NF-κB and FLIP in arsenic trioxide (ATO)–induced apoptosis in myelodysplastic syndromes (MDSs)

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Tumor necrosis factor (TNF)–α, a potent stimulus of nuclear factor-κB (NF-κB), is up-regulated in myelodysplastic syndrome (MDS). Here, we show that bone marrow mononuclear cells (BMMCs) and purified CD34+ cells from patients with low-grade/early-stage MDS (refractory anemia/refractory anemia with ring sideroblasts [RA/RARS]) have low levels of NF-κB activity in nuclear extracts comparable with normal marrow, while patients with RA with excess blasts (RAEB) show significantly increased levels of activity (P = .008). Exogenous TNF-α enhanced NF-κB nuclear translocation in MDS BMMCs above baseline levels. Treatment with arsenic trioxide (ATO; 2-200 µM) inhibited NF-κB activity in normal marrow, primary MDS, and ML1 cells, even in the presence of exogenous TNF-α (20 ng/mL), and down-regulated NF-κB–dependent antiprototic proteins, B-cell leukemia XL (Bcl-XL), Bcl-2, X-linked inhibitor of apoptosis (XIAP), and Fas-associated death domain (FADD)–like interleukin-1β–converting enzyme (FLICE) inhibitory protein (FLIP), leading to apoptosis. However, overexpression of FLIP resulted in increased NF-κB activity and rendered ML1 cells resistant to ATO-induced apoptosis. These data are consistent with the observed up-regulation of FLIP and resistance to apoptosis with advanced MDS, where ATO as a single agent may show only limited efficacy. However, the data also suggest that combinations of ATO with agents that interfere with other pathways, such as FLIP autoamplification via NF-κB, may have considerable therapeutic activity. (Blood. 2005;106:3917-3925)

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Introduction

No “standard” therapy for the treatment of myelodysplastic syndrome (MDS) has been established. Recent efforts have focused on pathophysiology-based approaches.

We and others have shown high rates of apoptosis in MDS marrow cells, especially with less advanced disease. Apoptosis occurs in both clonal and nonclonal precursors, as determined by fluorescent in situ hybridization (FISH). We have also shown up-regulation of tumor necrosis factor (TNF)–α in marrow plasma from patients with MDS. As the disease advances, the rate of apoptosis in clonal cells declines, and proliferation prevails. Concurrently, there appears to be a shift in the expression of TNF-α receptors R1 (p55) and R2 (p75) in favor of R2, which transmits cytoprotective signals via nuclear factor-κB (NF-κB), whereas R1 transmits both cytoprotective (via NF-κB) and proapoptotic signals (via TNF receptor–associated death domain/Fas-associated death domain [TRADD/FADD]/ caspase-8). In addition, we observed dysregulation of FADD-like interleukin-1β–converting enzyme (FLICE) inhibitory protein (FLIP) in MDS marrow. FLIP modulates death signals triggered by various pathways, including TNF-α and its receptors. The transcription factor NF-κB, which consists of homo- or heterodimers of the NF-κB/Rel family members, is activated in response to TNF-α, and regulates transcription of a great diversity of genes involved in differentiation, inflammatory responses, and regulation of apoptosis and cell growth, including the antiprototic gene products FLIP, cIAPs, Bcl-2, and Bcl-XL.

In most normal cells, NF-κB complexes are present in the cytoplasm but remain inactive due to their interaction with inhibitor of κB (IκB) proteins. Upon cell activation, IκB kinases phosphorylate IκBα, which is degraded and frees NF-κB to translocate to the nucleus. In contrast, many neoplastic cells (Hodgkin disease, lymphomas, and acute leukemias) show constitutive NF-κB activation, which contributes to abnormal proliferation, resistance to apoptosis, and disease progression.

As TNF-α, which is up-regulated in MDS, is a potent stimulus of NF-κB, and FLIP has been shown to be under NF-κB regulation, we hypothesized that this transcription factor was involved in the pathophysiology of MDS.

Arsenic trioxide (ATO), which has been shown to have therapeutic efficacy in patients with MDS, induces apoptosis through several mechanisms: release of cytochrome c, modulation of cellular redox potential, down-regulation of Bcl-2, and inhibition of NF-κB by interfering with IκB kinases, thereby preventing spontaneous and TNF-induced NF-κB translocation to the nucleus. The mechanism by which ATO induces therapeutic responses in MDS has not been defined. In this study, we assessed NF-κB activation in MDS marrow and determined the effect of ATO on NF-κB activity. NF-κB activity correlated with separations and assisted with Western blots; S. Seal generated the lentiviral constructs; B.S. provided patient samples and critically reviewed the data; and H.J.D. designed experiments, critically reviewed and analyzed results, and provided manuscript revisions.

