Impaired clearance of apoptotic cells leads to HMGB1 release in the bone marrow of patients with myelodysplastic syndromes and induces TLR4-mediated cytokine production

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ABSTRACT

Excessive pro-inflammatory cytokine production in the bone marrow has been associated with the pathogenesis of myelodysplastic syndromes. We herein investigated the involvement of toll-like receptors and their endogenous ligands in the induction/maintenance of the inflammatory process in the marrow of patients with myelodysplastic syndromes. We evaluated the expression of toll-like receptors in marrow monocytes of patients (n=27) and healthy controls (n=25) by flow-cytometry and also assessed the activation of the respective signaling using a real-time polymerase chain reaction-based array. We measured the high mobility group box-1 protein, a toll-like receptor-4 ligand, in marrow plasma and long-term bone marrow culture supernatants by an enzyme-linked immunosorbent assay and we performed cross-over experiments using marrow plasma from patients and controls in the presence/absence of a toll-like receptor-4 inhibitor to evaluate the pro-inflammatory cytokine production by chemiluminescence. We assessed the apoptotic cell clearance capacity of patients' macrophages using a fluorescence microscopy-based assay. We found over-expression of toll-like receptor-4 in patients' marrow monocytes compared to that in controls; this over-expression was associated with up-modulation of 53 genes related to the respective signaling. Incubation of patients' monocytes with autologous, but not with normal, marrow plasma resulted in over-production of pro-inflammatory cytokines, an effect that was abrogated by the toll-like receptor-4 inhibitor suggesting that the pro-inflammatory cytokine production in myelodysplastic syndromes is largely mediated through toll-like receptor-4. The levels of high mobility group box-1 protein were increased in patients' marrow plasma and culture supernatants compared to the levels in controls. Patients' macrophages displayed an impaired capacity to engulf apoptotic cells and this defect was associated with excessive release of high mobility group box-1 protein by dying cells. A primary apoptotic cell clearance defect of marrow macrophages in myelodysplastic syndromes may contribute to the induction/maintenance of the inflammatory process through aberrant release of molecules inducing toll-like receptor-4 such as high mobility group box-1 protein.

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Introduction

Myelodysplastic syndromes (MDS) constitute a group of clonal bone marrow (BM) disorders characterized by ineffective hematopoiesis, peripheral blood cytopenias and a high risk of transformation to acute myeloid leukemia.[1] Many models have been generated to unravel the complex pathophysiological process(es) leading to MDS development and progression. Excessive pro-inflammatory and inhibitory cytokine production in MDS BM has been recognized as a prominent pathogenic mechanism that disrupts hematopoiesis by inducing the apoptotic death of the BM progenitor/precursor cells.[2-4] In accordance with the aberrant cytokine production in the marrow microenvironment is the constitutively activated p38 mitogen activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) molecular pathways in BM cellular subsets of MDS patients.[5,6] However, the upstream pathways, the exact cellular source and the triggering events related to this cytokine excess in MDS BM remain unknown.

Toll-like receptors (TLRs) are a family of pattern recognition receptors which, upon ligand engagement, activate signaling pathways that result in production of numerous cytokines and inflammatory mediators.[7,8] This process can be especially useful in the case of pathogen-derived ligands representing essentially a first line of defense to microbe invasion. Nevertheless, TLRs can be activated by endogenous ligands released under stress conditions, such as heat-shock proteins, fibrinogen, extracellular matrix and high mobility group box 1 (HMGB1) protein; this process is apparently equally important, as it allows the host to respond to dangerous internal stimuli.[9] However, extended activation of TLRs by endogenous ligands has been associated with many inflammatory, autoimmune

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and malignant diseases by inducing and sustaining the inflammatory processes.[10,11] We have recently shown that TLR4 activation by HMGB1 in the BM of patients with chronic idiopathic neutropenia, a mild BM failure syndrome that shares common pathogenetic characteristics with MDS, contributes to perpetuation of the inflammatory BM milieu that induces the apoptotic death of the granulocytic progenitor cells.[12]

The possible involvement of TLRs in the pathophysiology of MDS has been very little studied.[13,14] In the present study we probed the possible involvement of TLRs in the generation and maintenance of the inflammatory BM microenvironment in MDS. Specifically, we studied basal surface TLR expression and degree of activation of TLR-related signal transduction pathways in BM monocytes and microenvironment cells of MDS patients and looked for potential endogenous TLR ligands. Furthermore, because tissue homeostasis is largely dependent on the effective clearance of the apoptotic cell load by tissue macrophages, we also investigated whether a macrophage failure to clear the excess of apoptotic cells in MDS BM might contribute to the inflammatory process through aberrant release of TLR-inducing self molecules.

Design and Methods

Patients and controls

We studied 27 adults with *de novo* MDS, 19 males and 8 females, aged 60-89 years (median age, 79 years). The patients' characteristics are presented in detail in *Online Supplementary Table S1*. As controls, we studied 25 hematologically healthy subjects who were undergoing lumbar or hip orthopedic surgery and who were age- and sex-matched with the patients. None of the patients or controls had infections at the time of the study or during the preceding 3 months. The study was approved by the Ethics Committee of the University Hospital of Heraklion and informed consent according to the Helsinki Protocol was obtained from all subjects.

Bone marrow samples

BM aspirates from the posterior iliac crest were diluted 1:1 with Iscove's modified Dulbecco's medium (IMDM; Gibco Invitrogen, Paisley, United Kingdom) supplemented with 100 IU/mL penicillin-streptomycin (PS; Gibco) and 10 IU/mL preservative-free heparin (Sigma-Aldrich, St. Louis, MO, USA). BM mononuclear cells (BMMC) were obtained following density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich) at 400g for 30 min. Undiluted BM samples from a second aspiration were collected into tubes containing ethylenediaminetetraacetic acid and centrifuged at 800g for 20 min to obtain the BM plasma.

Long-term bone marrow cultures

Long-term bone marrow cultures (LTBMC) were grown according to a standard assay described in the *Online Supplementary Design and Methods*. When cultures reached confluency (week 3), cell-free supernatants were harvested for HMGB1 measurement, while the adherent cell layers, representing an approximation of the marrow microenvironment cells, were trypsinized and assayed by flow-cytometry for TLR expression. Further details are provided in the *Online Supplementary Design and Methods*.

Generation of bone marrow-derived macrophages

BM-derived macrophages were generated from BMMC of MDS patients (n=5) and normal subjects (n=5) according to an established protocol with some modifications.[15] Detailed information regarding the experimental protocol is provided in the *Online Supplementary Design and Methods*. Macrophage monolayers were prepared by plating 500,000 trypsinized macrophages in each chamber of a two-chamber slide or 200,000 macrophages in each well of a 24-well plate, for 24 h before further experiments.

