Wei¹, R Chen², S Dimicoli¹, C Bueso-Ramos³, D Neubergh⁴, S Pierce¹, H Wang², H Yang¹, Y Jia¹, H Zheng¹, Z Fang¹, M Nguyen³, I Ganan-Gomez^{1,5}, B Ebert⁶, R Levine⁷, H Kantarjian¹ and G Garcia-Manero¹

The molecular bases of myelodysplastic syndromes (MDS) are not fully understood. Trimethylated histone 3 lysine 4 (H3K4me3) is present in promoters of actively transcribed genes and has been shown to be involved in hematopoietic differentiation. We performed a genome-wide H3K4me3 CHIP-Seq (chromatin immunoprecipitation coupled with whole genome sequencing) analysis of primary MDS bone marrow (BM) CD34 + cells. This resulted in the identification of 36 genes marked by distinct higher levels of promoter H3K4me3 in MDS. A majority of these genes are involved in nuclear factor (NF)- κ B activation and innate immunity signaling. We then analyzed expression of histone demethylases and observed significant overexpression of the JmjC-domain histone demethylase JMJD3 (KDM6b) in MDS CD34 + cells. Furthermore, we demonstrate that JMJD3 has a positive effect on transcription of multiple CHIP-Seq identified genes involved in NF- κ B activation. Inhibition of JMJD3 using shRNA in primary BM MDS CD34 + cells resulted in an increased number of erythroid colonies in samples isolated from patients with lower-risk MDS. Taken together, these data indicate the deregulation of H3K4me3 and associated abnormal activation of innate immunity signals have a role in the pathogenesis of MDS and that targeting these signals may have potential therapeutic value in MDS.

Leukemia advance online publication, 19 April 2013; doi:10.1038/leu.2013.91

Keywords: myelodysplastic syndromes; H3K4me3; CHIP-Seq; JMJD3; innate immunity

INTRODUCTION

The myelodysplastic syndromes (MDS) are a complex group of myeloid disorders characterized by peripheral blood cytopenias and an increased risk of transformation to acute myelogenous leukemia (AML).¹ Although we have witnessed significant improvements in our ability to diagnose and treat patients with MDS,² the prognosis of a large majority of patients with MDS is still poor.³ Molecular research in MDS is complicated by the absence of cell lines, few available animal models⁴ and the heterogeneous nature of the disease.⁵ The pathogenesis of MDS is the consequence of both genetic and epigenetic lesions, including alterations of DNA methylation and histone modifications.⁶ For instance, a large proportion of genetic mutations identified so far in MDS occur in DNA methylation regulators such as TET2, IDH1/2 and DNMT3A, as well as histone modifiers such as EZH2 and ASXL1.^{7,8} Histone 3 lysine 4 trimethylation (H3K4me3) is one of the best characterized histone marks and is known to be associated with an active gene transcription state.⁹ H3K4 methylation has been shown to be crucial for lineage determination of hematopoietic stem/early progenitor cells (HSPCs).¹⁰ JMJD3 is a JmjC domain protein involved in the control of histone methylation and gene expression regulation.¹¹ As MDS is the result of severely compromised hematopoiesis,¹ we hypothesized that H3K4me3 genomic distribution could be abnormal in the HSPCs of MDS and that its mapping will allow us to gain insight

into the pathophysiology of the disease. To study this, we performed CHIP-Seq (chromatin immunoprecipitation coupled with whole genome sequencing) to compare genome-wide H3K4me3 distribution at gene promoter regions between MDS and normal genomes. Three major findings are reported here. First, we identified 36 genes differentially marked by higher H3K4me3 levels at their promoters in MDS BM CD34 + cells. Second, a majority of these gene products are involved in innate immunity regulation and NF- κ B activation, suggesting a role for the deregulation of innate immunity signaling in MDS. Third, we identified that JMJD3 is involved in the transcription regulation of the genes identified by CHIP-Seq. Inhibition of JMJD3 in cultured primary MDS CD34 + cells isolated from patients with lower-risk MDS resulted in an increased formation of erythroid colonies. These data suggest that MDS CD34 + cells are characterized by deregulation of innate immunity signals and that this information could have prognostic and therapeutic value.

MATERIALS AND METHODS

Cell lines and culture

293 T cells were cultured in Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1% penicillin-streptomycin and 2 mM L-glutamine. OCI-AML3 cells were cultured in RPMI-1640, 10% fetal calf serum and 1% penicillin-streptomycin. All cells were obtained from ATCC (Manassas, VA, USA).

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Isolation and culture of bone marrow (BM) CD34⁺ cells

Human samples were obtained following institutional guidelines. MDS BM specimens were obtained freshly from patients referred to the Department of Leukemia at MD Anderson Cancer Center. Diagnosis was confirmed by a dedicated hematopathologist (CB-R) as soon as the sample was obtained. BMs from healthy individuals were obtained from AllCells (Emeryville, CA, USA). Isolation of CD34⁺ cells was performed using MicroBead Kit (Miltenyi, Bergisch Gladbach, Germany) following manufacturer's instructions. For colony-forming assays, MDS BM CD34⁺ cells were seeded at 10⁴ cells/ml, respectively, in 3.5-cm round culture dishes with methocult GF H4434 (Stem Cell Technology, Vancouver, Canada). Colonies were evaluated after 2 weeks of culture.

Chromatin immunoprecipitation

OCI-AML3 and fresh human BM CD34⁺ cells were cross linked before adding 0.125 M glycine and were processed following published CHIP protocol.⁹ Detailed protocol is described in Supplementary Materials and methods.

CHIP-PCR

Immunoprecipitated DNA was analyzed by quantitative real time PCR (Q-PCR) using the Quanti-Tect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). The amount of DNA fragment co-precipitated with antibody was calculated and compared with the amount of the same genomic fragment in total input DNA, resulting in percentage of input. A list of primers is shown in Supplementary Table S1.

Illumina sequencing of CHIP DNA and data analysis

Concentration of CHIP DNA was calculated using Picogreen DNA quantization kit following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). CHIP DNA libraries were prepared and then sequenced following the manufacturer's protocols (Illumina, San Diego, CA, USA). Sequence reads (<http://www.hgsc.bcm.tmc.edu/collaborations/ruichen/MDS/>) were mapped to the human reference genome (hg18) using BWA (Burrows-Wheeler Aligner).¹² To maximize the sensitivity of enriched region detection, data obtained from MDS patient and normal controls were pooled as patient and control, respectively. Enriched regions in MDS patients and normal controls were identified using MACS (model-based analysis of ChIP-Seq) with default parameters by comparing the pooled ChIP-Seq data to its matching input control.¹³ To ensure specificity, a *P*-value of 10^{−6} was used as cutoff to identify enriched regions across the genome. In-house perl scripts were developed to identify putative promoter peaks that were differently enriched in MDS samples versus normal controls.

Capture deep sequencing of genomic DNA

Illumina paired-end libraries were generated according to the manufacturer's protocol (Illumina). Three micrograms of pre-capture library DNA was used for each capture reaction. NimbleGen SeqCap EZ Hybridization and Wash Kits (Roche, Basel, Switzerland) were used following the manufacturer's protocol. In all, 14–17 cycles of PCR amplification were applied to the samples after hybridization, based on yield. After that, captured libraries were quantified by picogreen and sequenced on the Illumina HiSeq 2000 as 100-bp paired-end reads, following the manufacturer's protocols. Sequencing data were stored as fastq format, which contained reads and quality information. Fastq files were then mapped and aligned to human reference hg18 using BWA.¹² To refine the alignment and adjust the quality score provided by sequencer, genome analysis toolkit was used to realign reads and recalibrate the quality score.¹⁴ Single nucleotide polymorphisms (SNPs) were called using AtlasSNP2 (http://www.hgsc.bcm.tmc.edu/cascade-tech-software_atlas_snp-ti_hgsc). The posterior probability was set as 0.9 and the minimum number of variants reads as 3. To identify rare SNPs, candidate SNPs were filtered against dbSNP130, 1000 genome database and the Human Genome Sequencing Center (Baylor College of Medicine) internal database. Indels were called using AtlasIndel (http://www.hgsc.bcm.tmc.edu/cascade-tech-software_atlas2_snp_indel_calling_pipeline-ti_hgsc).

Quantitative RT-PCR

Total cellular RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Two hundred nanograms of total RNA were used

for reverse transcription (RT) reactions using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's protocol. For real-time PCR, primers and probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7500 Sequence Detection System. PCR reactions were performed using 20 × Assays-On-Demand Gene Expression Assay Mix and TaqMan Universal PCR Master mix according to the manufacturer's protocol. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as internal control.

siRNA transfection

Control siRNA and siRNAs targeting CSAR1, PTAFR, FPR2 and TYROBP genes were purchased from ABI Silencer Select Pre-Designed and Validated siRNA collection (Applied Biosystems). Transfection of OCI-AML3 cells was performed using Lonza nucleofector T kit (Lonza, Basel, Switzerland) with the siRNA pool containing 1 μmol of each siRNA or 4 μmol control siRNA. Cells were collected 24–48 h after transfection.

Recombinant retrovirus-mediated shRNA transduction

pGFP-V-RS plasmids expressing the 29mer shRNA against human JMJD3 or control shRNA were purchased from Origene (Rockville, MD, USA). Recombinant retrovirus expressing shRNA were packaged in 293FT (Invitrogen) and concentrated using the PEG-IT virus precipitation solution (System Biosciences, Mountain View, CA, USA). Transduction of OCI-AML3 and BM CD34⁺ cells with virus was performed by mixing virus with cell suspension followed by centrifugation at 30 °C for 90 min with 4 mg/ml polybrene in medium. After viral transduction, OCI-AML3 cells with stable expression of the shRNA against JMJD3 or a control scrambled shRNA were established in the presence of puromycin (10 μg/ml) in medium.

Immunocytochemical analysis

Immunocytochemical analysis was performed using a cytospin preparation of CD34⁺ enriched mononuclear cells according to standard procedures.¹⁵ The following antibodies were used: serine 276 phosphorylated p65 (p-p65, active form; Cell Signaling Technology, Cambridge, Danvers, MA, USA), and JMJD3 raised in rabbit against affinity-purified GST-JMJD3 (amino acids 798–1095) following previously published method.¹⁶ The antisera were affinity-purified on GST-JMJD3. Photomicrographs were captured using an Olympus BX41 dual head light microscope equipped with an Olympus Q-Color 5 digital camera (Olympus America, Center Valley, PA, USA), with a ×20 plan-apochromat objective. Digital images were obtained and adjusted using Adobe Photoshop CS3 (Adobe, San Jose, CA, USA).

Statistical methods

Overall survival was defined from date of sample to date of death or date last known alive; patients last known alive were censored for this analysis. To investigate associations between gene expression and overall survival, we considered splitting expression level at the 25th, 50th and 75th percentiles, generating three possible binary variables. Associations with clinical and demographic features at the time of sample were assessed only at the median expression level. For ordered clinical features such as the International Prognostic Scoring System (IPSS), the Kruskal–Wallis test was used; for binary features, such as gender, the Fisher's exact test was used. For continuous variables such as percentage of BM blasts or hemoglobin, the Wilcoxon's rank sum test was used.

RESULTS

Mapping of H3K4me3 with CHIP-Seq in primary MDS BM CD34⁺ cells

To study the potential differences in the distribution of H3K4me3 between MDS and normal cells, we compared genome-wide H3K4me3 locations at gene promoter regions of four MDS BM CD34⁺ cell and four normal BM CD34⁺ cell genomes. Patient characteristics are shown in Supplementary Table S2. H3K4me3 signals obtained in each of these eight genomes can be viewed at <http://www.hgsc.bcm.tmc.edu/collaborations/ruichen/MDS>. Overall CHIP-Seq data quality was confirmed by peak specificity analysis using saturation of recovery rate (Supplementary Figure S1A). Genome-wide H3K4me3 peak distribution in these samples

were preferentially located at the 5' end of known genes (Supplementary Figure S1B), which is consistent with previously reported H3K4me3 CHIP-Seq analysis.⁹ To maximize the sensitivity to call MDS-specific high H3K4me3 peaks, reads obtained from four MDS patients and four normal controls were pooled, respectively. Using MACS approach,¹³ 36 gene promoter regions (within 2 kb to transcriptional start site) with differentially higher levels (>four fold) of H3K4me3 in MDS samples were identified (Table 1). Examples of the CHIP-Seq identified H3K4me3 signals in MDS and control CD34+ cells for 11 of these 36 genes are shown in Figure 1a. To confirm the CHIP-Seq results, we randomly selected 11 of these 36 promoters and performed H3K4me3 CHIP-PCR, which confirmed the increased levels of H3K4me3 in MDS samples for all the promoters analyzed (Supplementary Figure S1C). Next, we measured RNA expression for 16 of these 36 genes in MDS CD34+ cells. Number of samples used for the analysis of each gene is listed in Supplementary Table S3. A summary of patient characteristics is shown in Supplementary Table S4. As shown in Figure 1b and in Supplementary Table S3, Q-RT-PCR analysis indicated that all of the 16 genes examined, except for PRX, were significantly overexpressed in MDS CD34+ cells. As expected, these results confirmed the positive correlation between transcription activity and the level of promoter H3K4me3.

Genes associated with distinct high levels of H3K4me3 at their promoters in MDS BM CD34+ cells are involved in innate immunity signaling and nuclear factor (NF)- κ B activation

Twenty-five of the 36 genes identified by CHIP-Seq have previously been reported to be associated with innate immunity regulation (Table 1). Ingenuity pathway analysis indicated that 7 of these 36 genes (C5AR1, FPR2, FCGR2A, MEFV, IL8-RB, TYROBP and PTAFR) encode upstream activators of NF- κ B (Supplementary Figure S2). We confirmed increased levels of promoter H3K4me3 and increased RNA expression in MDS BM CD34+ cells for all these genes (Figures 1b and c). Previous studies have reported NF- κ B activation in MDS in MDS BM CD34+ cells.¹⁷ We performed immunostaining of phosphorylated p65 (p-p65), a component of activated NF- κ B complex,¹⁸ in primary MDS and control BM CD34+ cell cytopins. This staining confirmed increase levels of p-p65 in MDS cells suggestive of NF- κ B activation (Figure 2a). To further study the effect of the CHIP-Seq identified genes on NF- κ B activity, we transfected OCI-AML3 cells with a pool of four siRNAs each individually against C5AR1, FPR2, TYROBP and PTAFR. This led to reduced RNA expression of the four targeted genes by 40–70%, respectively (Figure 2b). In the siRNA-transfected OCI-AML3 cells, reduced intensity of nuclear p-p65 was observed (Figure 2c). These data indicate that these genes are involved in NF- κ B activation.

JMJD3 (KDM6b) is overexpressed in MDS BM CD34+ cells

JMJD3 encodes a JmjC-domain protein with H3K27 demethylase activity and has also been demonstrated to be positively associated with H3K4me3 regulation.^{19,2011} JMJD3 is a known transcriptional target of NF- κ B after innate immunity stimulation.¹⁵ Because multiple of the genes identified by CHIP-Seq are involved in NF- κ B activation, we studied JMJD3 expression in MDS BM CD34+ cells. RNA levels of JMJD3 were examined in a cohort with 121 samples and was found to be overexpressed in 54% of the cases (>two fold increase) (Figure 3a). The average JMJD3 RNA level in the whole cohort was eightfold of control normal CD34+ cell samples ($P<0.0001$; Figure 3c and Supplementary Table S3). To investigate whether overexpression of JMJD3 in MDS CD34+ cells is specific among multiple histone demethylases, we analyzed the RNA expression of 19 other histone demethylases (Supplementary Figure S3A). The only other histone demethylase significantly overexpressed in MDS

CD34+ cells was JMJD1A, but its overall levels of upregulation and statistical significance were both lower than JMJD3 (Supplementary Figure S3B). To further characterize JMJD3 expression in MDS BM CD34+ cells, we performed immuno-histochemical staining of JMJD3 protein in primary BM CD34+ cytopins of patients with MDS ($N=7$) and healthy controls ($N=2$). Five of the seven MDS samples examined had strong JMJD3 signals in the cell nucleus, whereas both control CD34+ specimens had very weak nuclear JMJD3 signals (Figure 3b). These results are consistent with the RNA expression data described above. As JMJD3 has been shown to be expressed in macrophages,¹⁸ and to exclude macrophage contamination in the MDS CD34+ specimens analyzed, we stained cytopins from CD34-enriched MDS BM cells with CD68, a marker of human macrophage lineage.²¹ No CD68 signal was detected (Supplementary Figure S3C).

JMJD3 contributes to form a positive feedback loop between NF- κ B activation and genes identified by CHIP-Seq

To study the relation between JMJD3, NF- κ B activation and genes identified by CHIP-Seq, we transfected OCI-AML3 with siRNAs against C5AR1, FPR2, PTAFR and TYROBP. Together with the reduction in NF- κ B activation shown above (Figure 2b), we also observed a decrease in JMJD3 RNA expression (Figure 3c). This was accompanied by dissociation of the NF- κ B p65 subunit from the consensus NF- κ B-binding site of JMJD3 promoter (Figure 3d). These results indicated that multiple genes identified by CHIP-Seq in MDS could positively regulate the transcription of JMJD3 via NF- κ B.