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MDS disease stage, and ATO inhibited NF-κB translocation to the nucleus.

Materials and methods

Materials

ATO was obtained from CTI (Seattle, WA), and etanercept (Enbrel) was obtained from Immunix (Seattle, WA) and Agen (Thousand Oaks, CA). The competitive IκB kinase inhibitor, BMS345541, was kindly provided by Dr S. Nadler (Bristol-Myers Squibb, Princeton, NJ). TNF-α was purchased from PeproTech (Rocky Hill, NJ), TNF-related apoptosis-inducing ligand (TRAIL) from Alexis (San Diego, CA) and β-thionine sulphoneimine (BSO) from Sigma (Saint Louis, MO).

Cell cultures and sample preparation

The human myeloid leukemia cell line ML1 (a gift from Dr D. Banker, Fred Hutchinson Cancer Research Center [FHCRC]) was maintained in RPMI 1640 medium, containing 10% heat-inactivated fetal bovine serum in a humidified 5% CO₂ environment at 37°C. ML1 cells stably expressing FLIPlong and control-vector green fluorescent protein (GFP) were cultured under the same conditions as the wild-type cells.

Marrow aspirates were obtained from healthy volunteers and patients with MDS who had given informed consent according to procedures approved by the Institutional Review Board of the FHCRC. Bone marrow mononuclear cells (BMMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. CD34⁺ selection was performed by magnetic-activated cell sorting (MACS) according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Cell numbers varied considerably from patient to patient, and not all tests could be carried out on every patient. Samples for experiments were selected such that the spectrum of MDS categories was represented.

Lentivirus construct

We used a plasmid encoding the lentiviral vector pRRLMSCV.IRES2eGFP.sin, which was kindly provided by Dr H. P. Kim (FHCRC). To insert the FLIPlong coding sequence, the vector was digested with AgeI and BamHI. cDNA was prepared from total RNA extracted from KG-1a cells, which show constitutively high FLIP expression. AgeI and BamHI sites were added to the 5’ and 3’ ends, respectively, of the polymerase chain reaction (PCR) product prepared with the use of HiFi Taq polymerase (Invitrogen, Carlsbad, CA). PCR products were gel purified and digested with the above enzymes, ligated into the vector, and checked for sequence integrity. As a control, we used the lentiviral vector pRRLsin-cPTT-MSCV-GFP (GFP) (provided by Dr H. P. Kim, FHCRC). To produce lentivirus, 293T cells were cotransfected with the pRRLMSCV.FLIP.IRES2eGFP.sin/pRRLsin-cPTT-MSCV.GFP along with the constructs containing the gag/pol and the vesicular stomatitis virus G protein (VSV-G) envelope using calcium phosphate precipitation. Lentiviral supernatants were collected at 18, 30, and 42 hours after cotransfection in Dulbecco modified Eagle medium (DMEM; containing 10% heat inactivated fetal bovine serum [FBS] 1% penicillin/streptomycin [P/S] + 20 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid]), filtered through a 0.22-μm filter, concentrated by centrifugation for 24 hours at 6800g and resuspended in 1/100 of the volume in Iscove medium. ML1 cells were transduced either with FLIPlong or control vector (GFP).

Treatment and determination of apoptosis

Reagents including ATO, etanercept, and TNF-α were tested in ancillary experiments for optimum concentrations and time courses. Based on those data, cells were exposed to ATO (at concentrations of 2-200 μM) alone or in the presence of etanercept (at 5-10 μg/mL) for 1 hour, followed by TNF-α (20 ng/mL, a predetermined dose which induces NF-κB activation 31) for 16 to 20 hours. To determine apoptotic changes, cells were stained with Annexin V–fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Becton Dickinson, San Jose, CA) and propidium iodide or, alternatively, with CellQuest software (Becton Dickinson, San Jose, CA). To assess apoptosis induced by ATO, the proportion of apoptotic cells was calculated as follows: (drug-induced apoptosis – apoptosis in medium) × 100/(100 – apoptosis in medium) as described by Frelin et al. 31 Student’s t test was applied for statistical analysis using GraphPad Prism (version 3) software (GraphPad Software, San Diego, CA).