Phagocytosis assay

To evaluate the apoptotic cell clearance capacity of BM macrophages, a fluorescent microscopy-based phagocytic assay was developed. The experimental procedure is described in detail in the *Online Supplementary Design and Methods* and in *Online Supplementary Figure S1*.

Reverse transcription and quantitative polymerase chain reaction analysis

Reverse transcription, real-time polymerase chain reaction (PCR) and gene set enrichment analysis of the TLR-related genes were performed as reported in the *Online Supplementary Design and Methods*.

Crossover experiments to evaluate the effect of bone marrow plasma in TLR-mediated cytokine production by bone marrow monocytes

BMMC from MDS patients and healthy controls were suspended at a concentration of $2x10^6$ cells/mL in RPMI medium/10% fetal bovine serum. We next added 1 mL of this suspension into seven wells of a 24-well culture plate and incubated it at 37°C in 5% CO_2 in a humidified incubator for 3 h. Non-adherent cells were removed and 500 μL of the medium were added to each well containing the plastic adherent BM monocytes.[15] Cells in well 1 and well 4 were pre-incubated with 20 μg of Functional Grade anti-human TLR4 blocking monoclonal antibody (clone HTA125; eBioscience, San Diego, CA, USA) while cells in well 2 and well 5 were pretreated with Functional Grade mouse IgG2a isotype control monoclonal antibody (eBioscience) for 1 h in the incubator. Cells in well 3 and well 6 were left untreated. At the end of the incubation period 500 μL of autologous or normal BM plasma were added to wells 1-3 and wells 4-6, respectively, of the cultures containing BM monocytes from MDS patients. In cultures containing normal monocytes, autologous or BM plasma derived from MDS patients was added to wells 1-3 and wells 4-6, respectively. In all cases, 500 μL of RPMI medium were added to well 7. Following 24 h of incubation, the supernatants were collected and stored at -80°C for cytokine measurements. The levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α produced by the adherent BM monocytes, in the presence or absence of the specific TLR4 blocking monoclonal antibody or the non-specific control peptide, were evaluated by means of chemiluminescent technology (DPC-Immulite, Los Angeles, CA, USA). Thereafter, the percentage of inhibition of cytokine production induced by the TLR4 blocking monoclonal antibody was determined by dividing the difference in cytokine levels between the test culture and the culture treated with the TLR4 blocking monoclonal antibody by the cytokine level in the test culture.

Clonogenic assay to evaluate the effect of the apoptotic cells and HMGB1 protein on the colony-forming potential of bone marrow progenitor cells

To examine whether the impaired clearance of apoptotic cells by MDS macrophages might contribute to the ineffective hematopoiesis observed in MDS patients, we used a previously described two-stage culture procedure with some modifications.[16] In brief, macrophage layers from MDS patients (n=6) or healthy subjects (n=6) in six-well plates were irradiated (10 Gy) and then recharged with $2x10^4$ allogeneic normal $CD34^+$ BM cells in the

presence or absence of 2x10^6 apoptotic or live allogeneic normal peripheral blood mononuclear cells (PBMCs) in the presence or not of a TLR4-blocking monoclonal antibody. The clonogenic potential of the non-adherent cell fraction containing the CD34$^+$ cells was assessed in a week's time by means of a previously described clonogenic assay and the total colonies were scored and characterized as total colony-forming cells (CFC).[16] Finally, we evaluated the CFC numbers in the non-adherent cell fraction of normal macrophage cultures recharged with allogeneic normal CD34$^+$ BM cells in the presence or absence of rhHMGB1 at a concentration 300 ng/mL, corresponding to the mean cytokine levels measured in the BM plasma of MDS patients.

Statistical analysis

Data were analyzed using the GraphPad Prism Statistical PC program (GraphPad Software, San Diego, CA, USA). Grouped data were compared using the non-parametric Mann Whitney U test. The non-parametric Wilcoxon signed rank test for paired samples was used for the comparison of cytokine production in monocyte cultures treated with BM plasma in the presence or absence of the TLR4-blocking monoclonal antibody as well as the CFC numbers in cultures treated with apoptotic or live cells or HMGB1 protein. The two-way analysis of variance test (ANOVA) was used to test HMGB1 levels in macrophage layers co-cultured with different BMMC concentrations at different time-points. The homogeneity of the age and sex distribution of the patient and control groups was tested by the χ^2 test. Grouped data are expressed as mean ± 1 standard deviation.

Results

Increased expression of TLR4 in the CD14$^+$ cell fraction of bone marrow from patients with myelodysplastic syndrome

Results from the flow-cytometric evaluation of the proportion and the mean ratio of relative fluorescence intensity (MRFI) of surface TLR1, TLR2, TLR4 and intracellular TLR3 and TLR9 in the monocytic BM cell fraction and the monocytic and non-hematopoietic cell fractions of LTBMC adherent cells of MDS patients and controls are presented in *Online Supplementary Table S2*. A statistically significant increase was observed in the proportion of TLR4$^+$ cells within the CD14$^+$ cell fraction of BM cells of patients compared to controls (P<0.0001); this increase was paralleled by an up-regulation of TLR4 expression, as indicated by the increased TLR4 MRFI in MDS patients (P=0.0002). These abnormalities did not correlate with the disease severity because no statistically significant difference was documented between the Low/Intermediate-1 patients (n=23) and Intermediate-2 patients (n=4) in the proportion of TLR4 expressing CD14$^+$ cells (6.28±5.65% and 5.05±2.17%, respectively) or their MRFI (1.29±0.33 and 1.33±0.19, respectively). Similarly, no statistically significant differences were identified in the proportion or MRFI of TLR4-expressing CD14$^+$ cells among patients with different types of MDS (*data not shown*). Overall, a trend towards an increased expression of all TLRs tested was observed in MDS patients compared to controls, but the differences found were not statistically significant. Regarding the LTBMC adherent cells, there were significant increases in both the proportion and MRFI expression of TLR4 (P=0.0288 and P=0.0232, respectively) in the monocytic CD45$^+$/CD14$^+$ cell fraction of MDS patients compared to controls while a non-statistically significant increase was observed in all other TLRs tested. Similarly, in the non-hematopoietic (CD45-) adherent cell population, a non-statistically significant trend towards an increased expression of all TLRs was obtained in MDS patients compared to controls. Overall, these data show that the monocytes and BM microenvironment cells of patients with MDS display a degree of TLR up-modulation with a prominent increase of TLR4 in the monocytic cell populations.