In activated murine macrophages, genomic distribution of JMJD3 strongly correlates with positive regulation of the H3K4me3 levels at promoter regions, rather than with the changes of H3K27me3.²² Therefore, we studied the relationship between JMJD3 and genes identified by CHIP-Seq. We established an OCI-AML3 cell line that constitutively expresses a shRNA against JMJD3. These cells had a 50% decrease of JMJD3 RNA (Figure 4a). RNA expression for 6 of the 7 genes identified by CHIP-Seq involved in NF- κ B activation (except for MEFV) was reduced (ranging between 35% and 65%) compared with control cells (Figure 4a). Knock down of JMJD3 also led to decrease of NF- κ B p-p65 signal in nucleus OCI-AML3 cells (Supplementary Figure S4 and Figure 4b). To further characterize the impact of JMJD3 on histone methylation at the promoters of these genes, we measured the levels of H3K4me3 and H3K27me3. In JMJD3 knock-down cells, reduced H3K4me3 levels were observed in the promoters of IL8RB, TYROBP and FPR2 (Figure 4c), whereas increased H3K27me3 was detected only in the promoter of IL8RB (Figure 4d). We also observed that overall promoter H3K27me3 signals were weaker than H3K4me3. Finally, in primary MDS BM CD34+ cells, the expression levels of JMJD3 were associated in a positive fashion with the expression levels of the following CHIP-Seq-identified genes: IL8RB ($N=71$, $R=0.5$, $P<0.0001$), TYROBP ($N=80$, $R=0.42$, $P=0.0001$), PTAFR ($N=78$, $R=0.6$, $P<0.0001$) and FCGR2A ($N=58$, $R=0.5$, $P<0.0001$). Taken together, these results suggest that JMJD3 is involved in the transcription upregulation of the genes identified by CHIP-Seq involved in NF- κ B activation.

Deep sequencing of JMJD3 in MDS

To further explore potential alteration of JMJD3 in MDS, we performed capture deep sequencing of the JMJD3 gene and JMJD1A gene in 40 MDS whole BM mononuclear cell specimens (Supplementary Table S3). Two rare single nucleotides (SNPs) of JMJD3 (CCT-TCT: Pro1313-Ser; CCG-TG: Pro642-Leu) and one SNP of JMJD1A (TCT-GCT: Ser423-Ala) were identified (Supplementary Figure S5). However, all three SNPs were also present in BM CD3+

Table 1. Genes identified by CHIP-Seq to associate with high H3K4me3 at promoters in MDS CD34+ cells

Gene	Gene bank No.	CHR	Start end (HG18)	To TSS	Function and association with innate immune or TLR signaling
FCER1G	NM_004106	1	159452113 159453525	403	γ chain receptor of IgE Fc fragment. Synergize with TLR to augment production of inflammatory cytokines ³⁹
GBP5	NM_052942	1	89510023 89510813	-1096	
PTAFR	NM_000952	1	28374310 28375848	-1468	Platelet-activating factor receptor. Mediates recognition and intake of bacterial components ⁴⁰
S100A8	NM_002964	1	151629315 151630287	-858	S100 calcium-binding protein A 8. A chemoattractant and endogenous ligand of TLR4 ⁴¹
FCGR2A	NM_021642	1	159743029 159743795	1186	γ chain of receptor of IgG Fc fragment adaptor ⁴²
S100A9	NM_002965	1	151596426 151598773	-527	S100 calcium-binding protein A 9. A chemoattractant and endogenous ligand of TLR4 ⁴¹
FGR	NM_001042747	1	27823620 27824853	-1718	Src tyrosine kinase ⁴³
RGS18	NM_130782	1	190394360 190395631	146	
NCF2	NM_000433	1	181824396 181826329	-1966	Neutrophil cytosolic factor 2. Component of NADPH oxidase essential for innate immune response ⁴⁴
CD244	NM_016382	1	159098292 159099253	-977	Natural killer cell receptor 2B4. Enhances proliferation of NK cell ⁴⁵
IL8RA	NM_000634	2	218738864 218739249	-1097	CXCR1, IL-8 receptor 1 ⁴⁶
SLC11A1	NM_000578	2	218955150 218956435	155	Solute carrier family 11 ion transporter ⁴⁷
IL8RB	NM_001557	2	218700385 218701692	1395	CXCR2, IL-8 receptor 2 ⁴⁶
LMCD1	NM_014583	3	8517975 8518710	-535	
FYB	NM_199335	5	39254434 39255528	-990	Adaptor protein for tyrosine kinase FYN. Promotes activation NF- κ B ⁴⁸
GIMAP4	NM_018326	7	149895232 149896350	-158	
CLDN15	NM_014343	7	100665881 100667890	-1955	
FCN1	NM_002003	9	136948675 136949763	-955	Ficolin. Activates innate immunity via complement system ⁴⁹
TMEM203	NM_053045	9	139219681 139219933	-230	
IFITM2	NM_006435	11	298793 299540	687	IFN- γ -induced transmembrane protein 2. Mediates antiviral signal ⁵⁰
CLEC7A	NM_022570	12	10172300 10172678	-1835	Dectin, C-type lectin receptor. Activates downstream signaling through Sky/CARD9 ⁵¹
AQP9	NM_020980	15	56219153 56219627	1454	
BCL2A1	NM_004049	15	78049295 78050693	-1403	BCL2-related protein A ⁵²
ELMO3	NM_024712	16	65791023 65791506	495	Engulfment and cell motility protein 3 ⁵³
MEFV	NM_000243	16	3245967 3246996	-661	Pyrin. Modulator of innate immunity ⁵⁴
C5AR1	NM_001736	19	52504812 52505462	-131	C5A receptor. Complement system signaling activator ⁵⁵
FPR1	NM_002029	19	56945476 56946459	-1486	N-formylpeptide chemoattractant receptor 1. G protein-coupled receptor ⁵⁶
FPR2	NM_001005738	19	56956243 56956574	-21	N-formylpeptide chemoattractant receptor 2. G protein-coupled receptor ⁵⁶
PRX	NM_181882	19	45610885 45611447	-226	
C19orf33	NM_033520	19	43486092 43486480	-551	
FCAR	NM_133277	19	60078907 60079291	1547	Receptor for IgA Fc fragment ⁵⁷
LRG1	NM_052972	19	4490259 4491216	-777	
TYROBP	NM_003332	19	41090039 41091378	-987	ITAM-containing innate immunity signal mediator ⁵⁸
CFD	NM_001928	19	811439 812250	775	Complement factor D, C3 convertase activator ⁵⁹
C2orf54	NM_033409	20	697002 697468	-226	
CD93	NM_012072	20	23013407 23015099	-1570	Cell-surface glycoprotein and type I membrane protein. A receptor for C1q, can also be shedded and become a TLR ligand ⁶⁰

Abbreviations: BCL2, B-cell lymphoma 2; CARD, caspase-associated recruitment domain; CHIP-Seq, chromatin immunoprecipitation coupled with whole genome sequencing; CHR, chromosome; CXCR1, C-X-C motif chemokine receptor; H3K4me3, histone 3 lysine 4 trimethylation; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; ITAM, immunoreceptor tyrosine-based activating motif; MDS, myelodysplastic syndromes; NADPH, nicotinamide adenine dinucleotide phosphate; NK, natural killer; TLR, Toll-like receptor; TSS, transcriptional start site.

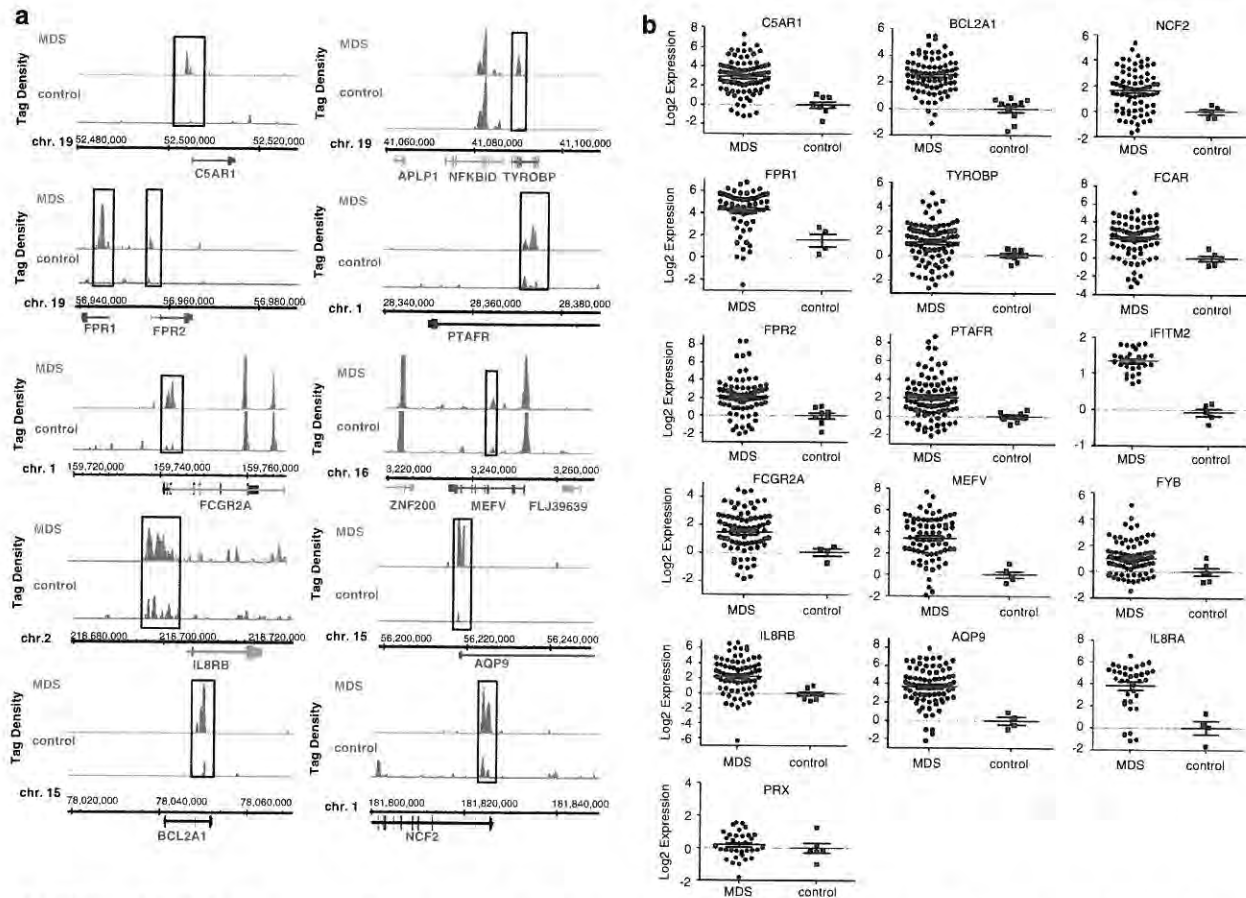


Figure 1. H3K4me3 CHIP-Seq in primary MDS bone marrow CD34⁺ cells. (a) Representative examples of H3K4me3 identified by CHIP-Seq in 11 genes in MDS (top) and control (bottom) BM CD34⁺ cells. Comparison between MDS and control samples indicate increased levels of H3K4me3 in the promoters of these genes in MDS CD34⁺ cells. (b) Logarithmic representation of Q-RT-PCR results of 16 genes identified by CHIP-Seq in MDS and control CD34⁺ cells. These results confirm increased RNA expression in genes marked by higher levels of promoter H3K4me3 in MDS.

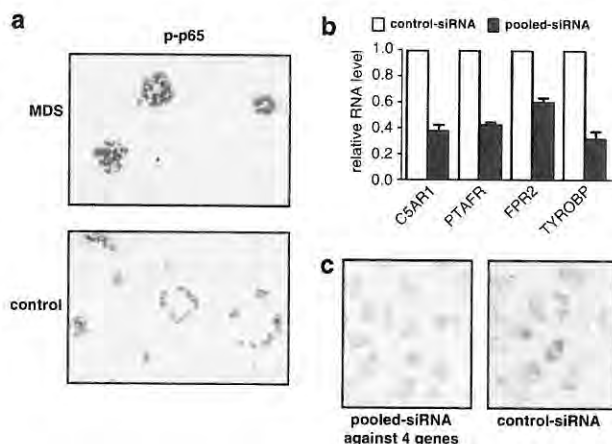


Figure 2. Genes identified by CHIP-Seq are involved in NF- κ B activation in MDS CD34⁺ cells. (a) Immuno-histochemical analysis of phospho-p65 in MDS (top panel) and controls (bottom panel) bone marrow CD34⁺ cells. (b) Knock down of C5AR1, FPR2, TYROBP and PTAFR in OCI-AML3 cells. (c) Immuno-histochemical analysis of phospho-p65 in the OCI-AML3 cells after knock down of the four genes described above (left panel) in comparison to control siRNA-transfected cells (right panel).

T cells from the same patients and therefore unlikely to be somatic.

Inhibition of JMJD3 positively regulates erythroid colony-forming units (CFU-E) formation in MDS BM CD34⁺ cells

Anemia is one of the most common clinical manifestations of MDS. Erythroid colony formation is known to be decreased in cultured MDS BM CD34⁺ cells.^{23,24} We observed that BM CD34⁺ cells isolated from lower-risk (IPSS low-risk and intermediate-1) and higher-risk (IPSS intermediate-2 and high-risk) MDS both had significantly lower numbers of CFU-E in methylcellulose medium (methocult)-supported clonogenic assays (Supplementary Figure S6A). We also used methocult assays to examine the impact of JMJD3 inhibition on the hematopoietic potential of MDS CD34⁺ cells. We transduced primary BM CD34⁺ cells isolated from patients with newly diagnosed lower-risk MDS ($N=4$) and higher-risk MDS ($N=4$) with a recombinant retroviral-mediated shRNA against JMJD3. Patient characteristics are described in Supplementary Table S4. JMJD3 shRNA resulted in reduced levels of JMJD3 RNA expression in primary MDS BM CD34⁺ cells examined (Supplementary Figure S6C). After 2 weeks of culture in methocult medium, discrepant effects on colony formation were observed between lower-risk and higher-risk samples. Three out of four lower-risk samples had an increased number of CFU-E after

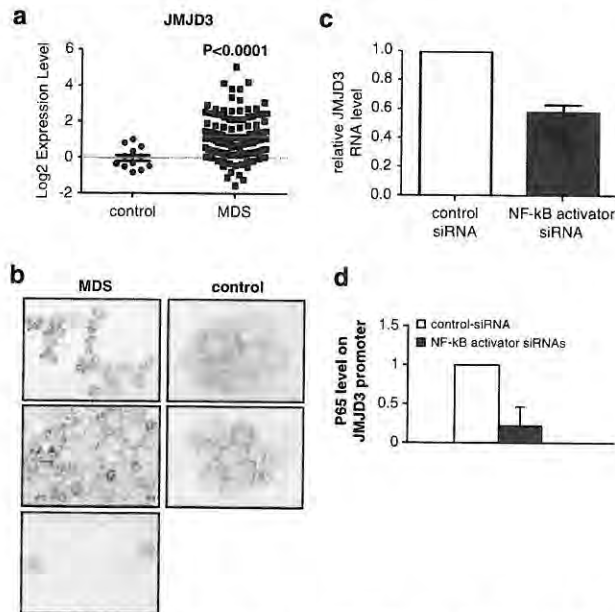


Figure 3. JMJD3 expression in MDS BM CD34+ cells. **(a)** Logarithmic representation of JMJD3 Q-RT-PCR results in MDS and control CD34+ cells in 121 MDS CD34+ samples. **(b)** Immuno-histochemical analysis of JMJD3 in cytopins of MDS (left three panels: Top two with strong JMJD3 staining and bottom one with weak JMJD3 staining) and controls (right two panels: both with weak JMJD3 staining) bone marrow CD34+ cells. **(c)** Q-RT-PCR analysis of JMJD3 RNA expression in OCI-AML3 cells transfected with siRNAs targeting C5AR1, FPR2, TYROBP and PTAFR or controls. **(d)** p65 CHIP-PCR analysis of JMJD3 promoter in the OCI-AML3 cells transfected with the siRNAs targeting C5AR1, FPR2, TYROBP and PTAFR or controls.

JMJD3 shRNA transduction (Figure 5a). On average, after JMJD3-shRNA transduction, 73 CFU-E were formed per 10^4 MDS BM CD34+ cells plated, which was a 52% increase compared with the number of CFU-E formed after control-shRNA transduction (Figure 5a). No significant effect on the formation of myeloid colonies was observed after JMJD3 inhibition (Figure 5a). Representative images of the methocult colonies formed from one low-risk sample are shown in Figure 5b. In contrast to the observations in lower-risk MDS, we did not observe positive effect on the formation of erythroid or myeloid colonies in any of the four BM CD34+ cells isolated from patients with higher-risk MDS (Supplementary Figure S6D). To further verify the effect of JMJD3 inhibition in lower-risk samples, we measured transcripts of several genes known to be positively associated with erythroid differentiation, including Glycophorin-A (GYPA), CD71 and EPOR. In the cells collected from methocult colonies, expression of all the three genes was increased after JMJD3 inhibition (Figure 5c). The most significant increase was observed in GYPA. The effect on gene expression further confirmed the positive impact of JMJD3 inhibition on the differentiation of erythroid lineage in MDS BM CD34+ cells of lower-risk type of MDS.