Cell lysates and nuclear extracts

Cell lysates for Western blots were obtained using Chaps Cell Extract Buffer (Cell Signaling Technology, Beverly, MA) according to manufacturer’s protocol. Nuclear extracts were prepared using fresh nonsorted BMMCs (5-10 x 10⁶ cells) or CD34⁺ cells selected by MACS (1-10 x 10⁶ cells); after washing with cold phosphate-buffered saline (PBS), cells were lysed with lysis buffer (20 mM HEPES, pH 7.6; 20% glycerol; 1.5 mM MgCl₂; 0.2 mM EDTA (ethylenediaminetetraacetic acid); 0.1% Triton X-100; 10 mM NaCl; 100 μg/mL aprotinin; 10 μg/mL leupeptin; 10 μg/mL pepstatin; 1 mM phenylmethylsulfonyl fluoride [PMSF]; and 1 mM dithiothreitol [DTT]). After centrifugation at 700g for 5 minutes, the pellet was resuspended in nuclear extract buffer (same as lysis buffer but with 0.5 M NaCl), then centrifuged at 10000g for 10 minutes. Supernatants were stored at –80°C. A Bio-Rad assay (Hercules, CA) was used to measure protein concentration.

Electrophoretic mobility shift assays

To determine NF-κB activation, electrophoretic mobility shift assay (EMSA) was carried out as previously described. 33 Briefly, NF-κB consensus oligonucleotide sequences from Santa Cruz Biotechnologies (Santa Cruz, CA) were labeled with T4 polynucleotide kinase and γ-[32P]-ATP. Protein nuclear extracts were incubated with 2 μg poly d(I-C) and 32P-labeled probe (5000 counts per minute [cpm]) in 20 mM HEPES, pH7.9; 40 mM KCl; 10% glycerol; 0.05 mM EDTA; and 1.6 mM MgCl₂ for 30 minutes at room temperature. For competitive inhibition, 50-fold molar excess of unlabeled (cold) probe was used. For supershift assays, antibody for p65 (sc-372; Santa Cruz Biotechnologies) was added to the reaction mixture 30 minutes before adding the probe. Protein/DNA complexes were resolved on 5% Tris (tris(hydroxymethyl)aminomethane)–borate–EDTA (TBE) gels in 1 X TBE. Gels were dried and exposed at –80°C to x-ray films. Protein/DNA complexes were analyzed densitometrically using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) for NF-κB activation. Results represent intensity ratios of patient samples against healthy controls.

Western blot analysis

Cell lysates (using 25 μg of protein) were resolved on 4% to 20% sodium dodecyl sulfate (SDS)–polyacrylamide gels and processed according to standard protocols. The antibodies used were p-IκBα (Ser32), p-Akt (Ser473), Bcl-XL, Bcl-2, XIAP (1:1000; Cell Signaling Technology), anti-FLIP (NF6; 1:500) and antiactin (Sigma, St Louis). The secondary antibodies (antirabbit and antimouse) were conjugated to horseradish peroxidase (dilution, 1:20 000). Signals were detected using the electrochemiluminescence (ECL) system (Pierce, Rockford, IL).

Quantitative real-time PCR

cDNA was prepared from a pool of total RNA obtained from 2 independent experiments with ML1 or primary MDS BMMCs using oligo-dT20 and 7-amino-actinomycin (7AAD) and examined on a FACScan (Becton Dickinson, Mountainview, CA). Results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA). To assess apoptosis induced by ATO, the proportion of apoptotic cells was calculated as follows: (drug-induced apoptosis – apoptosis in medium) x 100/(100 – apoptosis in medium) as described by Frelin et al. 31 Student’s t test was applied for statistical analysis using GraphPad Prism (version 3) software (GraphPad Software, San Diego, CA).
95°C for 30 seconds, and 60°C for 45 seconds in the ABI Prism 7700 sequence Detection System (Applied Biosystems, Foster City, CA). Cloning of FLIP long and FLIP short cDNA into a pcDNA3.1 plasmid was performed as previously described[10] to construct standard curves for each transcript analyzed. β2-microglobulin was cloned into pcr2.1 TOPOVector (Invitrogen) using a standard protocol. Values for each gene were normalized to expression levels of β2-microglobulin.

**Glutathione-SH determination**

Intracellular glutathione-SH (GSH) was measured using a glutathione detection kit according to the manufacturer’s protocol (Oncogene, San Diego, CA). In brief, cell lysates from 3 × 10⁶ cells were incubated with monochlorobimane, a high-affinity dye for glutathione in the presence of glutathione S-transferase. GSH levels were then determined fluorometrically by using a Biosystems Cytofluor4000 plate reader (PerSeptive Biosystems, Framingham, MA) and expressed as nmol GSH per mg protein. Protein concentration was assessed using the Bio-Rad protein assay.