Up-regulation of TLR4-mediated signaling in bone marrow CD14$^+$ cells from patients with myelodysplastic syndromes

To determine whether TLR4 over-expression in BM monocytes of MDS patients is associated with up-regulated TLR-mediated signaling, we screened 84 TRL-associated genes in immunomagnetically sorted CD14$^+$ BM cells from MDS patients (n=3; # 2, 5, and 23 in *Online Supplementary Table S1*) and healthy controls (n=3). As shown in Figure 1A, 53 out of 84 TLR-related genes displayed at least a 4-fold increase in mRNA expression in MDS patients compared to controls. The up-regulated genes were further characterized according to their function as genes encoding TLRs and TLR signaling molecules, adaptor and TLR interacting molecules, effectors and molecules regulating adaptive immunity, and signaling molecules associated with specific downstream pathways such as the NFκB pathway, the JUN N-terminal kinase (JNK)/p38 pathway, the Janus kinase and signal transducer and activator of transcription (JAK/STAT) pathway, the interferon (IFN)-regulatory factor (IRF) pathway, and cytokine-mediated pathways (*Online Supplementary Table S3*). Interestingly, genes involved in both myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways were found to be over-expressed in MDS patients compared to controls indicating activation of TLR4-mediated signaling, which is known to involve both the MyD88-dependent and MyD88-independent pathways leading finally to NFκB activation.[17] Indeed, a number of genes related to NFκB signaling and the JNK/p38 pathway were found to be up-regulated in MDS patients suggesting that TLR4 over-expression in patients' monocytes is associated with downstream activation of NFκB and JNK/p38 pathways (*Online Supplementary Table S3*).

The results of the gene set enrichment analysis for genes showing at least a 4-fold up-regulation in patients revealed interesting molecular functions, biological processes and cellular components that are significantly enriched in the differentially expressed genes under consideration (*Online Supplementary Table S4*). Interestingly, a number of genes fall in the cytokine activity molecular functional group (P=0.0009), a finding that further supports the involvement of BM monocytes in the generation of the inflammatory BM milieu in MDS.

To validate the data obtained from the PCR array analysis, we evaluated the mRNA expression of three representative genes, namely MyD88, TRIF/TICAM1 and TRAM/TICAM2, also representing key-adaptor molecules for MyD88-dependent and MyD88-independent TLR4 signaling, by means of individual quantitative RT-PCR reactions. The results, normalized to the expression of the RPL13A housekeeping gene, are illustrated in Figure 1B. The mean relative mRNA expression of MyD88, TRIF/TICAM1 and TRAM/TICAM2 in BM CD14$^+$ cells was significantly increased in MDS patients (2.39±1.26, 2.23±2.28 and 0.08±0.03, respectively) compared to con-

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trols (0.76±0.43, 0.89±0.60 and 0.01±0.009, respectively) (P=0.0001, P=0.0159 and P<0.0001, respectively).

Furthermore, we evaluated the mRNA expression of IRAKM and SHIP1, genes that negatively regulate TLR-mediated signaling and, therefore, contribute to the resolution of TLR-induced inflammatory reactions. MDS patients displayed increased expression of both IRAKM (0.80±0.35) and SHIP1 (0.57±0.17) compared to healthy controls (0.42±0.40 and 0.23±0.10; P=0.0251 and P<0.0001, respectively) (Figure 1C). These data indicate a compensatory control mechanism to TLR-mediated signaling but also suggest that the activated TLR signaling in patients' BM monocytes is unlikely to be due to inadequate suppressor mechanisms but is apparently due to constant stimulatory effects.

TLR4-dependent cytokine production by bone marrow monocytes following incubation with bone marrow plasma

The responses initiated by TLR4 activation are expected, in the end, to induce the production of a variety of cytokines. To investigate whether TLR4 up-regulation in patients' CD14+ BM cells is implicated in the production of pro-inflammatory cytokines in MDS BM under the influence of putative endogenous ligand(s), we performed a number of crossover experiments. Specifically, we evaluated the levels of IL-1β, IL-6 and TNFα in the supernatants of plastic adherent BM monocytes from MDS patients (n=7; #2, 4, 5, 13, 17, 23, and 24 in *Online Supplementary Table S1*) or normal subjects (n=6) following treatment with autologous or normal (for the experiments with MDS-derived monocytes) or MDS (for the experiments with normal monocytes) BM plasma, in the presence or absence of a specific TLR4 inhibitor or a non-specific control peptide. The baseline BM plasma concentration of the above cytokines was subtracted from all estimations.

In cultures of monocytes from MDS patients, the addition of autologous BM plasma induced significant increases in the production of IL-1β, IL-6 and TNFα compared to baseline (cultures treated with medium alone) (P=0.0156, P=0.0156 and P=0.0156, respectively). In the presence of

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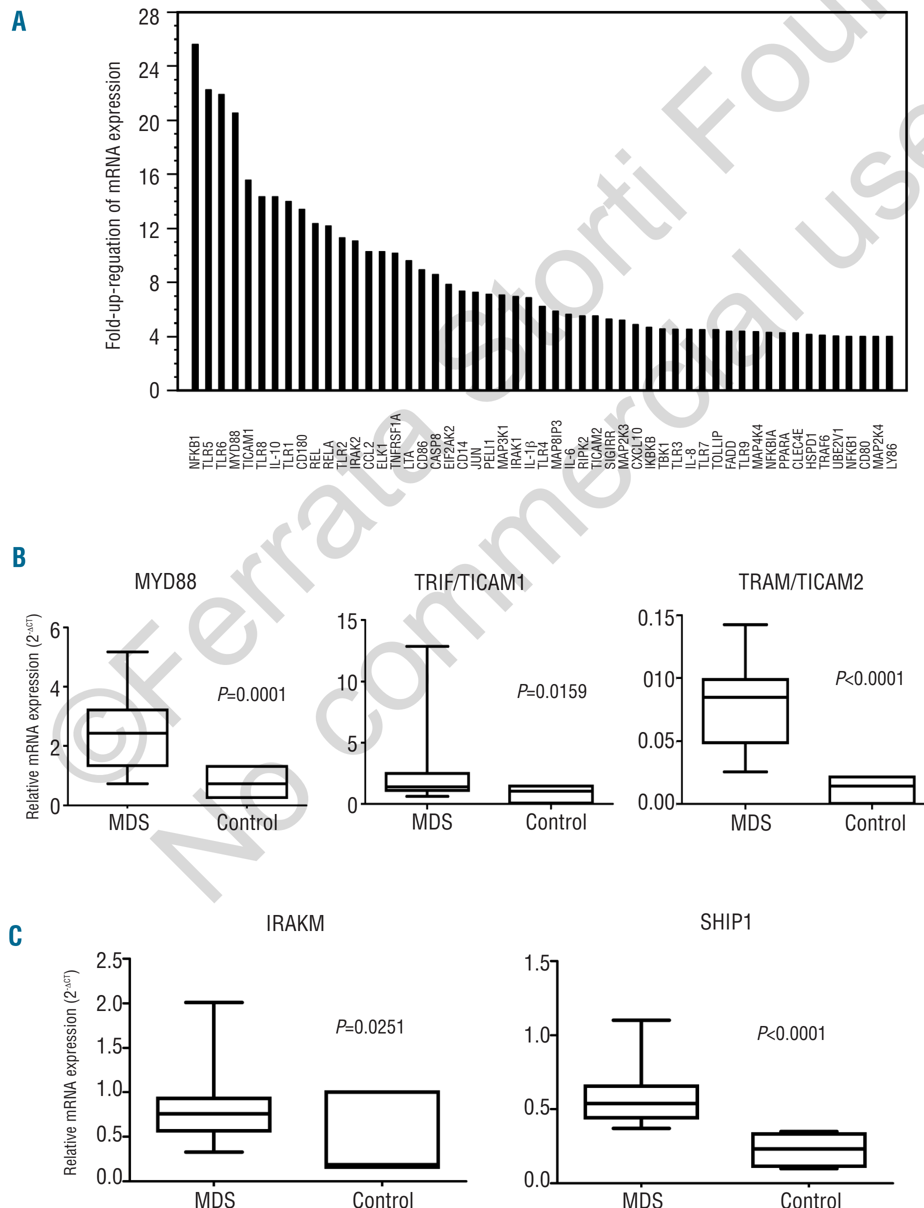