Potential clinical implications of CHIP-Seq-identified genes in MDS

We performed an exploratory analysis of clinical implications of expression of genes identified by CHIP-Seq in MDS BM CD34+ cells. A number of the genes analyzed showed potential correlation with overall survival. This included NCF2, AQP9 and MEFV (Figures 6a–c). We also explored the association between these genes and IPSS. PTAFR ($P=0.05$), NCF2 ($P=0.058$), AQP9 ($P=0.017$), MEFV ($P=0.012$) and FCAR ($P=0.019$), dichotomized at their median expression levels, were associated with risk by IPSS score.²⁵ These results are exploratory and need to be studied in larger cohorts.

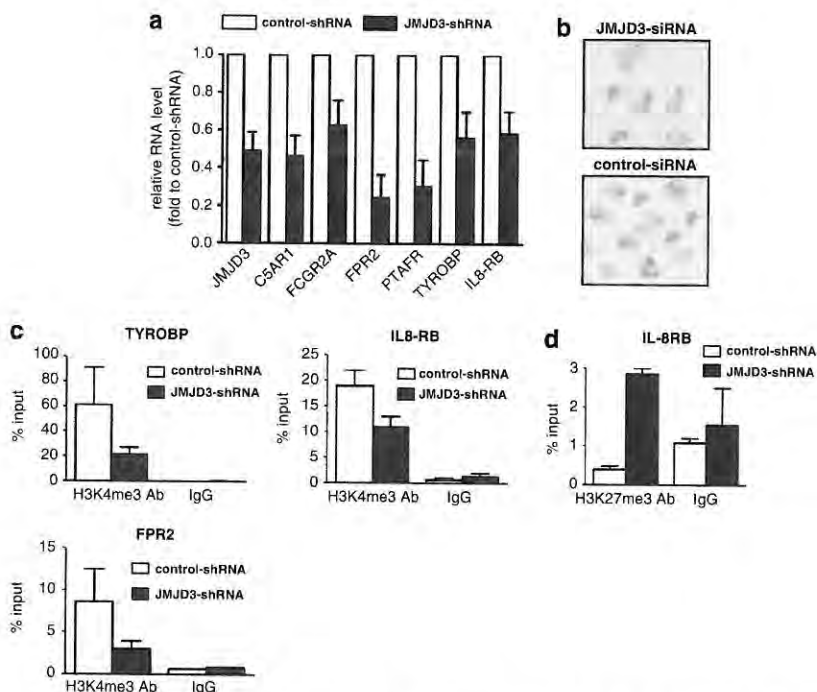


Figure 4. JMJD3 is involved in transcriptional regulation of genes identified by CHIP-Seq. **(a)** Q-RT-PCR analysis of RNA expression of six genes involved in NF- κ B activation in OCI-AML3 cells after JMJD3 knock down. **(b)** Immunohistochemical analysis of phospho-p65 in the OCI-AML3 cells with JMJD3 knock down (top panel) and in control cells (bottom panel). **(c)** H3K4me3 CHIP-PCR analysis of IL8RB, TYROBP and FPR2 in the OCI-AML3 cells after JMJD3 knock down. **(d)** H3K27me3 CHIP-PCR analysis of IL8RB in the OCI-AML3 cells after JMJD3 knock down.

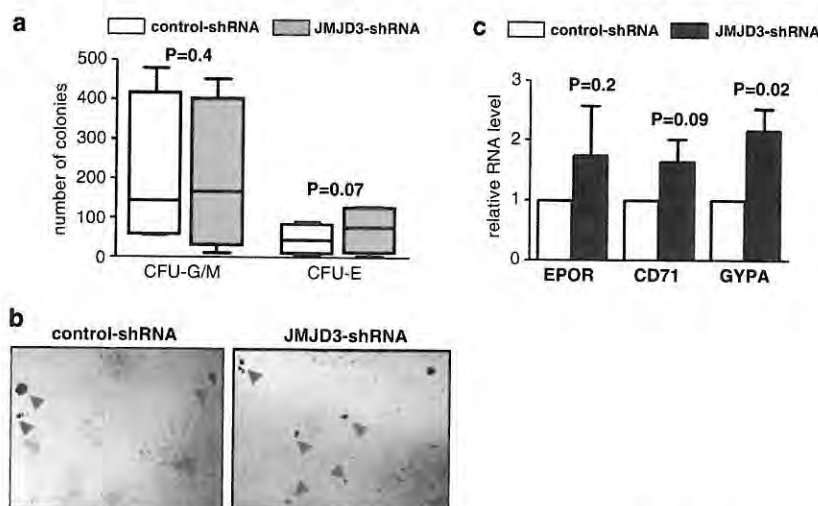


Figure 5. Effect of JMJD3 shRNA transduction in cultured MDS bone marrow CD34⁺ cells. **(a)** Numbers of myeloid colonies (CFU-G/M) and erythroid colonies (CFU-E) formed in methocult culture 2 weeks after transduction of JMJD3-shRNA and control shRNA in BM CD34⁺ cells isolated from patients with lower-risk MDS (low-risk and intermediate-1). **(b)** Representative microphotographs of colonies formed in methocult plates after transduction of control shRNA (left panel) and JMJD3-shRNA (right panel). Red arrows point to CFU-E. **(c)** Q-RT-PCR analysis of the RNA levels of CD71, EPOR and GYPA in cells collected from total colonies after shRNA transduction and methocult assays.

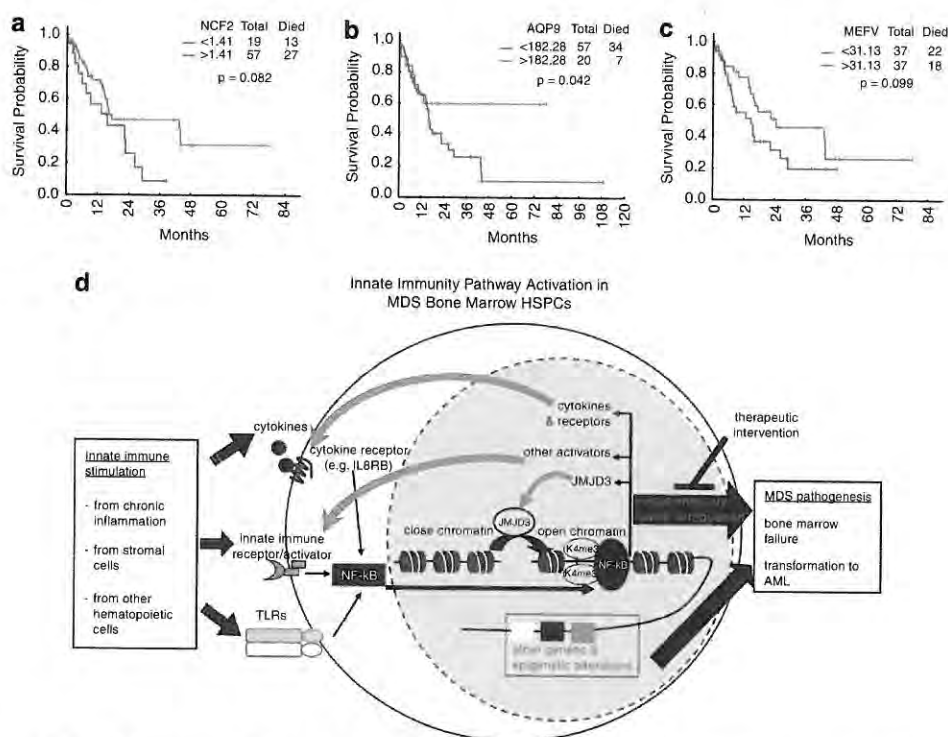


Figure 6. Survival impact of gene expression in MDS and proposed model of implications of innate immunity signaling in MDS. **(a–c)** Effect of mRNA expression of NCF2, AQP9 and MEKV on survival of patients with MDS. **(d)** Innate immunity stimulation derived from stroma, chronic inflammation or hematopoietic cells either in a para or autocrine fashion result in NF-κB activation in MDS CD34⁺ cells. NF-κB activation then triggers expression of multiple other effectors such as cytokines and importantly JMJD3. JMJD3 is a histone demethylase that contributes to the perpetuation of innate immunity signal and NF-κB activation. Signaling via this pathway probably cooperates with other known genetic and epigenetic lesions known to occur in MDS cells and contribute to bone marrow failure and transformation to AML that characterized MDS. Further analysis of this pathway could result in the development of inhibitors with therapeutic potential.

DISCUSSION

It has been demonstrated that the regulation of histone methylation/demethylation affects a wide range of essential biological processes, such as early development of embryonic

stem cell.²⁶ H3K4 methylation has been shown to impact lineage determination during normal hematopoietic differentiation.¹⁰ Based on this and because MDS is the result of BM hematopoietic stem cell dysfunction, we hypothesized that the

abnormalities of H3K4 methylation could contribute to MDS pathogenesis. In this study, we used genome-wide CHIP-Seq to map H3K4me3 distribution in MDS BM CD34+ cells. To our knowledge, this is the first reported use of this technology in primary samples of MDS. This analysis resulted in the identification of 36 genes characterized by increased levels of H3K4me3 in their promoters. As expected, there was a strong correlation between the CHIP-Seq-identified H3K4me3 increase and gene overexpression in MDS CD34+ cells.

The large majority of genes identified by CHIP-Seq are involved in the activation of innate immunity signaling. Of note, overexpression of several innate immunity regulatory genes in CD34+ cells of MDS has been previously reported.²⁷ The innate immune system is the first line of defense against pathogens.²⁸ Recently, emerging evidence has suggested a direct regulation of HSPCs by innate immunity signaling.²⁹ For instance, previous studies have indicated that the Toll-like receptors are expressed in primitive hematopoietic cells, and that acute Toll-like receptor stimulation alters myeloid/lymphoid ratios, whereas chronic stimulation results in stem cell exhaustion and BM failure.^{30,31} A small number of studies have also indicated a role for innate immunity deregulation in MDS. For instance, mir-145 and mir-146a have been demonstrated to regulate expression of TRAF-6, a key innate immunity regulator, in a model of del5q MDS.²⁹ The identification of deregulation for H3K4me3 and activation of innate immunity signal suggests that they have a potential interaction in HSPCs of MDS that should be further investigated. In this study, we provide evidence that JMJD3 may potentially regulate this interaction. JMJD3 is an epigenetic regulator and is also a known transcription target of NF- κ B after innate immune stimulation.²² The identification of JMJD3 overexpression further supports that in MDS CD34+ cells innate immunity and NF- κ B signaling are activated. Furthermore, its involvement in the positive regulation of multiple genes involved in NF- κ B activation suggests that JMJD3 forms a feedback loop between innate immunity effectors and the activation of NF- κ B. Mechanistically, we provide evidence that this feedback loop mediated by JMJD3 is associated with epigenetic regulation. After JMJD3 knock down, decreased levels of H3K4me3 were observed at the promoters of three of the involved genes, and one gene promoter (IL-8RB) presented detectable alterations of H3K27me3. These results are consistent with previous reports that although JMJD3 is an H3K27 demethylase, it is also a component of complexes harboring H3K4me3 methyltransferase activity.¹⁹ Furthermore, genomic distribution of JMJD3 coincides with H3K4me3 in transcriptional start site of macrophages.²² JMJD3 has also been shown to positively regulate gene transcription through histone methylation-independent mechanisms.^{32,33} Therefore, mechanisms by which JMJD3 regulates NF- κ B in MDS need to be further investigated.

JMJD3 has been described as essential for cell lineage determination during cell differentiation, including macrophage, skin cells and neurons.^{19,2011} In this study, we have shown that knock down of JMJD3 in BM CD34+ cells of lower-risk type of MDS resulted in increased erythroid differentiation, potentially the formation of erythroid blasts as suggested by increased GYPA expression.²³ This result suggests that JMJD3 can affect hematopoietic lineage determination. Furthermore, this result also has therapeutic implications as anemia is one of the most common clinical presentations of patients with MDS. In contrast to the observations in lower-risk samples, JMJD3 inhibition did not affect erythroid differentiation of BM CD34+ cells of patients with higher-risk MDS. This discrepancy suggests that other molecular lesions cooperate with the deregulation of JMJD3 and innate immunity signals to contribute in the progression of MDS (Figure 6). Although results of JMJD3 inhibition in MDS BM CD34+ cells in this study should be considered exploratory and need to be validated in a larger cohort of primary MDS patient

samples, they open new possibilities for the testing of potential new JMJD3 and/or innate immunity signal inhibitors in MDS. For instance, a new JMJD3 inhibitor has recently been described.³⁴

Finally, the observation that several of the genes described here may have potential prognostic value reinforces the importance of these results for the identification of patients with MDS at different risk. These results should be considered exploratory and need to be validated in a larger cohort of patients. Larger studies are needed to correlate potential associations between innate immunity deregulation and specific clinical and molecular alterations in MDS.

We realize that there are several limitations to this study. First, we have only analyzed one histone mark using genome-wide CHIP-Seq in MDS BM CD34+ cells. It is known that gene expression is regulated by a complex set of chromatin modifications.³⁵ For instance, it is well established that poised promoters in embryonic stem cells contain both H3K4me3 and H3K27me3³⁶ and that H3K4me2 is also critical for hematopoietic cell differentiation.¹⁰ Technically, it is still not possible to perform an analysis of multiple histone modifications in MDS CD34+ cells due to the very limited amount of cells available from an individual patient. Second, it has been shown that genetic manipulation of BM osteoprogenitor cells can result in an MDS phenotype in mice³⁷ and that MDS mesenchymal cells are abnormal.³⁸ Therefore, other cell populations that contribute to the pathogenesis of MDS, besides CD34+ cells, should also be comprehensively analyzed. Regarding the role of JMJD3 in the regulation of multiple genes identified by CHIP-Seq, the presence of JMJD3 in the promoters of these genes still needs to be analyzed. Unfortunately, despite previous reports, there are no optimal antibodies for the CHIP of JMJD3 in human samples, particularly in primary BM samples. Finally, we need to characterize the effect of enforced overexpression of JMJD3 in this setting. This is complicated due to the large size of JMJD3 gene and also to the lower transgene efficiency in MDS/AML cells compared with other cell types.

In summary, these results suggest that a deregulation of innate immunity signal is common in the BM stem/progenitor cells of MDS and may contribute to the pathogenesis of the disease. We propose a model in Figure 6. We also provide initial evidence that further characterization of JMJD3 and associated innate immunity-activating signals may have prognostic and therapeutic benefits in MDS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Overexpression of the Toll-Like Receptor (TLR) Signaling Adaptor MYD88, but Lack of Genetic Mutation, in Myelodysplastic Syndromes

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Abstract

MYD88 is a key mediator of Toll-like receptor innate immunity signaling. Oncogenically active MYD88 mutations have recently been reported in lymphoid malignancies, but has not been described in MDS. To characterize MYD88 in MDS, we sequenced the coding region of the MYD88 gene in 40 MDS patients. No MYD88 mutation was detected. We next characterized MYD88 expression in bone marrow CD34+ cells (N=64). Increased MYD88 RNA was detected in 40% of patients. Patients with higher MYD88 expression in CD34+ cells had a tendency for shorter survival compared to the ones with lower MYD88, which was significant when controlled for IPSS and age. We then evaluated effect of MYD88 blockade in the CD34+ cells of patients with lower-risk MDS. Colony formation assays indicated that MYD88 blockade using a MYD88 inhibitor resulted in increased erythroid colony formation. MYD88 blockade also negatively regulated the secretion of interleukin-8. Treatment of MDS CD34+ cells with an IL-8 antibody also increased formation of erythroid colonies. These results indicate that MYD88 plays a role in the pathobiology of MDS and may have prognostic and therapeutic value in the management of patients with this disease.

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Introduction

The myelodysplastic syndromes (MDS) are a complex group of myeloid disorders characterized by peripheral blood cytopenias, ineffective bone marrow hematopoiesis, and increased propensity of transformation to acute myelogenous leukemia (AML) [1]. Recent use of advanced DNA sequencing technologies has allowed the identification of multiple genetic lesions in MDS [2]. Despite these advances, the molecular pathogenesis of MDS remains unclear. The innate immunity system is well known as a conserved host defence mechanism that detects and eliminates pathogens [3]. Activation of innate immune signaling pathways can be initiated through the stimulation of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) [4], with conserved molecular patterns of microorganisms. These signals are mediated via downstream signaling mediators and eventually lead to activation of key intracellular molecular effectors such as NF- κ B and MAPK. The resulting immune responses, including release of inflammatory cytokines, cause elimination of pathogens. Although innate

immunity responses are mediated mostly by phagocytes such as macrophages and dendritic cells, emerging evidence has suggested that innate immune signalling activation can also directly impact hematopoietic stem and early progenitor cells (HSPCs) [5,6] and may be involved in the pathogenesis of MDS [7]. For instance, mir-145 and 146a are two microRNAs that have been shown to target the innate immune signal adaptors TIRAP and TRAF6 respectively [7]. Loss of these two microRNAs is involved in the 5q- syndrome subtype of MDS and overexpression of TRIAP and TRAF6 is associated with transformation to acute leukemia or marrow failure in a murine transplant system [8]. TRIAP and TRAF6 are both known to mediate MYD88 (Myeloid differentiation gene 88) dependent innate immune signals [4]. MYD88 mediated signaling is common to all Toll-like Receptors (TLR) except for the TLR3 pathway [9]. Of importance, oncogenically active MYD88 mutations have recently been identified as recurrent genetic lesions in chronic lymphocytic leukemia (CLL), B-cell lymphoma and Waldenström's macroglobulinemia [10–12]. To evaluate if MYD88 also plays a pathological role in myeloid

neoplasia, we studied MYD88 in primary samples of patients with MDS, including MYD88 mutation analysis in bone marrow mononuclear cells and the characterization of MYD88 RNA expression in bone marrow CD34+ cells and also investigated the impact of MYD88 blockade and downstream inflammatory interleukin IL-8 [13] in primary MDS CD34+ cells cultured in vitro.