**Flow cytometry**

Flow cytometric analysis of membrane TNF-α receptors R1 (p55) and R2 (p75) was performed using PE-conjugated monoclonal antibodies TNF R1 and R2 according to the manufacturer’s protocol (R&D Systems). Mouse immunoglobulin G (IgG)–PE isotype control was obtained form Becton Dickinson. Analysis was carried out with a FACScan flow cytometer (Becton Dickinson). Results were expressed as the mean fluorescence intensity of specific and isotype control antibody.

**Proliferation assay**

As a measure for the inhibitory effect of ATO on hematopoietic cells, we determined ³H-thymidine incorporation. Briefly, 5 × 10⁶ CD34⁺ cells were incubated with ATO at various concentrations in 96-well round-bottom cell culture plates for 24 hours, 0.0185 MBq (0.5 μCi) ³H-thymidine was added for the last 18 hours of incubation, and radioactivity was measured using a scintillation counter.

**Results**

**NF-κB activity in primary MDS cells**

We evaluated NF-κB activity in bone marrow cells from 25 patients with MDS who had not received therapy in the recent past; all experiments were performed with fresh samples. Results are summarized in Table 1 and illustrated in Figure 1. (For reference purposes, both French-American-British [FAB] and World Health Organization [WHO] classifications are shown in Table 1.)

The levels of constitutive NF-κB DNA binding activity as determined by EMSA varied dependent upon the MDS disease category. BMMCs from patients with advanced MDS (including refractory anemia with excess blasts [RAEB] and RAEB in transformation [RAEB-T] by FAB criteria) showed significantly higher baseline levels of NF-κB nuclear translocation than with low-risk MDS, including refractory anemia (RA) and RA with ringed sideroblasts (RARS) (Table 1; P < 0.008). In low-risk MDS, the pattern of NF-κB activity in unfractioned BMMCs resembled that in normal marrow controls (Figure 1A). The patient in this group with the highest NF-κB activity in unfractioned BMMCs was a patient with a RA/5q⁻ syndrome (patient 4). In 6 patients (2 RA; 2 RAEB; 2 CMML), sufficient cell numbers were available for EMSA of nuclear extracts from purified CD34⁺ precursors. In early-stage/low-risk MDS, CD34⁺ precursors consistently showed low NF-κB activity, but higher than observed in normal CD34⁺ precursors. In contrast, high NF-κB activity was observed in CD34⁺ cells from patients with RAEB (Figure 1B). Supershift experiments confirmed the presence of the p65 subunit of the NF-κB family in the nuclear extracts studied (data not shown).

NF-κB activity in bone marrow mononuclear cells; nuclear extracts were prepared as described in “Materials and methods” and electrophoretic mobility shift assays were carried out. Protein binding levels were determined by densitometry using ImageQuant software. Results represent intensity ratios of patient samples against normal. Results are illustrated graphically in Figure 1. Levels in RAEB were statistically significantly higher than in RA/RARS (P = 0.008).

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**Table 1. Disease characteristics**

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The flow score considers phenotypic aberrancies as determined by 16 monoclonal anti-myeloid/monocyte antibodies, scatter properties, myeloid to lymphoid ratio of marrow cells, and blast count. The score has been shown to add prognostic information beyond that provided by the International Prognostic Scoring System, and in transplanted patients to correlate with posttransplantation relapse. ³⁴ ³⁵

FAB indicates French-American-British classification; WHO, World Health Organization; RA, refractory anemia; RCDM, refractory cytopenia with multilineage dysplasia; RARS, RA with ringed sideroblasts; RCDM-RS, RCDM with ringed sideroblasts; RAEB, RA with excess of blasts; RAEB-T, RAEB in transformation; CMML, chronic myelomonocytic leukemia; and IAML, transformed acute myeloid leukemia. NA indicates not available.

NF-κB activity in bone marrow mononuclear cells; nuclear extracts were prepared as described in “Materials and methods” and electrophoretic mobility shift assays were carried out. Protein binding levels were determined by densitometry using ImageQuant software. Results represent intensity ratios of patient samples against normal. Results are illustrated graphically in Figure 1. Levels in RAEB were statistically significantly higher than in RA/RARS (P = 0.008).