Figure 1. Relative expression of genes involved in TLR signaling in BM CD14+ cells from MDS patients compared to normal controls. (A) Columns represent the fold up-regulation of genes involved in TLR signaling in BM CD14+ cells of MDS patients (n=3; #2, 5, 23 in *Online Supplementary Table S1*) compared to healthy individuals (n=3) using a real time PCR array. The figure depicts genes exhibiting at least a 4-fold up-regulation in MDS compared to normal samples. Fold-change was calculated as the ratio between the MDS and normal relative gene mRNA expression. (B) Each box plot depicts the relative mRNA expression of *MYD88*, *TRIF/TICAM1* and *TRAM/TICAM2* in the BM CD14+ cells of patients and controls assessed by individual real time RT-PCR to validate the array data. (C) Box plots of the relative *IRAKM* and *SHIP1* mRNA expression, representing negative regulators of TLR signaling, as estimated by real-time RT-PCR. The expression of genes depicted in graphs (B) and (C) was calculated according to the threshold cycle (Ct) relative quantification $2^{-\Delta CT}$ method, using *RPL13A* as the housekeeping gene for normalization, where ΔCt=[Ct(gene)-Ct(RPL13A)]. MDS and control groups were compared by the non-parametric Mann-Whitney U test and the P values are shown. Median values are indicated by the horizontal lines in the boxes. The whiskers extend to the minimum and maximum values in the groups tested.

the TLR4 inhibitor, the levels of IL-1β, IL-6 and TNFα decreased significantly (9.33±2.62 pg/mL, 136.31±52.01 pg/mL, and 6.92±1.30 pg/mL, respectively) compared to cultures treated with autologous BM plasma alone (26.42±11.33pg/mL, 503.86±159.45 pg/mL, and 57.43±5.56 pg/mL, respectively; *P*=0.0156, *P*= 0.0156 and *P*=0.0156, respectively) (Figure 2) with a percentage of inhibition of 62.03±10.26%, 70.92±12.28%, and 87.93%±2.10, respectively. In the same set of experiments, the addition of BM plasma from healthy subjects did not have a significant effect on IL-1β, IL-6 or TNFα production compared to baseline; the presence of the TLR4 inhibitor did not have a significant effect on cytokine production either (Figure 2). The mean percentage of TLR4 inhibitor-mediated reduction of IL-1β, IL-6 and TNFα production by patients' monocytes was significantly lower in cultures treated with normal plasma (6.29±12.55%, 1.85±15.29%, and 3.23±29.52%, respectively) than with autologous plasma (*P*=0.0006, *P*=0.0006, and *P*=0.00006, respectively).

In cultures of monocytes from normal subjects, the addition of autologous BM plasma did not result in a significant increase in the production of IL-1β, IL-6 and TNFα compared to baseline (cultures treated with medium alone) and the addition of the TLR4 inhibitor did not result in any significant effect in cytokine levels (4.49±1.61 pg/mL, 59.62±5.94 pg/mL, 4.78±1.23 pg/mL, respectively) compared to cultures treated with autologous plasma alone (5.66±1.47 pg/mL, 62.72±7.36 pg/mL, 5.09±0.74 pg/mL) (Figure 2). In the same set of experiments however, the addition of BM plasma from MDS patients resulted in a sig-