Materials and Methods

MYD88 Gene Pyrosequencing Analysis

Pyrosequencing analysis was performed in 38 patients with MDS. Exons 3 and 4 of MYD88 were amplified by polymerase chain reaction using primers listed on **Table S1**. These primers were chosen based on published data [10–12]. For pyrosequencing assay, the reverse primer was biotinylated. This biotinylated strand was captured on streptavidin sepharose beads (Amersham Biosciences, Uppsala, Sweden) and annealed with a sequencing primer. Pyrosequencing was performed using PSQ HS 96 Gold SNP reagents and the PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). Programmed polymorphic sites were set at specific nucleotides (see table below) to detect any mutations. Mutations were detected as abnormal program patterns (pyrosequencing peak).

MYD88 Gene Barcode PCR-deep Sequencing Analysis

The complete coding region of MYD88 gene was amplified using ten pairs of PCR primers in 40 patients with MDS (38 described above and two additional ones). Characteristics of these patients are listed in **Table 1**. First round PCR products were then amplified in 2nd round PCR using universal primers with Illumina adaptor and 40 patient-specific barcode sequences. All PCR products were then pooled together and sequenced using the Illumina HiSeq 2000 (Illumina, San Diego CA). All PCR primers are listed in **Table S2**. MYD88 sequencing dataset can be accessed at NIH Short Read Archive using ID SRP026064.

Real-Time RT-PCR

Total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For each sample, 200 ng of total RNA were used for reverse transcription (RT) reactions using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol. For real-time PCR, primers and probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7500 Sequence Detection System. PCR reactions were performed using 20×Assays-On-Demand Gene Expression Assay Mix and TaqMan Universal PCR Master mix according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Quantitative values were obtained from the cycle number (C_T value), at which an increase in fluorescent signal associated with an exponential accumulation of PCR products was detected. The amount of target gene was normalized to the endogenous reference GAPDH to obtain the relative threshold cycle (ΔC_T) and then related to the C_T of the control to obtain the relative expression level ($2^{-\Delta\Delta C_T}$) of target gene.

Cell Lines and Reagents

KG1 cells were obtained from ATCC (Manassas, VA) and were cultured in IMDM, 20% fetal calf serum and 1% penicillin-streptomycin. TLR-agonists MALP2 (2 µg/ml) and PAM3CSK4 (0.1 µg/ml) were purchased from Invivogen (San Diego, CA). MYD88 peptide inhibitor (Pepinh-MYD) or peptide control

Table 1. Clinical Characteristics of Patients in MYD88 Mutation Analysis.

Characteristics (N = 40)	No. of Patients	%
Age, years		
Median	68	
Range	28–84	
Sex		
Male	24	60
Female	16	40
BM blast, %		
Median	5	
Range	0–33	
WBC, $\times 10^3/\mu\text{L}$		
Median	3	
Range	0.8–116.1	
Hemoglobin, g/dL		
Median	10	
Range	6.8–15	
Platelet, $\times 10^3/\mu\text{L}$		
Median	95	
Range	1–488	
Neutrophil, $\times 10^3/\mu\text{L}$		
Median	52	
Range	0–85	
Prior Malignancy		
Yes	11	27.5
No	29	72.5
Prior Chemotherapy		
Yes	6	15
No	34	85
Prior Radiotherapy		
Yes	2	5
No	38	95
Dx		
RARS/RCMD/RCMD-Rs	8	20
RAEB	13	32.5
CMML	3	7.5
AML/AMML	5	12.5
MPD	3	7.5
other	8	20
IPSS		
H	4	15
I-2	9	35
I-1	10	38
L	3	12
NA	14	
Cytogenetics		
Dip	23	57.5
5q-	3	7.5
other	11	27.5
IM	3	7.5

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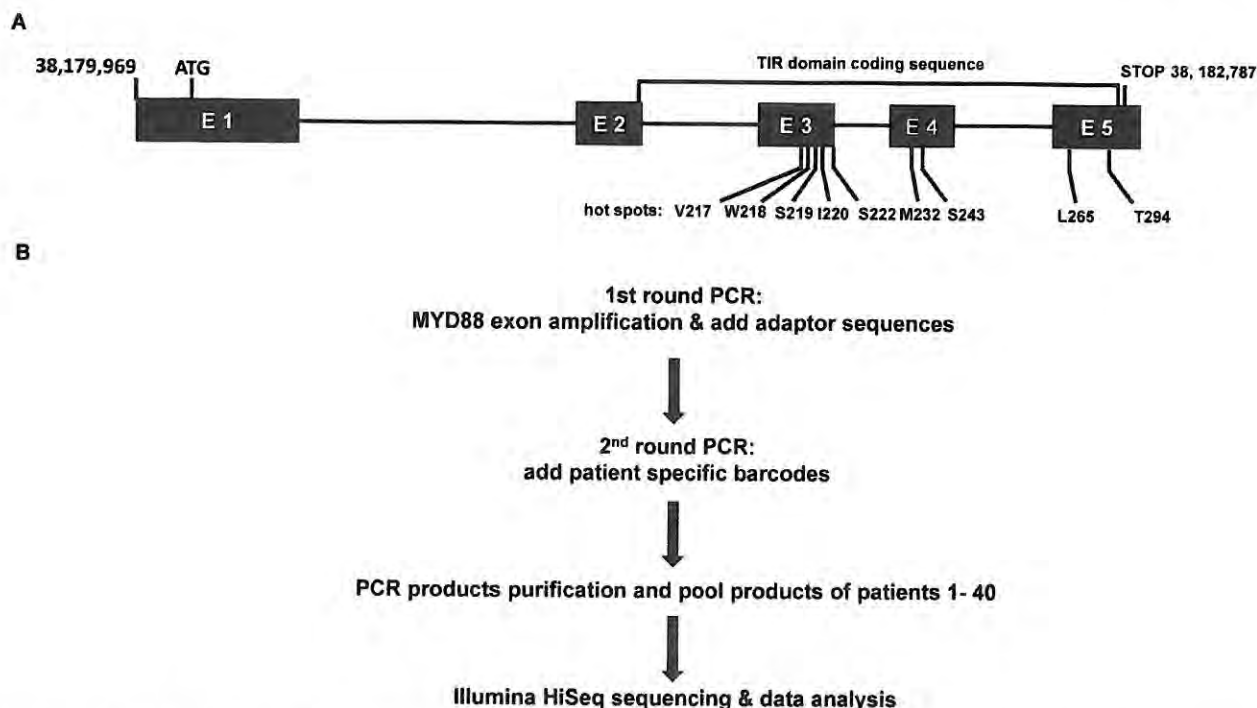


Figure 1. Barcode PCR-deep sequencing analysis MYD88 gene in bone marrow samples of 40 patients with MDS. (A) Genomic Sequence of MYD88 is analyzed in this study. Coding regions are illustrated by capitalized characters, and locations of 10 pairs of primers used in the 1st round of PCR are highlighted in different colors. Sequence encoding the TIR domain is underlined. First codon (ATG) and stop-codon are squared by red lines. **(B)** Graphic illustration of the barcode PCR-deep sequencing approach used in this study.
doi:10.1371/journal.pone.0071120.g001

(5 μ M) were purchased from Invivogen (San Diego, CA). IL-8 neutralization antibody (10–20 μ g/ml) was purchased from ABCAM (Cambridge, MA).

Isolation and Culture of Bone Marrow CD34⁺ Cells

MDS bone marrow specimens were obtained freshly from patients referred to the Department of Leukemia at MD Anderson Cancer Center following protocol PA12-0445, which is approved by Institutional Review Board (IRB) 5 of MD Anderson Cancer Center. Written informed consent from donors were obtained for use of this sample in research. Diagnosis was confirmed by a dedicated hematopathologist as soon as sample was obtained. Bone marrows from healthy individuals were obtained from AllCells (Emeryville, CA). CD34⁺ cells were isolated from fresh bone marrow specimens of patients with MDS using MicroBead Kit (Miltenyi, Bergisch Gladbach, Germany) following manufacturer's instructions. Primary bone marrow CD34⁺ cells were cultured in IMDM (Gibco-Invitrogen), 20% BIT 9500 (bovine serum albumin, insulin, transferrin) (Stem Cell Technology, Vancouver, Canada), human thrombopoietin (hTPO) 50 ng/ml (Stem Cell Technology), IL3 10 ng/ml (Stem Cell Technology), Stem cell factor (SCF) at 100 ng/ml (Stem Cell Technology), FLT3L at 100 ng/ml (Stem Cell Technology). For colony forming assays, healthy and MDS BM CD34⁺ cells were seeded at 1000 cells/ml and 10000 cells/ml respectively in 3.5 cm round culture dishes with methocult GF H4434 (Stem Cell Technology, Vancouver, CA). Colonies were counted after two weeks of culture.

NF- κ B Activity Evaluation by Luciferase Assay

KG1 cells were transfected with pGL4.32[luc2P/NF- κ B-RE] (Promega, Madison, WI) using the Nucleofector Device and Nucleofector T kit (Lonza, Basel, Switzerland). These cells were pre-treated with 5 μ M MYD88 peptide inhibitor or peptide control (Invivogen, San Diego, CA). pRL-TK Vectors expressing the Renilla luciferase was also cotransfected as internal control. One day after transfection, TLR2 agonist MALP2 (2 μ g/ml) or PAM3CSK4 (0.1 μ g/ml) (Invivogen, San Diego, CA) was added into medium. Luciferase assays were performed 20 hours later with the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) on 3010 luminometer (BD Biosciences, San Diego, CA). NF- κ B activity was calculated by the ratio of firefly luminescence from the pGL4.32[luc2P/NF- κ B-RE] reporter to the renilla luminescence from the control pRL-TK Vectors reporter.

ELISA

IL-8 protein level in bone marrow plasma and in culture medium was analyzed using Human CXCL8/IL-8 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

Statistical Methods

Descriptive statistics were used for baseline characteristics. Non-parametric analyses were used; binary variables were compared using the Fisher's exact test, the Kruskal-Wallis test was used for categorical variables, and continuous variables by the Wilcoxon rank sum test. MYD88 levels were evaluated as a continuous and categorical/dichotomous variable, using expression levels at the 25th, 50th and 75th percentiles. Associations were considered of potential interest if the *p*-value was less than 0.10. Overall survival (OS) was defined from the date of sample acquisition to date of

Table 2. Clinical Characteristics of Patients in MYD88 RNA Expression Analysis.

Characteristics (N= 64)	No. of Patients	%
Age, years		
Median	69	
Range	33–89	
Sex		
Male	22	34
Female	42	66
BM blast, %		
Median	7	
Range	1–26	
WBC, $\times 10^3/\mu\text{L}$		
Median	3	
Range	0.8–67	
Hemoglobin, g/dL		
Median	10	
Range	7–14.2	
Platelet, $\times 10^3/\mu\text{L}$		
Median	67	
Range	8–488	
Neutrophil, $\times 10^3/\mu\text{L}$		
Median	47	
Range	0–86	
Prior Malignancy		
Yes	24	37
No	40	63
Prior Chemotherapy		
Yes	10	15
No	54	85
Prior Radiotherapy		
Yes	3	5
No	61	95
Dx		
RA/RCMD/RCMD-Rs/MDS-U	27	40
RAEB	28	43
CMML	9	14
IPSS		
H	9	13.8
I-2	18	27.7
I-1	26	41.5
L	11	16.9
Cytogenetics		
Dip, -Y	28	44.6
5/7-	15	23.1
other	18	27.7
IM	3	4.6

doi:10.1371/journal.pone.0071120.t002

death or date of last follow-up (censored). Kaplan-Meier analysis was used to construct OS curves; curves were compared by log-rank test. An adjusted Cox proportional hazards model was

constructed using clinically important variables to examine the effect of potential confounders. We have updated this in the methods section.

Results

Absence of Genetic Mutations in the Coding Region of MYD88 Gene in 40 Patients with MDS

Mutations of MYD88 have been identified in CLL [10], B cell lymphoma [11,12] and Waldenström's macroglobulinemia¹², which affect several conserved amino acids in the intracellular Toll/Interleukin-1 receptor (TIR) domain (Figure 1A). We first examined these previously described “hot spots” of MYD88 in MDS using pyrosequencing approach. Pyrosequencing was performed in whole bone marrow mononuclear cells isolated from 38 patients of MDS. Characteristics of these patients are described in Table 1. Sequenced codons include: V217, W218, S219, I220, S222, M232, S243, L265, and T294. Using this technique, no mutation was detected in any of the 38 MDS samples analyzed. We then expanded the sequencing efforts by using an approach that combines bar-code PCR amplification and Illumina parallel sequencing (Figure 1B). Characteristics of these patients are listed in Table 1. Eleven of these patients had prior malignancies, including one with prior Burkitt's lymphoma in remission. The entire coding region of MYD88 of 40 MDS samples, including the 38 samples used in pyrosequencing, were amplified and sequenced. No genetic mutation of MYD88 was detected in these 40 patients with this approach either.

MYD88 is Overexpressed in Bone Marrow CD34+ Cells of MDS

We examined RNA expression levels of MYD88 in bone marrow CD34+ cells of patients with MDS. Analysis was performed in a cohort of 64 patients (Table 2). Characteristics of these patients are described in Table 2. Eighteen of these samples were also used for the sequencing analysis of MYD88 described earlier. We compared their MYD88 expression to 7 control normal CD34+ samples. Quantitative RT-PCR indicated that 27% of MDS patients (N = 17) had a more than 2 fold increase of MYD88 RNA, and 14% (N = 9) had a 1.3–1.9 fold increase. In total 41% (N = 26) of patients overexpress MYD88 (Figure 2A). Among these 64 patients, 35 patients (54%) were previously untreated MDS and the other 29 (45%) patients had received prior MDS treatments at the point of sample collection. Both untreated and treated patients showed increased MYD88 expression compared to controls (Figure S1A). We also analyzed MYD88 expression in these 64 patients based on their IPSS scores. MYD88 is overexpressed in patients with lower-risk (low-risk and INT-1) MDS compared to higher-risk (INT-2 and high-risk) group ($p = 0.1$) (Figure S1B).

MYD88 Expression and Patient Survival in MDS

We next evaluated correlations between MYD88 expression and patient characteristics in 62 of the 64 samples with available complete clinical information. Patients with higher MYD88 RNA expression in bone marrow CD34+ cells (split by median value) had a propensity for shorter survival ($p = .09$, HR 1.9, 95% CI 0.89–4.26) (Figure 2B). Of interest, higher MYD88 expression correlated significantly with shorter overall survival in the multivariate model adjusted for IPSS risk score and patient age ($p = .027$, HR 2.46, 95% CI 1.1–5.45). These results suggest that MYD88 expression level may hold potential prognostic value in MDS, independent of known prognostic risk factors.