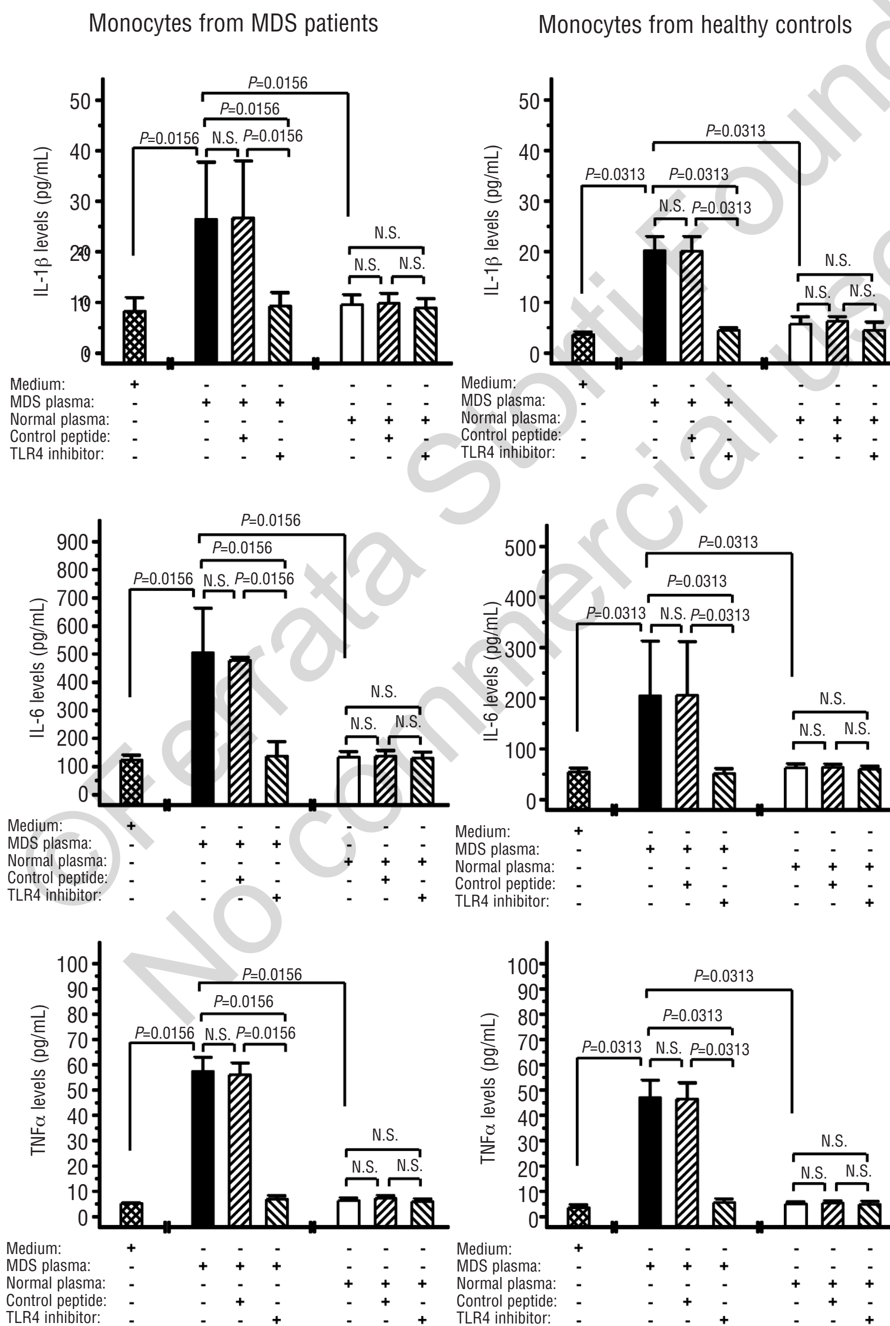

Figure 2. Crossover experiments for the evaluation of the effect of BM plasma from MDS patients or healthy subjects on pro-inflammatory cytokine production by BM monocytes from patients or healthy subjects. The graphs on the left depict the mean (plus one standard deviation) of production of IL-1β, IL-6 and TNFα by BM monocytes from MDS patients (n=7; #2, 4, 5, 13, 17, 23, 24 in *Online Supplementary Table S1*) following treatment with medium, autologous or normal BM plasma in the presence or absence of a specific TLR4 inhibitor (anti-human TLR4 blocking monoclonal antibody) or a non-specific control peptide (IgG2a isotype control monoclonal antibody). Similarly, the graphs on the right depict the cytokine levels in monocyte cultures derived from healthy subjects (n=6) following treatment with medium, autologous or MDS-derived BM plasma in the presence or absence of the TLR4 inhibitor or the control peptide. Cytokine levels were evaluated by means of a chemiluminescence assay. Comparisons were performed using the non-parametric Wilcoxon signed rank test for paired samples and the *P* values are indicated. N.S. denotes a non-statistically significant difference.

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nificant increase in the production of IL-1β, IL-6 and TNFα production compared to baseline (P=0.0313, *P*=0.0313 and *P*=0.0313, respectively). The addition of the TLR4 inhibitor significantly decreased the levels of IL-1β, IL-6 and TNFα (4.45±0.56 pg/mL, 51.73±9.27 pg/mL, and 5.71±1.29 pg/mL, respectively) compared to cultures treated with BM plasma (MDS-derived) alone (20.18±2.80 pg/mL, 204.53±108.09 pg/mL, and 46.96±6.94 pg/mL, respectively; *P*=0.0313, *P*=0.0313 and *P*=0.0313, respectively) (Figure 2). Overall, the percentage of TLR4 inhibitor-mediated reduction of IL-1β, IL-6 and TNFα production was significantly greater in monocyte cultures treated with MDS-derived BM plasma (77.74±2.76%, 68.49±16.55%, and 87.43±4.66%, respectively) compared to that in cultures treated with autologous normal plasma (9.59±59.90%, 3.52±17.75%, and 4.78±27.66%, respectively) (*P*=0.0022, *P*=0.0022, *P*=0.0022, respectively). No significant differences were observed in any of the sets of experiments in the levels of cytokines between the cultures pre-treated with the non-specific control peptide before the addition of the BM plasma (autologous or normal) and the cultures treated with BM plasma alone. Furthermore, no statistically significant differences were found between patients' and control cultures in the production of cytokines following treatment with medium alone, indicating that intrinsic cell differences are unlikely to have a major role in the overproduction of pro-inflammatory cytokines by patients' monocytes. All the above data strongly suggest that soluble factor(s) present in the BM of MDS patients apparently induce the production of pro-inflammatory cytokines by MDS and normal BM monocytes via a TLR4-mediated pathway.

Increased HMGB1 levels in supernatants of long-term bone marrow cultures and bone marrow plasma from patients with myelodysplastic syndromes

Recent evidence suggests that HMGB1, apart from its intracellular actions of stabilizing nucleosomes and facilitating transcription, can also be released extracellularly and may induce pro-inflammatory cytokine production upon ligation to TLR4 through activation of the NFκB and JNK/p38 pathways.[18-21] In order to probe the hypothesis that HMGB1 might be involved in the activation of TLR4 in BM monocytes of MDS patients, we compared protein levels in LTBMC supernatants of MDS patients (n=27) and healthy individuals (n=25). HMGB1 levels were significantly higher in patients (3.02±2.94 ng/mL) than in controls (0.96±1.26 ng/mL; *P*=0.0186) (Figure 3) corroborating the hypothesis that HMGB1 protein might constitute an endogenous TLR4-activating ligand in MDS BM. The increased levels of HMGB1 in the BM plasma of MDS patients (n=7; #2, 4, 5, 13, 17, 23, and 24 in *Online Supplementary Table S1*) (327.04±48.51 ng/mL) compared to healthy controls (n=6) (90.75±20.93) (*P*=0.0012) further substantiates the above hypothesis. Notably, the increased HMGB1 levels in LTBMC supernatants did not differ significantly between the Low/Intermediate-1 (3.05±3.03 ng/mL, n=23) and Intermediate-2 (2.86±2.80 ng/mL, n=4) MDS patients. Similarly, there were no significant differences in HMGB1 levels between patients with different types of MDS (*data not shown*).

Impaired apoptotic cell clearance by bone marrow macrophages in patients with myelodysplastic syndromes leads to HMGB1 release

HMGB1 is passively released from necrotic and damaged cells; however, it remains inside cells undergoing apoptosis and this mechanism seems to act protectively, preventing apoptotic death from being immunogenic and pro-inflammatory.[22,23] It has been shown however that inadequate removal of apoptotic cells by professional phagocytes may lead to secondary cell necrosis resulting in extracellular release of HMGB1.[24] To probe the hypothesis that increased HMGB1 levels in the MDS BM microenvironment might be the result of ineffective clearance of apoptotic cells by BM macrophages, we co-cultured BM-derived macrophages from MDS patients (n=5; # 2, 4, 5, 23, and 24 in *Online Supplementary Table S1*) or normal subjects (n=5) with autologous apoptotic BM cells and we calculated the phagocytic/efferocytic indices. BM macrophages from MDS patients did indeed display decreased apoptotic cell phagocytosis capacity (12.00±2.00%) in comparison to those from healthy individuals (36.70±4.81%; *P*=0.0079).

To examine the biological consequences of the impaired clearance of apoptotic cells by MDS-derived BM macrophages in terms of HMGB1 protein release, which might result in TLR4 activation, we loaded increasing numbers, i.e. $4x10^5$, $2x10^6$ and $4x10^6$, apoptotic or freshly isolated BMMCs on autologous macrophage monolayers from MDS patients (n = 3; # 2, 5, and 23 in *Online Supplementary Table S1*) in the presence or absence of the

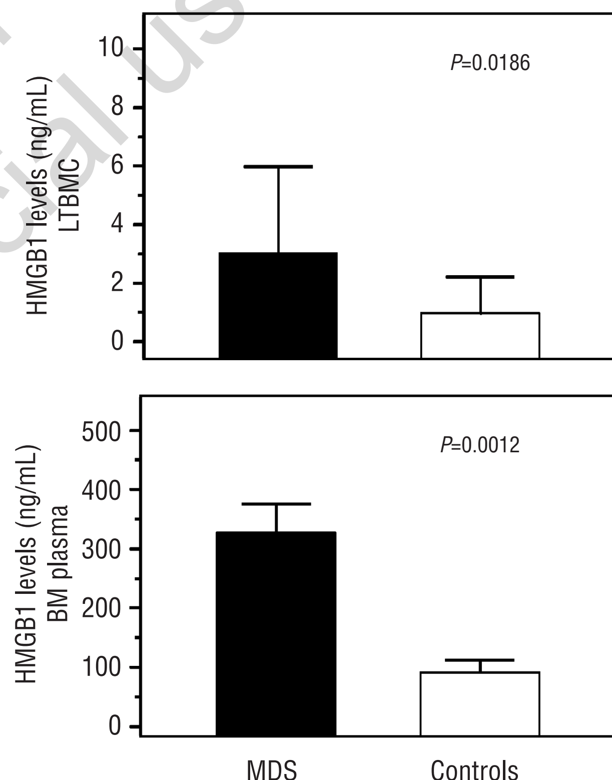

Figure 3. Levels of HMGB1 in LTBMC supernatants and BM plasma. The bars represent the mean (plus one standard deviation) concentration of HMGB1 protein in the supernatants of confluent LTBMCs from MDS patients (n=27) and healthy individuals (n=25) (upper graph) and in BM plasma from MDS patients (n=7; # 2, 4, 5, 13, 17, 23, 24 in *Online Supplementary Table S1*) and healthy controls (n=6) (lower graph). Measurements were made by means of an ELISA. Comparisons were made by the non-parametric Mann Whitney test and the *P* values are indicated.

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TLR4-blocking monoclonal antibody for 12, 24 and 36 h for each cell concentration. Experiments were performed in triplicate. At the end of each incubation period, the supernatants were collected and assayed for HMGB1 by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 4A, HMGB1 release by BM macrophages from MDS patients was dependent on the apoptotic cell load (P<0.001) and incubation time (P=0.0417). In particular, HMGB1 levels in macrophage cultures containing $4x10^5$, $2x10^6$ and $4x10^6$ apoptotic cells were 7.37±0.61, 12.54±2.34 and 22.09±3.28 ng/mL at 12 h, 7.86±.52, 20.09±6.98 and 32.22±8.94 ng/mL at 24 h, and 8.58±1.05, 24.12±12.61 and 36.43±11.99 ng/mL at 36 h. Incubation of the same macrophage layers with freshly isolated autologous BMMCs resulted in a dose-dependent (P<0.001) but not a time-dependent increase of HMGB1 levels compared to baseline. Specifically, HMGB1 levels in cultures containing $4x10^5$, $2x10^6$ and $4x10^6$ fresh BMMC cells were 4.51±0.17, 8.96±1.24 and 15.56±6.15 ng/mL at 12 h, 6.22±0.08, 10.42±3.69 and 20.10±6.74 ng/mL at 24 h, and 6.83±1.55, 10.76±3.25 and 19.30±8.24 ng/mL at 36 h. For each incubation period (12, 24 and 36 h) HMGB1 levels were significantly lower in cultures containing fresh BMMCs compared to the corresponding cultures containing apoptotic BMMCs (P=0.011, P=0.01261 and P=0.0147, respectively) (Figure 4B). In normal subjects (n=3), a statistically significant difference in HMGB1 levels between cultures containing live and apoptotic cells was detected only in the supernatants of cultures with the highest apoptotic cell concentration (*data not shown*) suggesting that the capacity of normal macrophages to clear apoptotic cells efficiently is apparently saturated at the highest apopotic cell load resulting in release of HMGB1 from the remaining late apoptotic/necrotic cells. Furthermore, the presence of a TLR4 inhibitor in the cultures did not have any effect on HMGB1 levels (*data not shown*) suggesting that HMGB1 production/release is mediated through a TLR4-independent mechanism.

Taken together, these data suggest that impaired apoptotic cell clearance by BM macrophages in MDS may lead to a TLR4-independent release of HMGB1 by the secondary necrotic cells at a concentration proportional to the apoptotic cell load. HMGB1 may, in turn, induce a TLR4-dependent inflammatory cytokine release by BM macrophages.