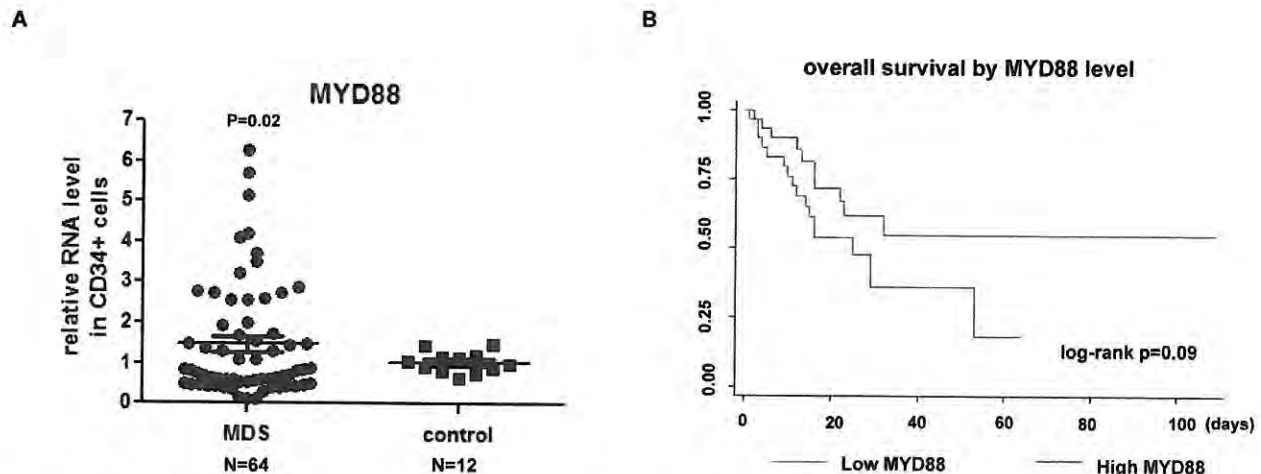


Figure 2. Analysis of MYD88 RNA expression in bone marrow CD34+ cells of patients with MDS. (A) Q-PCR results of MYD88 RNA in MDS and control CD34+ cells. **(B)** Impact of MYD88 RNA level in overall MDS patient survival.
doi:10.1371/journal.pone.0071120.g002

Blockade of MYD88 in MDS CD34+ Cells

To study the role of MYD88 signaling in MDS, we used a 26-amino acid inhibitory peptide that blocks MYD88 homodimerization [14] [15]. Due to the lack of representative MDS cell lines, we first used KG1 cells, a CD34 positive cell line derived from AML [16] [17], to examine the effect of the MYD88 peptide inhibitor. RNA levels of MYD88 are higher in KG1 cells than in 293T cell and several other leukemia cell lines such as K-562, HL-60 or U937 (data not shown). PAM3CSK4 and MALP2 are two TLR2 agonists that increase NF- κ B activity in a MYD88 dependent fashion [18], [19]. These two compounds increased NF- κ B promoted luciferase activity in KG1 (Figure S2). When cells were pre-incubated with the MYD88 peptide inhibitor prior to the addition of TLR2 agonists, NF- κ B activation was largely reduced (Figure 3A). This result indicates that the MYD88 peptide inhibitor can block efficiently the TLR2-MYD88 dependent downstream signals.

We next applied MYD88 inhibitor on the primary bone marrow CD34+ cells isolated from patients with lower-risk MDS (IPSS low or intermediate-1) (N=7). Patient characteristics are described in Table S3. After two weeks of culture in methylcellulose supported erythroid and myeloid differentiation medium, and in comparison to peptide control, treatment with MYD88 inhibitor resulted in increase of the number of erythroid colonies (CFU-E) in 5 of the 7 samples (Figure 3B). In average, MYD88 inhibition led to a 2 fold increase of the numbers of erythroid colonies (Figure 3C) without an obvious effect on the numbers of myeloid colonies. The impact of MYD88 blockade on differentiation of erythroid lineage in lower-risk MDS samples was further evaluated by examining the expression of genes known to be activated during erythroid differentiation. MYD88 inhibitor treatment resulted in increased expression of CD71, EPOR, GYPA and GYPB (Figure 3D) [20] [21]. We next examined expression of GATA1 and GATA2 genes, two crucial regulators of hematopoietic differentiation, whose ratio of expression (GATA1/GATA2) has been described to positively correlate with erythroid differentiation [22] [23] [24]. We observed that while expression of both genes increased with MYD88 blockade, the increase of GATA1 was significantly higher than GATA2 (Figure 3E), leading to an increased ratio of GATA1/GATA2 (Figure 3E). These results further support the positive effect on erythroid differenti-

ation by MYD88 blockade in low-risk MDS CD34+ cells. We also characterized effect of MYD88 blockade on the growth of MDS CD34+ cells in another three samples that were cultured in liquid hematopoietic progenitor cell expansion medium. No obvious impact on cell growth was observed (Figure S2). We next examined the effect of MYD88 inhibitor on the hematopoietic colony formation capacity of the CD34+ cells isolated from patients with clinically classified high-risk MDS (N=3). In contrast to the observations in low-risk samples, no positive impact on erythroid colony formation was observed in any of the three high-risk samples.

Blockade of IL-8 in MDS CD34+ Cells

Interleukin-8 (IL-8) is a key inflammatory cytokine dependent on MYD88 [25]. IL-8 has been shown to be one of the most significantly overexpressed cytokines in MDS BM CD34+ cells [26]. We analyzed IL-8 protein levels in the bone marrow plasma samples of patients with MDS (N=33) and observed that IL-8 concentration was elevated in comparison to control counterparts (P=0.03) (Figure 4A). We then examined IL-8 expression in the above described seven lower-risk MDS CD34+ samples treated with the MYD88 inhibitor. Treatment with MYD88 inhibitor resulted in a decrease of IL-8 RNA by 20% (Figure 4B). We next used ELISA to examine the IL-8 protein concentration in the supernatant of culture medium of MDS CD34+ cells (N=4) treated with MYD88 inhibitor or control peptide. With the addition of MYD88 inhibitor, IL-8 protein concentration in culture medium was reduced by 30%, ranging between 16% to 40% in 4 samples (Figure 4C). These results suggest that MYD88 blockage negatively regulates the expression and secretion of IL-8 in MDS CD34+ cells. We then examined the effect of direct IL-8 blockade in MDS CD34+ cells by applying an IL-8 specific neutralization antibody in cells from patients with low-risk MDS (N=5) (Table S4). A similar effect on hematopoietic differentiation of CD34+ cells was observed with the IL-8 antibody as with the MYD88 inhibitor. Colony formation assays indicated that IL-8 antibody treatment resulted in increased numbers of erythroid colonies in 4 of the 5 cases in comparison to isotype control treatment (Figure 4D). The average increase of the number of CFU-E in all 5 samples treated with IL-8 antibody was 1.7 fold in comparison to isotype control. Consistent with the positive impact

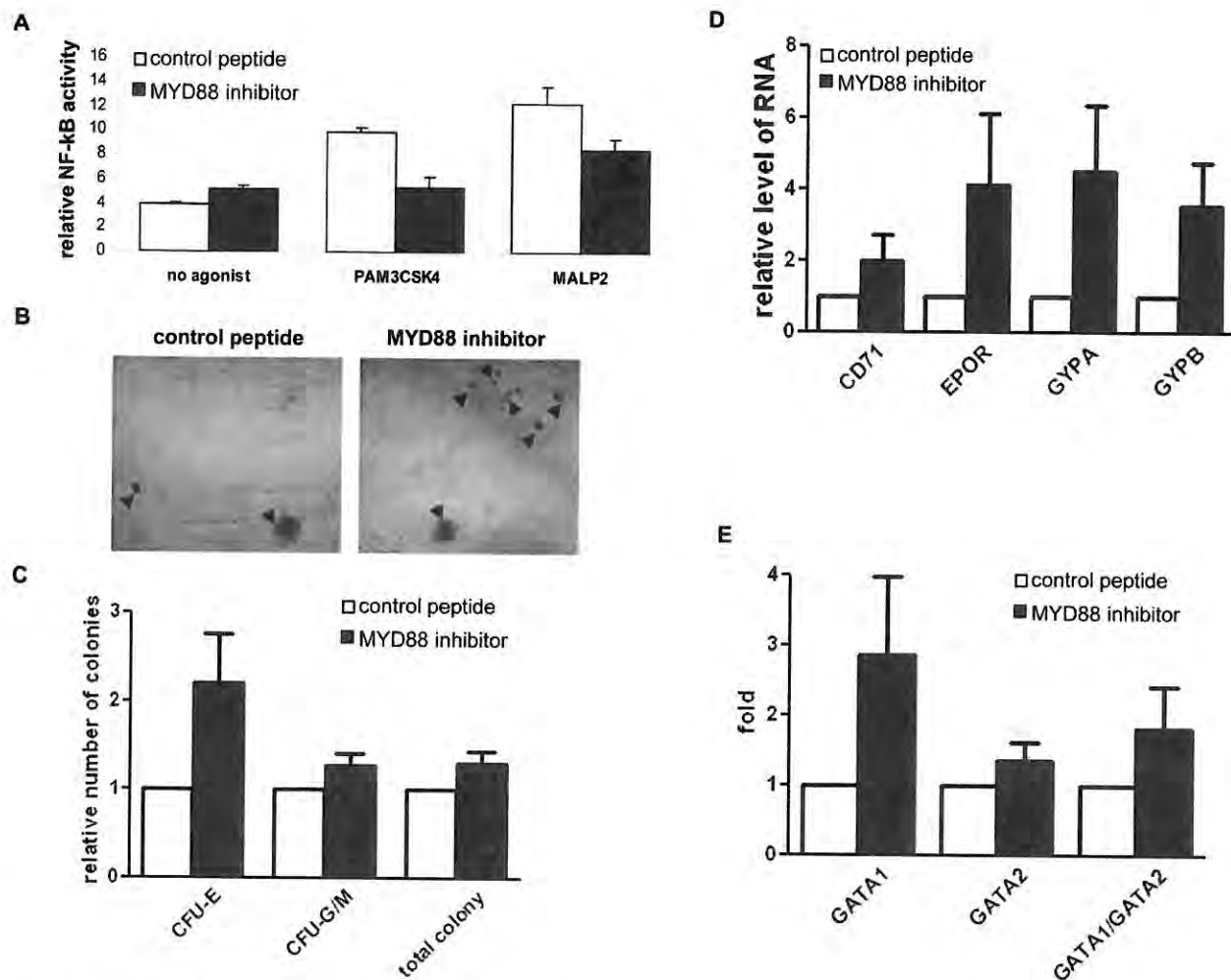


Figure 3. Effect of MYD88 blockade in cultured lower-risk MDS bone marrow CD34+ cells. (A) Analysis of the activity of the MYD88 inhibitor in KG-1 cells. Luciferase assays indicates that MYD88 inhibitor reduces the NF- κ B activity that is stimulated by the TLR2 agonists PAM3CSK4 and MALP2. (B) Representative microphotographs of colonies derived from MDS CD34+ cells in methocult plates after two weeks of culture, treated with control peptide or MYD88 inhibitor peptide. Arrows point to CFU-E. (C) Colony counting after two weeks of methocult culture and treatment of MYD88 inhibitor peptide in comparison to control peptide. (D) Q-PCR analysis indicated elevated RNA levels of erythroid differentiation genes EPOR, CD71 and Glycophorin A and B in MYD88 inhibitor peptide treated cells in comparison to control peptide treatment. Cells were collected from methocult plates after two weeks of culture. (E) Q-PCR analysis of GATA1, GATA2 or GATA2/GATA1 ratio in MYD88 inhibitor peptide treated cells. doi:10.1371/journal.pone.0071120.g003

on the numbers of erythroid colonies, increased CD71 and EPOR RNA expressions were also observed with IL-8 antibody treated samples (Figure 4E). No obvious increase in the RNA expression of Glycophorin A or B genes was observed after treatment with IL-8 antibody (data not shown).

Discussion

Recently, emerging evidence has suggested a role of the TLR mediated innate immune signal in the tumorigenesis such as in gastric cancer through mechanisms independent of chronic inflammation [27]. Similar to the findings in solid tumors, deregulated innate immune signal has also been found to directly impact hematopoietic stem/early progenitor cells (HSPCs) [7] and contribute to leukomogenesis including MDS pathogenesis. [7,28] Stimulation of TLRs is known to lead to activation of intracellular pathways such as NF- κ B and p38MAPK [3], which have both been documented as important molecular signals for tumorigenesis

including in MDS [29,30]. We recently report that TLR2 [26], the histone demethylase JMJD3 and multiple innate immune genes that are regulated by JMJD3 are overexpressed in BM CD34+ cells of MDS [31]. Of importance, JMJD3 is a known transcriptional target of TLR signaling stimulated NF- κ B activity [32].

As an important signal adaptor for the TLR signaling, MYD88 has been previously identified as a key contributor during the pathogenesis of solid tumors including ovarian and cutaneous malignancies [33,34]. More recently, genetic lesions of MYD88 have recently been found to play an important role in lymphoid neoplasms, including ABC- diffuse large B cell lymphomas, central nervous system lymphomas, Waldenstrom macroglobulinemia, and also in T lymphomas [10–12]. Of importance, biological evidence has indicated that these recurrent genetic mutations of the MYD88 gene in lymphoma, particularly the hot-spot alteration of L265P in the TIR domain, is a gain-of-function

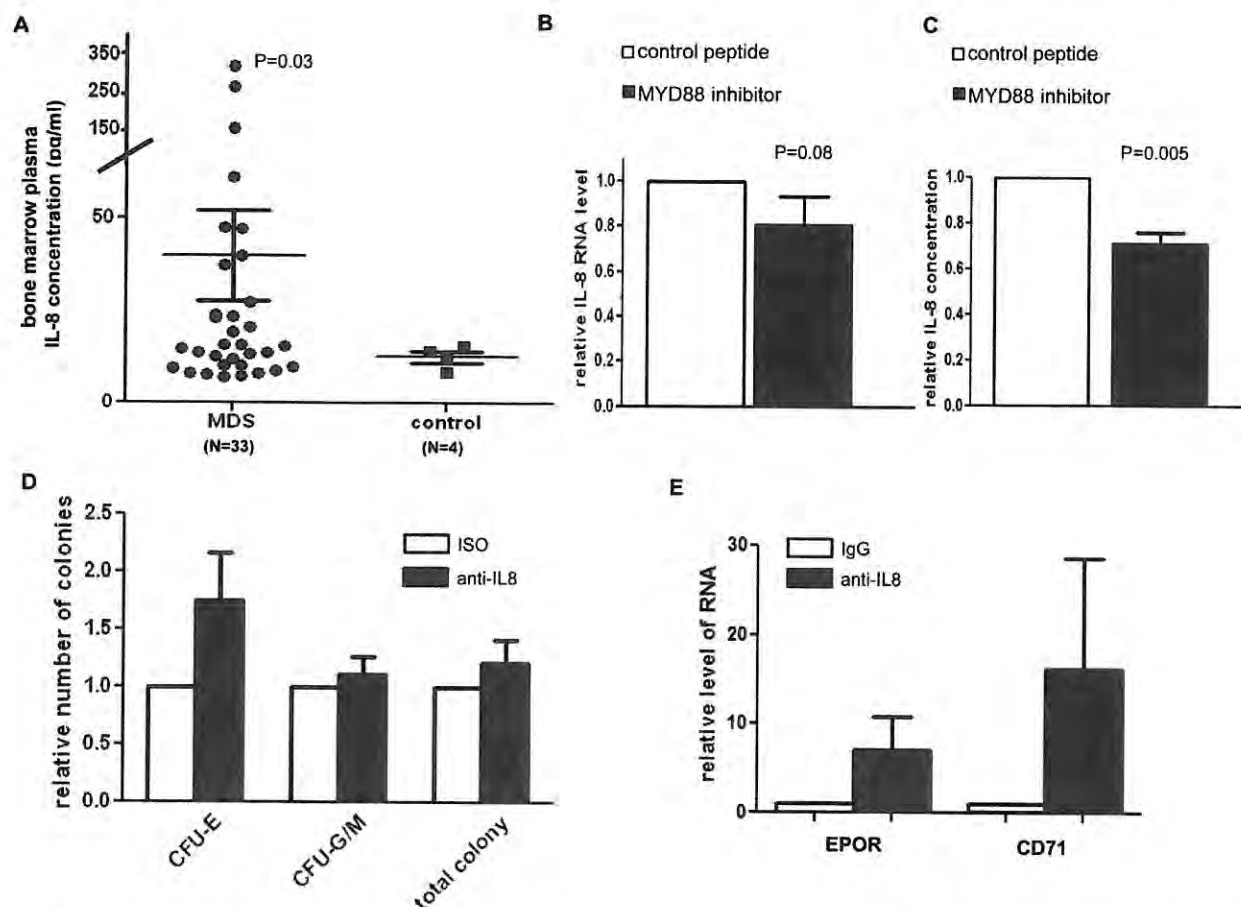


Figure 4. Analysis of the implication of IL-8 in MDS. (A) IL-8 protein concentration is elevated in bone marrow plasma samples of patients with MDS in comparison to healthy controls. (B) MYD88 inhibitor peptide treatment in cultured bone marrow CD34+ cells of patients with MDS leads to reduced IL-8 RNA levels. (C) Colony counting after two weeks of methocult culture in cells treated with IL-8 antibody or isotype control (ISO) indicates that treatment with IL-8 antibody leads to increased number of erythroid colonies in cultured CD34+ cells of patients with low-risk MDS. (D) Q-PCR analysis indicated elevated RNA levels of erythroid differentiation genes EPOR, and CD71 in IL-8 antibody treated cells in comparison to isotype control. Cells were collected from methocult plates after two weeks of culture. doi:10.1371/journal.pone.0071120.g004

mutation. These results implicate that MYD88 as a proto-oncogene in these lymphoid neoplasms. In this study, we were not able to detect any genetic mutation of MYD88 in bone marrow samples of MDS. In parallel to our study, the absence of L265P mutations in acute myeloid leukemia (AML), myelomonocytic leukemia, acute lymphoblastic leukemia (ALL), and multiple myeloma has been recently reported [35]. Of note, only one of the 40 patients examined for MYD88 mutation had prior history of lymphoma. This result suggests that genetic lesions of MYD88 could be specifically important for the pathogenesis of lymphoid but not myeloid neoplasms.