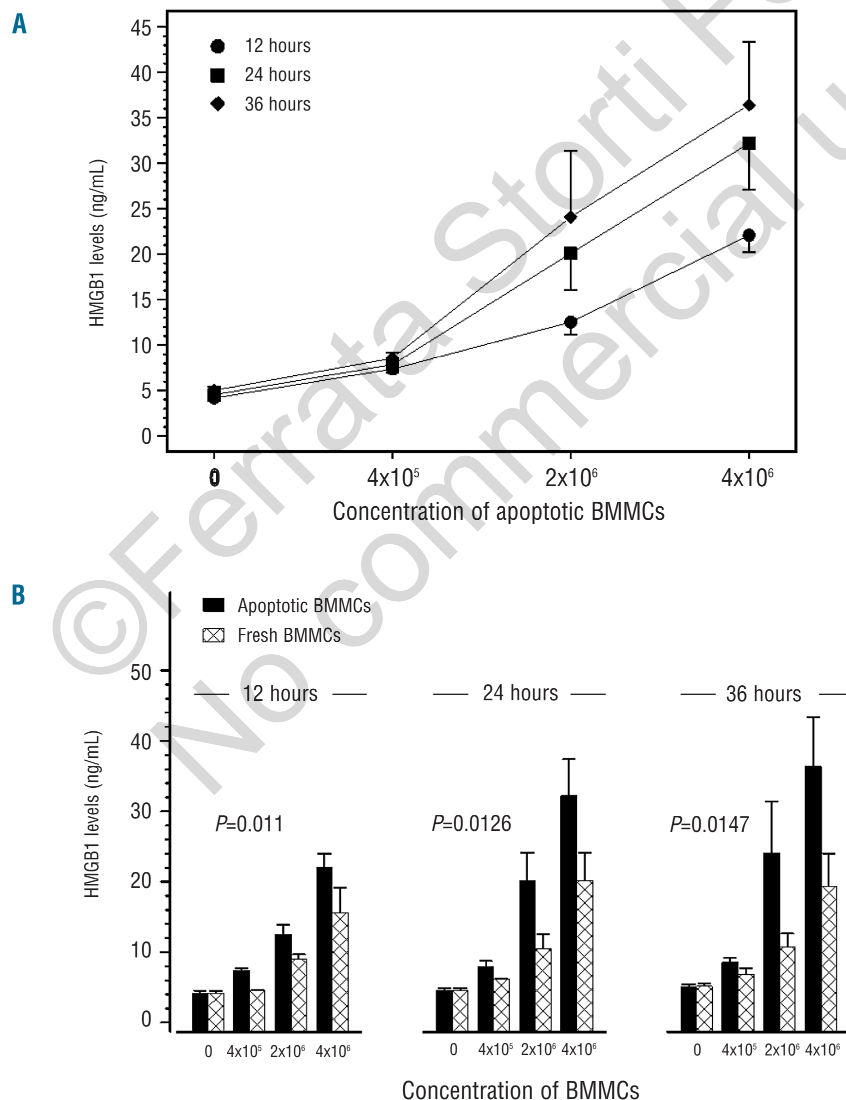

Figure 4. Time course of HMGB1 release in the supernatants of MDS macrophages loaded with increasing numbers of apoptotic BMMCs. (A) BM-derived macrophages from MDS patients (n=3; # 2, 5, 23 in *Online Supplementary Table S1*) were co-cultured with $4x10^5$, $2x10^6$ and $4x10^6$ apoptotic autologous BMMCs for 12, 24 and 36 h. At the end of each incubation period the supernatants were assayed for HMGB1 by means of an ELISA. The dots represent the mean (plus or minus one standard error) HMGB1 concentration for a defined experimental condition. HMGB1 concentration was dependent on the number of the loaded apoptotic cells (P<0.0001) and the incubation time (P=0.0417). Statistical analysis of HMGB1 levels according to the apoptotic cell load and incubation time was performed by means of the two-way analysis of variance test. (B) The bars represent the mean HMGB1 levels (plus one standard error) in the supernatants of co-cultures of BM macrophages with apoptotic or fresh autologous BMMCs from MDS patients. The concentration of the apoptotic/fresh cell load and the incubation time are indicated. For each incubation period HMGB1 levels were significantly higher in cultures with apoptotic compared to those with fresh BMMCs. Analysis was performed by means of the two-way analysis of variance test and the P values are shown.

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Impaired clonogenic potential of normal CD34+ cells in the presence of apoptotic cells or HMGB1

To investigate whether the impaired clearance of apoptotic cells by MDS macrophages might contribute to the ineffective hematopoiesis observed in MDS patients, we recharged monocyte cultures from MDS patients (n=6) or healthy subjects (n=6) with allogeneic normal CD34+ cells in the presence or absence of apoptotic or live allogeneic PBMCs. The results are presented in *Online Supplementary Figure S2*. The presence of apoptotic cells significantly decreased the numbers of CFC produced by the non-adherent cells of recharged MDS-derived macrophage cultures (7.00±2.45 CFC per $2x10^4$ CD34+ cells) compared to the respective cultures containing only CD34+ cells (48.0±14.20 CFC per $2x10^4$ CD34+ cells) (P=0.0313) (*Online Supplementary Figure S2A*). In contrast, numbers of CFC produced by the non-adherent cell fraction of normal macrophage cultures did not differ significantly between cultures treated or not with apoptotic cells (106.0±21.69 CFC per $2x10^4$ CD34+ cells and 114.0±5.37 CFC per $2x10^4$ CD34+ cells, respectively) (*Online Supplementary Figure S2B*). The presence of the TLR4 inhibitor significantly increased the numbers of CFC produced by the non-adherent cells of MDS-derived macrophage cultures (34.0±7.27 CFC per $2x10^4$ CD34+ cells) compared to the respective cultures with the apoptotic cells only (P=0.0313) (*Online Supplementary Figure S2A*). As expected, the presence of the TLR4 inhibitor did not have a significant effect on the clonogenic potential of the non-adherent cells in cultures derived from normal macrophages. Interestingly however, when the normal macrophage cultures were recharged with allogeneic normal CD34+ cells in the presence of a higher concentration of apoptotic PBMCs, i.e. 4 $x10^6$, significantly fewer CFC were produced by the non-adherent cells (66.0±9.25 CFC per $2x10^4$ CD34+ cells) in comparison to cultures not containing apoptotic cells (P=0.0313) apparently implying that the increased apoptotic cell load exceeds the clearance capacity of normal macrophages (*Online Supplementary Figure S2B*).

The presence of live PBMCs in MDS-derived macrophage cultures did not have any significant effect on the clonogenic potential of non-adherent cells (43.0±17.46 CFC per $2x10^4$ CD34+ cells) compared to the respective cultures containing CD34+ cells only; likewise, the presence of a TLR4 inhibitor did not exert any significant effect on CFC formation (49.0±15.72 CFC per $2x10^4$ CD34+ cells) (*Online Supplementary Figure S2A*). Finally, in cultures of macrophages from healthy subjects recharged with allogeneic normal CD34+ cells, the presence of rhHMGB1 significantly decreased the clonogenic potential of the non-adherent cells (46.0±32.79 CFC per $2x10^4$ CD34+ cells) compared to cultures not treated with rhHMGB1 (86.0±48.10 CFC per $2x10^4$ CD34+ cells) (P=0.0313) (*Online Supplementary Figure S2C*).

Taken together, all these data suggest that the impaired clearance of apoptotic cells by MDS macrophages negatively affects BM hematopoiesis in MDS patients through a TLR4-mediated mechanism that probably involves the HMGB1 protein.

Discussion

The recognition of accelerated apoptotic cell death as an important element of the pathogenesis of MDS provides a satisfying explanation for the paradox of a hypercellular BM with peripheral cytopenias but raises further questions as regards the underlying mechanisms that trigger and sustain the apoptotic process. It has become clear, however, that not only the MDS clone cells but also the BM microenvironment cells and the abnormal interactions thereof are involved in the apoptotic mechanisms through disturbed production of growth-promoting cytokines and aberrant release of inhibitors and pro-inflammatory mediators.[25-27] The clarification of the mechanisms underlying the abnormal BM milieu in MDS is of particular importance not only for better understanding of the disease pathogenesis but also for the development of novel therapeutic approaches targeting cytokines, signal transduction pathways and abnormal cellular interplay.

In this study we provide for the first time evidence that pro-inflammatory cytokine production in MDS is largely mediated through TLR4 activation on BM macrophages. We initially showed an over-expression of TLR1, TLR2, TLR3 and TLR9 in the monocytic cell fraction of BMMC and BM microenvironment cells of MDS patients compared to healthy controls, albeit not at a statistically significant level. Only TLR4 was found to be significantly up-regulated in the monocytic component of the BMMC and LTBMC adherent cell population of MDS patients. This finding is in accordance with a previous study showing over-expression of TLR4 in almost all BM cell lineages, including monocytes, of MDS patients.[13]

A variety of pro-inflammatory cytokines such as TNFα and IFNγ present in the MDS BM microenvironment have been reported to up-modulate TLR4.[13,28,29] The increased mRNA levels of 53 components of TLR-mediated signaling in association with increased expression of the TLR negative regulators IRAKM and SHIP1 suggests a specific ligand-mediated TLR4 up-modulation in MDS patients rather than a non-specific cytokine-mediated effect. We specifically observed increased expression of genes related to the MyD88-dependent and MyD88-independent cascades as well as downstream genes implicated in the NFκB and MAPK pathways, two functionally important pathways in MDS pathophysiology.[5,6] TLR4-specific activation in BM monocytes is, therefore, anticipated to result in a vivid pro-inflammatory cytokine production. We did indeed find that exposure of MDS-derived monocytes to autologous BM plasma significantly increased IL-1β, IL-6 and TNFα production and this increase was abrogated in the presence of a TLR4 inhibitor, suggesting a TLR4-mediated effect. These findings demonstrate the pathophysiological significance of TLR4 up-regulation in BM monocytes of MDS patients and highlight a novel mechanism for the induction and maintenance of the inflammatory process in the MDS marrow environment. This finding corroborates the results of those studies suggesting a major contribution of monocytes/macrophages to the inflammatory milieu of MDS.[30,31]

Gene expression microarray technology has been used to probe the molecular pathogenesis of MDS and identify genes/molecular pathways underlying evolution of the disease. A number of genes have been identified that are differentially expressed between MDS patients and healthy controls.[32] It is difficult, however, to relate our findings to published microarray data because of the different cellular populations used in different studies.[33,34] Interestingly, deregulated cytokine and innate immune signaling due to interstitial deletion on chromosome 5 in humans and chromosome 11 and 18 in mice has led to the MDS phenotype.[35]

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Overexpression of Toll–IL-1 receptor domain–containing adaptor protein (TIRAP) and TNF receptor–associated factor-6 (TRAF6) due to knockdown of miR-145 and miR-146a in mouse hematopoietic progenitor cells has resulted in BM dysplasia highlighting the importance of inappropriate activation of innate immune signals in the pathogenesis of MDS.[36]

As regards possible TLR4 ligands in MDS, it is well known that TLR4 can be activated by both bacterial and self-derived products associated with tissue inflammation and damage. The possibility of an underlying bacterial infection leading to TLR4 activation in our patients is unlikely because patients with acute infection were excluded from this study. We therefore infer that endogenous ligand(s) trigger the TLR4-mediated signaling in MDS. The increased levels of HMGB1 protein in MDS LTBMC supernatants compared to controls indicate a possible association between HMGB1 and TLR4 activation in patients' monocytes.[22] Based on the existing knowledge described in the Results section regarding the mode of release and action of HMGB1,[23,24,37] one could hypothesize that the increased levels of HMGB1 in MDS might be due to its active release from activated BM monocytes under the influence of the locally produced pro-inflammatory cytokines.[18] This hypothesis does, however, seem rather unlikely because HMGB1 mRNA was not over-expressed in MDS BM CD14^{+} cells according to the PCR array data. A TLR4/IRAK4-dependent mechanism for HMGB1 production[38] also seems unlikely in MDS because the addition of a specific TLR4 inhibitor in patients' cultures did not exert any significant effects on protein levels.

In an attempt to identify additional mechanisms underlying the increased HMGB1 levels in MDS BM, we found that MDS-derived macrophages had an impaired capacity for apoptotic cell clearance and this defect was associated with increased release of HMGB1 in culture supernatants. HMGB1 levels increased proportionally to the apoptotic cell load and time in culture. Interestingly, HMGB1 was also detected in co-cultures of patients' macrophages with freshly isolated autologous BMMC, probably reflecting the increased proportion of apoptotic cells within the BMMC fraction of MDS patients. Increased HMGB1 extracellular levels have been described in a number of inflammatory and malignant diseases;[37] however, an association with MDS has not been recognized so far.

An impaired capacity to clear apoptotic cells has been associated with the pathogenesis of a range of disorders including autoimmune and degenerative diseases.[39,40] However, to our knowledge this is the first study indicating a clearance defect in MDS. The pathogenic significance of impaired apoptotic cell clearance in MDS was indicated by a set of experiments demonstrating that the clonogenic potential of BM progenitor cells significantly decreases in cultures of normal CD34^{+} cells in the presence of an excess apoptotic cell load through a TLR4-mediated mechanism that apparently involves HMGB1.

The presence of an efficient phagocytic mechanism is particularly important in MDS because of the increased rate of cell apoptosis in the BM. Rapid and efficient removal of apoptotic cells by phagocytes is crucial for BM homeostasis not only because it prevents the release of potentially immunogenic intracellular contents but also because it produces anti-inflammatory mediators that suppress inflammation and facilitate the clearance of apoptotic cells.[40] An impaired apoptotic cell clearance machinery in MDS may, therefore, represent an important contributory mechanism for the development of an inflammatory BM microenvironment in MDS. Whether the phagocytes with impaired clearance capacity in MDS belong to the abnormal clone is an open field for further research.

In conclusion, data from this study indicate novel pathophysiological mechanisms for the immunologically disturbed BM microenvironment in MDS. Although the inflammatory cytokines that trigger apoptosis in MDS BM are known to originate from both hematopoietic and stromal cells,[41] data from this study demonstrate that the inflammatory cytokine production in MDS BM is largely TLR4-dependent through a vicious loop that involves locally derived inflammatory mediators up-regulating TLR4 in BM monocytes which in turn, under the influence of endogenous ligands such as HMGB1, further induce and sustain the inflammatory process. Apart from highlighting a novel role for TLR4 and HMGB1 in the pathophysiology of the impaired BM environment in MDS, which could potentially have important therapeutic implications through targeting these molecules and the related signaling pathways, this study provides evidence that not only increased cell apoptosis but also defective clearance of apoptotic cells may have distinct roles in the pathobiology of MDS. Because the clearance of apoptotic cells is a multistep process involving recognition receptors, complex signaling events, molecular pathways and engulfment genes, further studies are definitely needed for the clarification of the intrinsic defects in macrophages that might be responsible for the reduced apoptotic cell clearance in MDS BM.

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Authorship and Disclosures

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