While no genetic lesion in the coding region of the MYD88 gene was detected in the MDS patients, we observed that RNA expression of MYD88 was abnormally increased in a substantial percentage of MDS patients in their CD34+ cells. This result is consistent with previous reports that aberrant activation of innate immune signals in MDS, including overexpression of several TLRs [36] and loss of TRAF6 targeting microRNA in the 5q- syndrome [8]. This study also suggests that MYD88 expression level was associated to the overall survival (OS) in MDS patients. The overexpression of MYD88 in MDS also suggests that although no genetic lesion has been detected in the coding regions of the gene,

sequencing effort of MYD88 should also be expanded to the regulatory sequences of this gene, including promoter and potential microRNA targeting sequences that are important for its transcriptional and translational regulation. Because disease and treatment heterogeneity of patients with MDS, we performed a tentative analysis removing 21 patients with low risk morphologies (6 refractory anemia, 12 refractory cytopenia multilineage dysplasia [RCMD], and 3 RCMD-RS [ring sideroblasts]). The differences in expression compared to control persisted ($p=0.1$) but the impact on survival was lost ($p=0.4$) (data not shown). However, most likely this effect is due to the decrease in sample size. That said, larger analysis of specific morphological subsets are indicated. Indeed one limitation of the study is the relative small sample size analysed. Another limitation is that the number of overlapping samples used for both MYD88 RNA and IL-8 Elisa analysis is very small, which makes it difficult to analyze correlation between MYD88 expression and IL-8 level. Both should be further evaluated in a larger cohort of patients in the future.

Another key finding of this study is that blockage of MYD88 and downstream IL8 in MDS CD34+ cells, particularly in CD34+ cells isolated from patients with lower-risk MDS (IPSS low-risk

and intermediate-1), could positively regulate differentiation of the erythroid lineage. This observation in lower-risk samples is consistent with the observation that MYD88 expression levels tended to be higher in patients with lower-risk MDS (IPSS low-risk and intermediate-1) (Figure S1B). This effect is also consistent with our prior results of JMJD3 inhibition in MDS BM CD34+ cells [31], and that inhibition of TRAF6 in both murine and human HSPCs could prompt erythroid differentiation [8] [37]. Although the underlying molecular mechanisms are still unclear, and because anemia is one of the most common cytopenia in MDS, these observations suggest that inhibiting innate immune signals may have therapeutic role in MDS.

Overall, results of this study demonstrate that MYD88, a key innate immune signal adaptor, is potentially involved in the pathogenesis of MDS. Blockade of MYD88 mediated innate immunity signalling including IL-8 could potentially improve erythropoiesis. These results further support the hypothesis that deregulation of innate immune signal contributes to the development of MDS and this is at least partially through direct impact on hematopoietic stem/progenitor cells. Further dissecting the innate immune signalling and development of pharmacological intervention of this pathway may benefit patients with MDS.

Supporting Information

Figure S1 (A) MYD88 RNA expression level in untreated (N = 35) and treated (N = 29) MDS CD34+ cells compared to

control (N = 12) CD34+ cells. (B) MYD88 RNA expression levels in BM CD34+ cells of different IPSS groups compared to control. (PDF)

Figure S2 Lack of effect on cell growth for MYD88 blockade in MDS CD34+ cells. (PDF)

Table S1 Primers used for MYD88 Pyrosequencing. (PDF)

Table S2 Primers used in bar-code sequencing of MYD88. (PDF)

Table S3 Characteristics of the 7 low-risk patient MDS whose CD34+ cells treated with MYD88 inhibitor peptide. (PDF)

Table S4 Characteristics of the 5 low-risk patient MDS whose CD34+ cells treated with IL-8 antibody. (PDF)

Author Contributions

Conceived and designed the experiments: SD YW CBR RC GGM. Performed the experiments: SD YW CBR HY YJ HZ ZF MN HW. Analyzed the data: CD SP SD YW RC CW GGM. Contributed reagents/materials/analysis tools: CBR MN HY. Wrote the paper: SD YW CBR CD GGM.

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ORIGINAL ARTICLE

Toll-like receptor alterations in myelodysplastic syndrome

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Recent studies have implicated the innate immunity system in the pathogenesis of myelodysplastic syndromes (MDS). Toll-like receptor (TLR) genes encode key innate immunity signal initiators. We recently identified multiple genes, known to be regulated by TLRs, to be overexpressed in MDS bone marrow (BM) CD34+ cells, and hypothesized that TLR signaling is abnormally activated in MDS. We analyzed a large cohort of MDS cases and identified TLR1, TLR2 and TLR6 to be significantly overexpressed in MDS BM CD34+ cells. Deep sequencing followed by Sanger resequencing of *TLR1*, *TLR2*, *TLR4* and *TLR6* genes uncovered a recurrent genetic variant, TLR2-F217S, in 11% of 149 patients. Functionally, TLR2-F217S results in enhanced activation of downstream signaling including NF- κ B activity after TLR2 agonist treatment. In cultured primary BM CD34+ cells of normal donors, TLR2 agonists induced histone demethylase JMJD3 and interleukin-8 gene expression. Inhibition of TLR2 in BM CD34+ cells from patients with lower-risk MDS using short hairpin RNA resulted in increased erythroid colony formation. Finally, RNA expression levels of TLR2 and TLR6, as well as presence of TLR2-F217S, are associated with distinct prognosis and clinical characteristics. These findings indicate that TLR2-centered signaling is deregulated in MDS, and that its targeting may have potential therapeutic benefit in MDS.

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INTRODUCTION

The myelodysplastic syndromes (MDS) are a complex group of malignant myeloid disorders arising from bone marrow (BM) hematopoietic stem and progenitor cells (HSPCs).¹ The pathophysiology of MDS is not fully understood. We recently performed a genome-wide chromatin immunoprecipitation (CHIP)-Seq analysis of H3K4me3 in MDS.² This analysis identified multiple genes marked by increased H3K4me3 in BM CD34+ cells. A large majority of the genes identified are known to be involved in Toll-like receptor (TLR)-mediated innate immunity signaling and NF- κ B activation.² TLRs are a family of pattern-recognition receptors that function as key initiators of innate immunity signaling.³ In the same study, we also identified that the histone demethylase JMJD3 (KDM6B) is significantly overexpressed in MDS BM CD34+ cells and has an important role in the regulation of expression of genes involved in innate immunity.² Prior work by De Santa *et al.*⁴ in murine macrophages has indicated that JMJD3 expression is regulated by TLR-mediated NF- κ B activation. These findings suggested a potential role for TLR function in MDS.

On the basis of these findings, we performed gene expression and mutational analysis of eight human TLRs in a large cohort of MDS. Several major findings are reported here. First, we identified that TLR1, TLR2 and TLR6 are significantly overexpressed in MDS BM CD34+ cells. Of importance, both TLR1 and TLR6 are known to form functional heterodimers with TLR2. Second, deep sequencing identified a rare genetic variant of TLR2 (F217S) present in 11% of BM mononuclear cells (BM-MNCs) of patients with MDS. The incidence of TLR2-F217S in MDS is significantly higher than its known frequency in normal population. Third, we

demonstrated that inhibition of TLR2 in cultured BM CD34+ cells from patients with lower risk of MDS results in increased formation of erythroid colonies. Finally, clinical analysis indicated that RNA expression levels of TLR2 and TLR6, as well as the presence of TLR2-F217S, were associated with distinct clinical characteristics. These results indicate that TLR2-mediated innate immune signaling has a role in the pathophysiology of MDS, and that its targeting may have therapeutic potential.

MATERIALS AND METHODS

Isolation and culture of BM CD34+ cells

Human samples were obtained following institutional guidelines. MDS BM specimens were freshly obtained from patients who were referred to the Department of Leukemia at MD Anderson Cancer Center following institutional guidelines. Diagnosis was confirmed by a dedicated hematopathologist (CB-R) as soon as sample was obtained. BMs from healthy individuals were obtained from AllCells (Emeryville, CA, USA). Isolation of CD34+ cells was performed using MicroBead Kit (Miltenyi, Bergisch Gladbach, Germany). Primary BM CD34+ cells were cultured in IMDM (Invitrogen, Carlsbad, CA, USA), 20% BIT 9500 (bovine serum albumin, insulin and transferrin), human thrombopoietin 50 ng/ml, interleukin (IL)-3 10 ng/ml, stem cell factor 100 ng/ml, FLT3L 100 ng/ml (Stem Cell Technology, Vancouver, CA, USA). For colony-forming assays, healthy and MDS BM CD34+ cells were seeded at 1000 and 10 000 cells per ml, respectively, in 3.5-cm round culture dishes with methocult GF H4434 (Stem Cell Technology).

Cell lines and culture

293T cells were cultured in DMEM, 10% fetal calf serum, 1% penicillin-streptomycin and 2 mM L-glutamine. OCI-AML3 cells were cultured in

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RPML-1640, 10% fetal calf serum and 1% penicillin-streptomycin. All cells were obtained from American Type Culture Collection (Manassas, VA, USA).

Quantitative reverse transcription-PCR

Total cellular RNA was extracted using Trizol (Invitrogen) according to manufacturer's protocol. An amount of 200 ng of total RNA was used for reverse transcription (RT) reactions using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). For real-time PCR, primers and probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7500 Sequence Detection System. PCRs were performed using 20 × Assays-On-Demand Gene Expression Assay Mix and TaqMan Universal PCR Master mix according to the manufacturer's protocol. GAPDH was used as the internal control.

Capture deep sequencing of genomic DNA

Illumina paired-end libraries were generated according to the manufacturer's protocol (Illumina, San Diego, CA, USA). An amount of 3 µg of pre-captured library DNA was used for each capture reaction. NimbleGen SeqCap EZ Hybridization and Wash Kits (Roche, Basel, Switzerland) were used following the manufacturer's protocol. A total of 14–17 cycles of PCR amplification were applied to the samples after hybridization, based on yield. After that, captured libraries were quantified by picogreen and sequenced on the Illumina HiSeq 2000 as 100-bp paired-end reads, following the manufacturer's protocols. Sequencing data were stored as fastq format that contained reads and quality information. Fastq files were then mapped and aligned to human reference hg18 using Burrows-Wheeler Alignment Tool.⁵ To refine the alignment and adjust the quality score provided by sequencer, GATK was used to realign reads and recalibrate the quality score.⁶ Single-nucleotide polymorphism (SNPs) were called using AtlasSNP2 (<http://www.biomedcentral.com/1471-2105/13/8>). The posterior probability was set as 0.9 and the minimum number of variant reads as 3. To identify rare SNPs, candidate SNPs were filtered against dbSNP130, 1000 genome database and the Human Genome Sequencing Center (Baylor College of Medicine) internal database. Indels were called using AtlasIndel (http://www.hgsc.bcm.tmc.edu/cascade-tech-software_atlas2_snp_indel_calling_pipeline-ti.hgsc).

Recombinant retrovirus-mediated short hairpin RNA transduction pGFP-V-RS plasmids expressing the 29mer short hairpin RNA (shRNA) against human TLR2 or control shRNA were purchased from Origene (Rockville, MD, USA). Recombinant retrovirus expressing shRNA was packaged in 293T cells (Invitrogen) and concentrated using the PEG-IT virus precipitation solution (System Biosciences, Mountain View, CA, USA). Transduction of OCI-AML3 and BM CD34+ cells with virus was performed by mixing virus with cell suspension followed by centrifugation at 30 °C for 90 min with 4 µg/ml polybrene in medium. After viral transduction, OCI-AML3 cells with stable expression of the shRNA against TLR2 or a control scrambled shRNA were established in the presence of puromycin (10 µg/ml) in medium.

Luciferase assay

293T cells were transfected with pGL4.32[luc2P/NF-κB-RE] (Promega, Madison, WI, USA) or pAP1-Luc (Agilent, Santa Clara, CA, USA) together with pCMV6-AC-GFP-TLR2 wild-type (Origene) or P217S mutant. pRL-TK vectors expressing the Renilla luciferase were cotransfected as internal control. Lipofectamine 2000 (Invitrogen) was used for transfection. One day after transfection, TLR2 agonist MALP2 (1 µg/ml) or PAM2CSK4 (0.3 µg/ml; Invivogen, San Diego, CA, USA) were added to the medium. Luciferase assays were performed 8 h later with the Dual-Luciferase Reporter Assay kit (Promega) on 3010 luminometer (BD Biosciences, San Diego, CA, USA). Luciferase activity was calculated using the ratio of firefly luminescence from the pGL4.32[luc2P/NF-κB-RE] or pLuc-AP1 reporter to the renilla luminescence from the control pRL-TK vectors reporter.

Western blot and immunoprecipitation

293T cells were transfected with pCMV6-AC-GFP-TLR2 wild-type (Origene) or P217S mutant. TLR2 agonist MALP2 (1 µg/ml) or PAM2CSK4 (0.3 µg/ml; Invivogen) were added into the medium 1 day after transfection. Cells were collected after 5–30 min. Cells were lysed using RIPA lysis buffer (Cell Signaling, Danvers, MA, USA) or processed for immunoprecipitation using cross-link IP kit (Thermo Scientific, Rockford, IL, USA). Antibodies used include phospho-p38 and IRAK1 (4511 and 4504, Cell

Signaling), phospho-IRAK1 (S376) (PAB0497, Abnova, Walnut, CA, USA), phospho-IRAK1 (T209) (ab61799, Abcam, Cambridge, MA, USA), poly-Ub-K63 (05-1308, Millipore, Billerica, MA, USA), TLR2 (TA306125, Origene) and β-actin (A5441, Sigma-Aldrich, St Louis, MO, USA).

Multicolor flow cytometry

All monoclonal antibodies, including CD34, CD117, CD64, CD14, CD71, CD45 and HLA-DR, were obtained from BD Biosciences (San Jose, CA, USA). Samples were incubated with monoclonal antibodies for 25 min at 4 °C and acquired on FACSCanto II instruments (BD Biosciences). Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA, USA).

CHIP-PCR

Immunoprecipitated DNA was analyzed by quantitative real-time PCR (qPCR) using the Quanti-Tect SYBR Green PCR kit (Qiagen, Valencia, CA, USA). The amount of DNA fragment co-precipitated with antibody was calculated and compared with the amount of the same genomic fragment in total input DNA, resulting in percent input.

Statistical methods

Overall survival (OS) was defined from date of sample to date of death or date last known alive; patients last known alive were censored for this analysis. To investigate associations between gene expression and OS, we considered splitting expression level at the 25th, 50th and 75th percentiles, generating three possible binary variables. Associations with clinical and demographical features at the time of sampling were assessed only at the median expression level. For ordered clinical features, such as International Prognostic Scoring System, the Kruskal-Wallis test was used; for binary features, such as gender, the Fisher's exact test was used. For continuous variables, such as percentage BM blasts or hemoglobin, the Wilcoxon rank sum test was used.

Other reagents

Human lymphoblastoid cell DNA was purchased from Sigma-Aldrich. According to manufacturer's instructions, cells were collected from UK Caucasian blood donors. The DNA was then extracted from lymphoblastoid cell lines derived by Epstein Barr Virus that can be continuously propagated in culture.

RESULTS

Overexpression of TLR2 and its functional partners TLR1 and TLR6 in BM cells of MDS

We first profiled RNA expression of eight human TLRs (TLR1–4 and TLR6–9) in a cohort of MDS BM CD34+ cells ($N=40$). RNA expression levels of TLRs in MDS in comparison with control BM CD34+ cells are shown in Figure 1a. This initial analysis indicated that TLR1, TLR2 and TLR6 were significantly overexpressed in MDS samples compared with controls (Figure 1a). Although the expression of TLR4, TLR7, TLR8 and TLR9 was also increased in some MDS samples, the overall difference between MDS and controls was not significant (Figure 1a). We then expanded the analysis of RNA expression of TLR 1, TLR2 and TLR6, and summarized their expression in total 103 cases of MDS BM CD34+ samples analyzed. Summary of clinical characteristics of this sample cohort are in Supplementary Table 1. Compared with controls, average expression levels in MDS samples were increased by 10-fold for TLR1 (74% over twofold increase, $N=87$, $P<0.0001$, Figure 1b), by 37-fold for TLR2 (82% over twofold increase, $N=83$, $P<0.0001$, Figure 1c) and by 168-fold for TLR6 (73% over twofold increase, $N=94$, $P=0.0001$, Figure 1d). This result is of potential biological significance because both TLR1 and TLR6 are known to form functional heterodimers with TLR2.^{7,8}

Identification of TLR2 mutation (F217S) in MDS BM-MNCs

To further study the role of TLRs in MDS, we performed capture deep sequencing of genes encoding TLR1, TLR2, TLR4 and TLR6 in

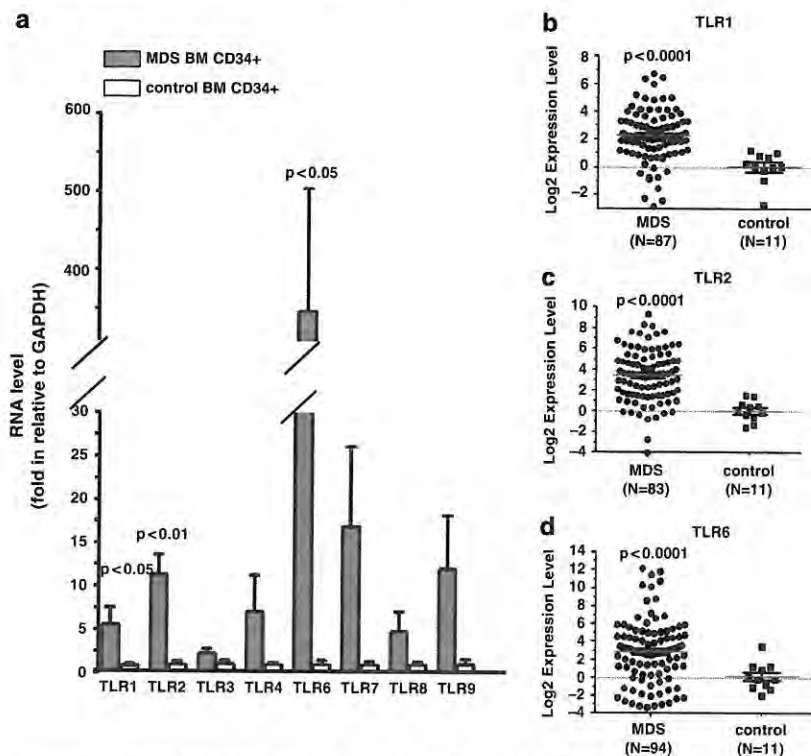


Figure 1. TLR RNA expression in primary MDS BM CD34⁺ cells. **(a)** qRT-PCR analysis of RNA expression of eight *TLR* genes in MDS and control BM CD34⁺ cells. **(b–d)** Logarithmic representation of qRT-PCR results of TLR1, TLR2 and TLR6 in MDS and control CD34⁺ cells. Number of samples with evaluable RNA expression are marked underneath of each graph.

MDS BM-MNCs. We first studied a cohort of 40 patients and identified three rare SNP variants located in the coding region of TLR2 and one SNP in TLR4 (Supplementary Figure 1A). We subsequently analyzed these four SNPs using Sanger sequencing. One rare TLR2 SNP (rs139227237, g.154624709T>C) was detected as a heterogeneous alteration in 11% ($N=17$) of a cohort of 149 MDS BM-MNC samples (Supplementary Table 2 and Figure 2a). At the protein level, this SNP results in a conserved hydrophobic phenylalanine (F217) located in the leucine-rich repeats of extracellular region to be converted into a hydrophilic serine (Figure 2b). To evaluate whether TLR2-F217S is a somatic alteration in MDS, we analyzed the corresponding BM CD34⁺ cells available from 16 of the 17 patients known to carry this alteration in BM-MNC. Fourteen of these patients did not carry F217S in CD34⁺ cells (Figure 2a). Of interest, two CD34⁺ samples contained this SNP (Supplementary Figure 1B). We also sequenced 20 CD34⁺ samples isolated from patients without TLR2-F217S. No TLR2-F217S was detected in any of these CD34⁺ cells (Supplementary Figure 1C). We next analyzed a group of random normal control DNA samples ($N=47$) that were derived from human lymphoblastoid cells isolated from healthy donors. None of the normal control samples was positive for TLR2-F217S (data not shown).

Biological implications of TLR2-F217S in innate immunity signal activation

To characterize the impact of TLR2 mutation F217S on the activation of innate immunity signaling, we expressed green fluorescent protein (GFP) fused with wild-type or TLR2-F217S in 293T cells (Figure 2c), and analyzed NF- κ B activity. 293T cells express endogenous TLR1 and TLR6 but not TLR2, and therefore are often used to characterize genetically altered forms of TLR2.⁹ In luciferase reporter assays, in the absence of TLR2 agonist,

expression of wild-type or F217S mutant TLR2 led to similar levels of NF- κ B activation (Figure 2d). When a TLR2 agonist (PAM2CSK4, PAM3CSK4 or MALP2) was added, NF- κ B activation was increased in TLR2-F217S vs wild-type transfected cells (Figure 2d). Similar effects on AP1 activity and associated phospho-p38MAPK were also observed in TLR2-F217S with PAM2CSK4 treatment but not without TLR2 agonist (Supplementary Figures 1D and 1E). To further assess the effect of TLR2-F217S on innate immunity signaling, we examined the status of IRAK1 protein, a key downstream signal mediator of TLRs.¹⁰ In the absence of TLR agonist, a slight increase of a slowly migrating IRAK1 band (~180 kDa) was observed in both wild-type and F217S-transfected 293T cells (Figure 2e). After MALP2 and PAM2CSK4 treatment, the density of this high-molecular IRAK1 band was higher in TLR2 (F217S) than in wild-type transfected cells (Figure 2e). IRAK1 immunoprecipitation followed by immunoblotting indicated that this higher molecular form of IRAK1 contained phospho- and polyubiquitin-IRAK1 (Figure 2e), two active isoforms of this protein.¹¹ Taken together, these results indicated that TLR2-F217S is a gain-of-function mutation that augments TLR2-mediated downstream signaling.

TLR2 stimulation in BM CD34⁺ cells activates IL-8 and JMJD3

One major group of genes that are known to be regulated by TLR2 signals are inflammatory cytokines.³ The levels of several of these cytokines are also known to be elevated in MDS BMs.^{12,13} We therefore investigated whether some of these cytokines were actively expressed in BM CD34⁺ cells and whether deregulation of TLR2 in MDS BM CD34⁺ cells could affect their expression. We first assessed the basal expression of several of these cytokines in normal BM CD34⁺ cells. Results indicated that basal RNA level of IL-8 was significantly higher than that of other inflammatory cytokines analyzed, including IL-1 β , IL-4, IL-6 and TNF- α .

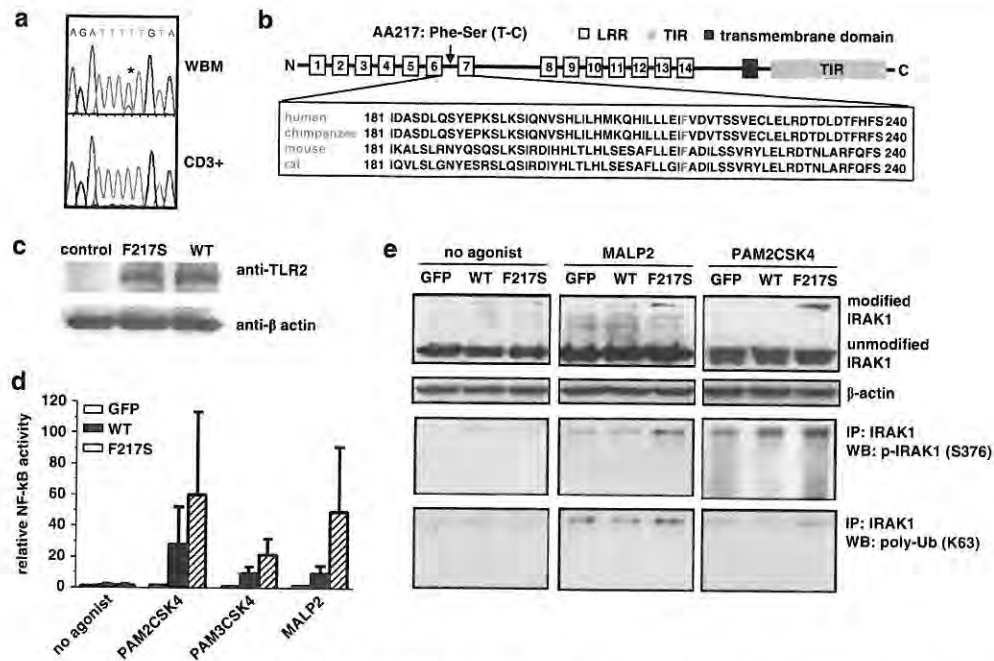


Figure 2. Mutational analysis and identification of TLR2-F217S in MDS. **(a)** Representative Sanger sequencing traces showing nucleotides around coding region of TLR2-F217 in whole BM (WBM) DNA (top) and CD3+ genomic DNA (bottom) of one MDS sample. * In the WBM sample trace marks the location of the mutation. **(b)** Schema of *TLR2* gene and sequence alignments between human and other species. **(c)** Western blots of GFP fusion protein expression of TLR2 wild-type (WT) and F217S in 293T cells. **(d)** Luciferase analysis of activation of NF-κB by WT and F217S TLR2 in transfected 293T cells. Cells were exposed to either no agonist or to PAM2CSK4, PAM3CSK4 or MALP2 known TLR2 agonists. Pooled data from three separate experiments. **(e)** Western blot characterization of IRAK1 modification in 293T cells transfected with either WT or F217S TLR2 and treated with no agonist, MALP2 or PAM2CSK4. Top: western blot analysis of IRAK1; middle: IRAK1 immunoprecipitation followed by phospho-IRAK1 western blot; bottom: IRAK1 immunoprecipitation followed by polyubiquitin(K63).

(Supplementary Figure 2A). Subsequently, we compared IL-8 RNA expression in MDS and control BM CD34+ cells. RT-PCR analysis of 109 MDS samples indicated that IL-8 expression was significantly elevated in MDS samples compared with normal CD34+ cells (Figure 3a and Supplementary Figure 2B). To evaluate whether TLR2 activation could contribute to the activation of IL-8 in BM CD34+ cells, we treated cultured primary BM CD34+ cells with MALP2 and PAM3CSK3. Both these TLR2 agonists induced higher IL-8 levels of RNA expression (Figure 3b).

We have recently identified that the histone demethylase JMJD3 is overexpressed in BM CD34+ cells of MDS, which contributes to the abnormal epigenetic activation of multiple genes involved in innate immunity activation.² JMJD3 has been previously demonstrated to be regulated by TLRs in murine macrophages.³ To examine if TLR2 activation can also activate JMJD3 in BM CD34+ cells, we assessed the effects of MALP2 and PAM3CSK3 on the expression of JMJD3. Results indicated that in cultured BM CD34+ cells, and in parallel to the increase in IL-8 expression, JMJD3 RNA levels also significantly increased after treatment with both TLR2 agonists (Figure 3b). We previously reported that in OCI-AML3 cells, knockdown of JMJD3 negatively affects the expression of multiple innate immunity genes.² We therefore examined if IL-8 RNA expression was also altered in OCI-AML3 cell line that constitutively expresses an shRNA against JMJD3. In parallel to JMJD3 knockdown, we observed a decrease of IL-8 RNA (Figure 3c). We next evaluated potential impact of JMJD3 knockdown on histone methylation by measuring the levels of H3K4me3 and H3K27me3 in the promoter of IL-8. CHIP-PCR revealed that there was a decrease in the level of H3K4me3 and an increase in the level of H3K27me3 in IL-8 promoter (Figures 3d and e). These results indicated that TLR2

could activate the expression of both IL-8 and JMJD3. Furthermore, histone methylation of IL-8 promoter could be regulated by JMJD3. Consistent with the results from cultured cells, we also observed in primary MDS BM CD34+ cells ($N=42$) that RNA levels of IL-8 and JMJD3 were both positively correlated with TLR2 expression ($R=0.88$ and 0.61 , respectively, $P<0.0001$), and, furthermore, IL-8 level was also positively correlated with JMJD3 expression ($R=0.66$, $P<0.0001$).

TLR2 activation negatively regulates erythroid differentiation in cultured BM CD34+ cells

Stimulation of TLR2 has been shown to alter hematopoietic differentiation in cultured mouse HSPCs.⁴ To explore the impact of TLR2 activation in human BM CD34+ cells, we treated BM CD34+ cells isolated from healthy individuals ($N=4$) with TLR2 agonists (MALP2 and PAM3CSK4) and analyzed their effects on hematopoietic differentiation: 48 h after treatment, flow cytometry assays indicated that MALP2 caused a significant decrease of a erythroid precursor cell population, which was defined by strong CD71 expression and absence of HLA-DR (Figures 4a and b). PAM3CSK4 had similar but less significant effect (Figures 4a and b). The negative influence of TLR2 agonists on erythroid lineage was further confirmed by colony formation assays in methylcellulose medium (methocult). After 2 weeks in methocult culture, MALP2 treatment led to a 55% reduction in erythroid colony-forming units (CFU-E) (Figure 4c). Consistent with the results of flow cytometry, PAM3CSK4 had a similar but less significant effect on CFU-E formation (Figure 4c). We also assessed the impact of IL-8 treatment in colony formation of normal BM

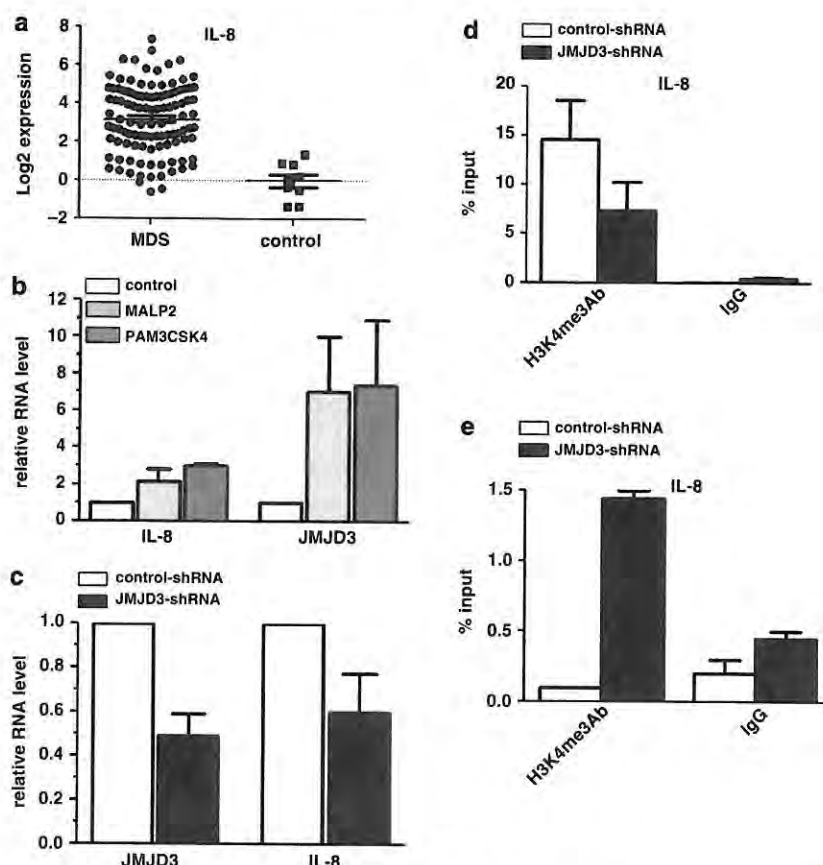


Figure 3. Activation of *IL-8* and *JMJD3* gene expression by TLR2 signaling in primary BM CD34+ cells. **(a)** Logarithmic representation of qRT-PCR result of *IL-8* gene in MDS and control CD34+ cells. **(b)** qRT-PCR analysis of *IL-8* and *JMJD3* RNA expression in cultured BM CD34+ cells after treatment with TLR2 agonist MALP2 and PAM3CSK4. **(c)** qRT-PCR analysis of *IL-8* RNA expression in OCI-AML3 cells after *JMJD3* knockdown. **(d)** H3K4me3 CHIP-PCR analysis of *IL-8* promoter in the OCI-AML3 cells after *JMJD3* knockdown. **(e)** H3K27me3 CHIP-PCR analysis of *IL-8* promoter in OCI-AML3 cells after *JMJD3* knockdown.

CD34+ cells and observed similar negative effect on CFU-E formation as TLR2 agonist treatments (Figure 4c).

Inhibition of TLR2 positively regulates CFU-E formation in MDS BM CD34+ cells

To evaluate the effect of TLR2 inhibition on hematological colony formation in cultured MDS BM CD34+ cells, we transduced primary BM CD34+ cells isolated from patients with newly diagnosed lower- (N=4) and higher-risk MDS (N=3) with a recombinant retroviral-mediated shRNA against TLR2. Patients' characteristics are described in Supplementary Table 3. TLR2-shRNA resulted in reduced levels of TLR2 RNA expression in primary MDS BM CD34+ cells examined (Supplementary Figure 3A). After 2 weeks of culture in methocult medium, all four lower-risk samples had an increased number of erythroid colonies (CFU-E) after TLR2-shRNA transduction (Figure 5a). On average, 64 CFU-E were formed per 10⁴ MDS BM CD34+ cells plated in response to the transduction of TLR2-shRNA, which was a 35% increase compared with the number of CFU-E formed after control shRNA transduction (Figure 5a). No significant effect on the formation of myeloid colonies (CFU-G/M) was observed after TLR2 inhibition (Figure 5a). Representative images of the methocult colonies formed from one low-risk sample are shown in Figure 5b. In contrast to lower-risk MDS, we did not observe a positive effect on the formation of erythroid or myeloid colonies in any of the three BM CD34+ cells isolated from patients with

higher-risk MDS (Supplementary Figure 3B). To further verify the effect of TLR2 inhibition in lower-risk samples, we measured transcripts of several genes known to be positively associated with erythroid differentiation, including Glycophorin-A (GYPA), CD71, EPOR and GATA1. In the cells collected from methocult colonies, expression of all four genes was increased after TLR2 inhibition (Figure 5c). The effect on gene expression further confirmed the positive impact of TLR2 inhibition on the differentiation of erythroid lineage in MDS BM CD34+ cells of lower-risk type of MDS.

Clinical implications of TLR in MDS

To further investigate implications of TLR1, TLR2 and TLR6 deregulation in MDS, we analyzed the associations of RNA expression in MDS BM CD34+ cells (N=103) with relevant clinical characteristics. Increased levels of TLR2 expression were associated with low-risk MDS by International Prognostic Scoring System (P=0.01) (Figure 6a), diploid cytogenetic (P=0.04) and a diagnosis of chronic myelomonocytic leukemia (P=0.04). In contrast, expression of TLR6 gradually increased with International Prognostic Scoring System risk: high-risk patients had highest TLR6 expression, intermediate-1 and -2 patients had intermediate expression and low-risk group had lowest TLR6 expression (P=0.015) (Figure 6b). Patients that had lowest TLR6 expression were associated with diploid cytogenetic (P=0.02) and a diagnosis of chronic myelomonocytic leukemia (P=0.0004),

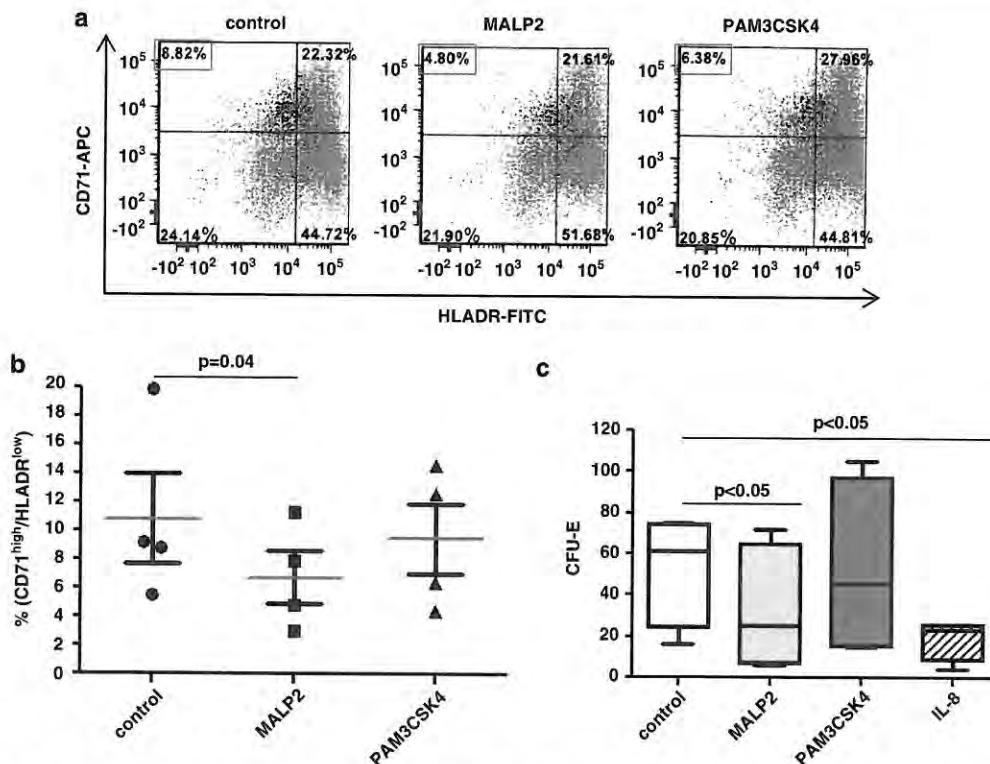


Figure 4. Effect of TLR2 activation in *in vitro*-cultured primary normal BM CD34⁺ cells. (a) Flow cytometry analysis of normal CD34⁺ cells after being treated with MALP2 or PAM3CSK4 for 48 h. Compared with control, treatment resulted in a decreased percentage of early erythroid progenitor cells marked with CD71⁺ and HLA-DR. (b) Summary of flow cytometry analysis of CD71⁺ and HLA-DR cells in primary BM CD34⁺ cells of four different donors. (c) Methocult medium supported colony formation assays revealed the decreased formation of erythroid colonies (CFU-E) from primary BM CD34⁺ cells treated by TLR2 agonist MALP2, PAM3CSK4 or IL-8.

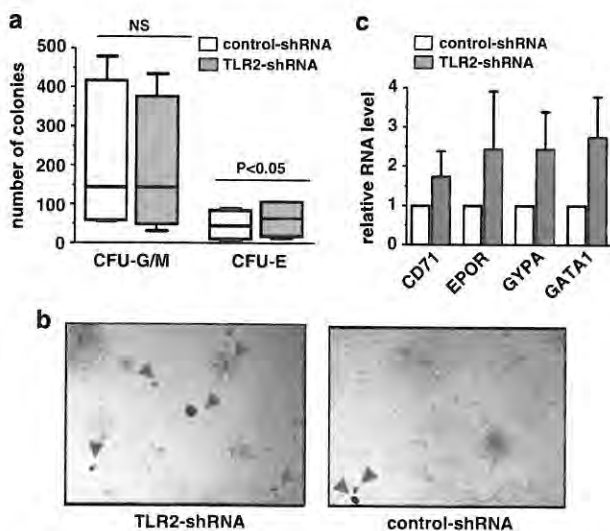


Figure 5. Effect of TLR2-shRNA transduction in cultured MDS BM CD34⁺ cells. (a) Numbers of myeloid colonies (CFU-G/M) and erythroid colonies (CFU-E) formed in methocult culture 2 weeks after transduction of TLR2-shRNA and control shRNA in BM CD34⁺ cells isolated from patients with lower-risk MDS (low-risk and intermediate-1). (b) Representative microphotographs of colonies formed in methocult plates after transduction of TLR2-shRNA (left panel) and control shRNA (right panel). Red arrows point to CFU-E. (c) qRT-PCR analysis of the RNA levels of CD71, EPOR, GYPA and GATA1 in cells collected from total colonies after shRNA transduction and methocult assays.

Furthermore, patients with higher TLR6 expression (above median) had increased percentage of BM blasts (7.8 vs 3.2%; $P < 0.0001$). In terms of OS, we observed that patients with higher TLR2 expression (above 25% median) had significant longer survival than patients with lower TLR2 expression (43.9 vs 13.7 months; $P = 0.03$; Figure 6c). TLR6 expression had a tendency to be negatively correlated with OS (22.7 vs 72.8 months; $P = 0.18$) (Figure 6d). Finally, patients with TLR2-F217S had a significant higher frequency of chromosome 7 deletion ($P = 0.03$; Figure 6e).

DISCUSSION

Recent advance indicates that abnormal activation of innate immunity signals occurs in MDS. For instance, deregulation of TRAF-6, a key innate immunity signal mediator, together with the loss of miR-146a, is involved in the development of del5q MDS.^{14,15} We also recently reported that the histone demethylase JMJD3 and multiple innate immunity genes regulated by JMJD3 are overexpressed in BM CD34⁺ cells of MDS.² Of importance, expression of JMJD3 has been previously shown to be controlled by TLR-mediated NF- κ B activation in murine macrophage cells.⁴ Based on this, we hypothesized that TLRs could be abnormally upregulated in MDS.

First, based on the analysis of RNA expression in a large cohort of patient samples, we document significant overexpression of TLR1, TLR2 and TLR6 in MDS BM CD34⁺ cells. Although TLRs are known to be present in differentiated hematopoietic cells, such as macrophages and dendritic cells, emerging evidence has recently indicated that functional TLRs are also actively expressed in primitive HSPCs, including hematopoietic stem cells and their early progenies such as multipotent progenitors, common

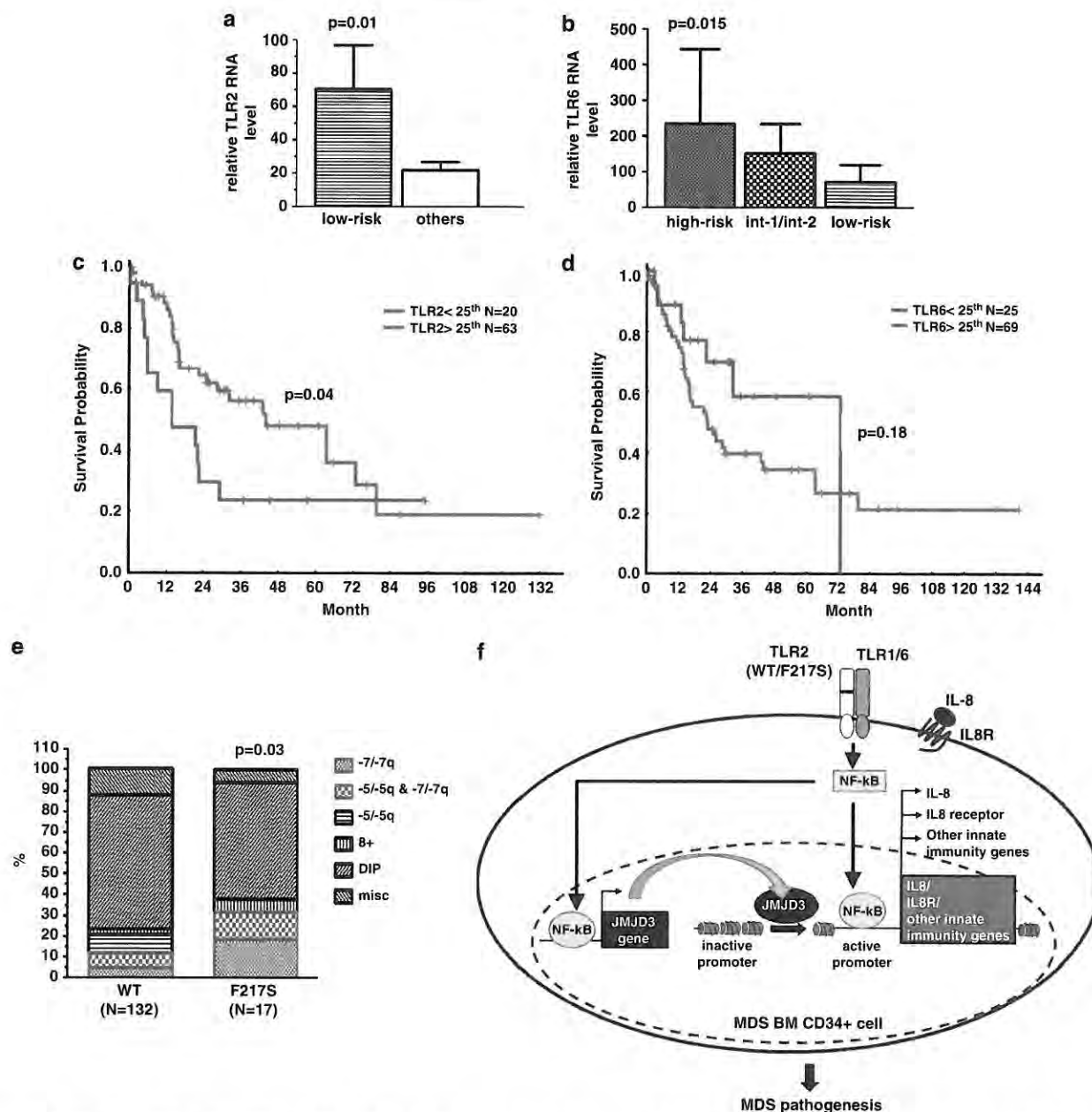


Figure 6. Clinical analysis and proposed model of TLR2-mediated innate immunity signaling in MDS. **(a)** Higher TLR2 expression level in low-risk patients compared with others. **(b)** Distinct TLR6 expression levels in high-risk, intermediate-1 and -2 and low-risk patients. **(c and d)** Correlation of TLR2 and TLR6 with OS of patients. **(e)** Patients with TLR2-F217S are with higher frequency of 7 – /7q – . **(f)** Proposed model of a potential TLR2-JMJD3-IL-8 signaling axis is abnormally activated in BM CD34 + cells of MDS. This abnormal activation includes overexpression of TLR1, TLR2 and TLR6, as well as genetic alteration (F217S) of TLR2. This signal axis leads to consequent activation of JMJD3 and IL-8. JMJD3 can also positively regulate both genes of IL-8 and its receptor in MDS BM CD34 + cells. Impact of this innate immunity signaling contributes to the pathogenesis of MDS.

lymphoid progenitors and common myeloid progenitors.^{16–18} It is known that both TLR1 and TLR6 bind to TLR2,^{7,8} and together they form TLR2-centered functional pattern-recognition receptor complexes that recognize conserved molecular patterns. These conserved molecular patterns are derived from Gram-positive bacteria and mycobacteria or from endogenous molecules released during tissue damage or cell death.^{3,19,20} Therefore, the identification of overexpression of TLR1, TLR2 and TLR6 in MDS BM CD34 + cells is of potential biological significance. For instance, an autosomal syndrome with monocytopenia and increased susceptibility to mycobacteriosis has been reported in MDS.²¹

TLR2 has been demonstrated to be overexpressed in other types of neoplasms and has been shown to have oncogenic function independent of chronic inflammation in a gastric cancer model.²² Other studies using smaller patient cohorts have previously reported the overexpression of other TLRs such as TLR4 in MDS BM CD34 + cells.²³

We have also identified a mutation of TLR2-F217S in 11% of patients. We demonstrate that TLR2-F217S induces more robust activation of NF- κ B, p38MAPK and IRAK1 modifications upon stimulation with TLR2 agonists in transfected 293T cells (Figure 2 and Supplementary Figure 4). These results are in support of the

central hypothesis that TLR2 signaling is abnormally activated in MDS. The question is whether TLR2-F217S is a somatic mutation in MDS. First, none of the 47 normal human control DNA samples had TLR2-F217S. This result is consistent with records in an annotated SNP database (1000 Genome project <http://www.ncbi.nlm.nih.gov/projects/snp>), which indicates that the incidence of TLR2-F217S in population is 0.3%. TLR2-F217S was found in 2 of the 19 CD3+ T-cell specimens studied. One explanation is that T cells emerged from the original MDS clone. Defective T cells, such as NKT cells, have been previously reported in MDS.^{24,25} To further clarify this, we will need to analyze non-hematological controls, such as germline cells, in large cohort of patients. A potential model is that TLR2-F217S can not only be a somatic mutation in patients with MDS but also a polymorphism that could be associated with increased risk for development of MDS. Large-scale population analysis in both patients and healthy individuals, as well as detailed biological characterization in hematopoietic cells, will need to be performed to better define the implications of TLR2-F217S in the pathogenesis of MDS.

In this study, we also observed that stimulation of TLR2 in human BM CD34+ cells leads to activation of multiple genes known to be overexpressed in the same cell compartment of MDS. Emerging evidence has recently suggested a direct regulation of HSPCs by innate immunity signaling.²⁶ In mouse HSPCs, acute TLR stimulation has been shown to alter myeloid/lymphoid ratios, whereas chronic stimulation results in stem cell exhaustion and BM failure.^{16,17} Hyperactivation of TLR signaling has also been documented in BM failure syndromes such as Fanconi anemia.^{27,28} In this report, we demonstrate that TLR2 activation in primary human BM CD34+ cells activates expression of histone demethylase JMJD3 and the inflammatory cytokine IL-8. We also demonstrate that JMJD3 can positively regulate expression of IL-8. In a recent study, we have previously shown that JMJD3 activates expression of the gene encoding IL-8 receptor (IL8RB), which is overexpressed in MDS BM CD34+ cells.² Together, these results suggest that in BM CD34+ cells of MDS, there is a potential TLR2-JMJD3-IL-8 signaling axis. One molecular consequence of the deregulation of this signaling is overexpression IL-8. Overexpressed IL-8 then influences the fate of CD34+ HSPCs through its receptor (IL8RB), which is overexpressed in the same cell, forming a potential autocrine of IL-8 to contribute to the pathogenesis of MDS (Figure 6f).

Results of this study also have clinical implications. First, knockdown of TLR2 in MDS CD34+ cells resulted in increased erythroid differentiation. Although the precise biochemical mechanisms by which TLR2 inhibition promotes erythroid differentiation is still unclear, these observations have obvious therapeutic implications, suggesting that targeting TLR2 could improve hematopoiesis in patients with MDS. Second, RNA expression levels of TLR2 and TLR6, as well as the presence of genetic alteration of TLR2-F217S, were associated with several important clinical characteristics. Of interest, there is a potential discrepancy between the effect of TLR2 and TLR6 expression. This discrepancy potentially reflects the pathological and molecular heterogeneity of MDS. Mechanistically, this discrepancy suggests that TLR6 can potentially contribute to the pathogenesis of MDS through mechanisms independent of its interaction with TLR2. Indeed, recent studies have suggested that TLR6 can interact with innate immunity receptors other than TLR2 to sense microbial invading and stimulate host-defensive response.²⁹ The results of clinical analysis should be considered exploratory and need to be validated in a larger cohort of patients.

In summary, this study identifies that a TLR2-centered innate immunity pathway is deregulated in MDS, including both gene overexpression and genetic alteration. These results, together with our recent identification of JMJD3 and other genes that are involved in innate immunity regulation, further support a role of innate immunity signals in the BM stem/progenitor cells of MDS.

Further characterization of TLR2-associated innate immunity signals, particularly the potential TLR2-JMJD3-IL-8 axis, may have prognostic and therapeutic benefits in MDS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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