**Effect of TNF-α and TRAIL on NF-κB activity**

TNF-α and, to a lesser extent, TRAIL have been reported to trigger NF-κB activation,²²,²³,³¹,³⁵ and both are up-regulated in MDS.⁵,³⁶ The addition of exogenous TNF-α to MDS BMMCs enhanced NF-κB nuclear translocation above baseline levels in both normal
and MDS BMMCs; however, the extent of NF-κB translocation differed. TRAIL, on the other hand, did not alter the constitutive NF-κB pattern (Figure 1A). These results suggest that the NF-κB pathway may be less relevant for TRAIL-induced apoptosis in this model, and are in agreement with data by others who showed that NF-κB activity did not correlate with responses to TRAIL in primary leukemic cells.

TNF-α signaling is mediated by 2 cell surface receptors, TNF R1 and R2. TNF R1 signals trigger not only proapoptotic (via caspase-8) but also antiapoptotic pathways (through NF-κB activation), while R2 lacks a cytoplasmic death domain and signals only through NF-κB. Since TNF R1– and R2–transmitted signals may lead to different end results, and evidence for altered TNF receptor expression in transformed cells has been reported, we determined the cell surface expression of TNF R1 and R2 in BMMCs from patients with MDS and healthy controls. TNF R1 was expressed at high levels among patients with early-stage disease (RA), while patients with advanced disease showed a pattern similar to that in normal BMMCs (Figure 3A–B). Furthermore, in CD34+ cells from patients with RA the ratio of R1 to R2 was significantly higher than in CD34+ cells from patients with RAEB (Figure 3C). The R1/R2 ratio in normal CD34+ cells lay in between RA and RAEB CD34+ cells; the values did not differ significantly from either. TNF R1 overexpression in early-stage MDS would be consistent with higher apoptotic rates, and a shift in favor of R2 may lead to predominantly antiapoptotic signals, mediated through NF-κB activation in advanced MDS.

Inhibitory effect of ATO on NF-κB activation
As stable “MDS cell lines” are lacking, we used leukemia-derived ML1 cells as a model to study the effects of ATO on NF-κB activation. ML1 showed constitutive NF-κB activation, and NF-κB nuclear translocation was enhanced in response to TNF-α (Figure 4A, lanes 1 and 2). In the presence of ATO (5-200 μM), nuclear translocation of NF-κB, as determined by EMSA, was reduced in normal marrow, ML1 cells, and primary MDS cells (Figure 4A-D), and no IκBα phosphorylation was detectable by Western blot. This pattern remained unchanged in the presence of TNF-α blockade by etanercept (Figure 4E). To determine if NF-κB inhibition in cells treated with ATO plus etanercept was modified by the neutralizing effects of etanercept on TNF-α–induced NF-κB activation, cells were exposed to etanercept and excess exogenous TNF-α. With exposure to TNF-α and etanercept (without ATO), there was only a minimal decrease in NF-κB nuclear translocation (Figure 4A, lane 4; and Figure 4D, lane 5), and phosphorylation of IκBα was lower
when ML1 cells were treated with ATO + TNF-α in the presence of etanercept than with ATO and TNF-α only (Figure 4E). However, etanercept by itself did not enhance ATO-induced NF-κB inhibition (Figure 4A, lanes 6 and 7; Figure 4B, lanes 6 and 8). Thus, in this model NF-κB inhibition was primarily due to a direct effect of ATO, not further modified by TNF-α signals.

Recent reports suggest that the phosphatidylinositol (PI) 3-kinase/Akt pathway is involved in TNF-α-mediated NF-κB activation and in constitutive NF-κB activation in leukemic cells, apparently via activation of IkBα kinases. Conversely, others have shown that Akt can be regulated by NF-κB. In ML1 cells, ATO down-regulated Bcl-XL, Bcl-2, and XIAP, all molecules under NF-κB control, with potent antiapoptotic function (Figure 4F), and phosphorylation of Akt decreased, to a limited extent. These effects were time dependent and became most prominent after 18 hours. Bcl-XL in ML1 cells was also down-regulated in the presence of the specific IkBα kinase inhibitor, BMS345541 (Figure 4E), in agreement with results obtained with other specific pharmacologic antagonists of NF-κB. Thus, ATO had broad effects, not only on well-characterized antiapoptotic molecules, but also on alternative survival pathways such as PI3 kinase/Akt.

**Apoptosis induced by ATO in the presence of etanercept**

In ML1 cells and in primary MDS marrow cells, ATO induced apoptosis in a dose-dependent fashion (2-10µM). ATO-induced apoptosis was significantly higher in MDS cells than in cells from healthy controls (Figure 5A). The addition of the soluble TNF receptor, etanercept (5 to 10µg/mL), did not significantly change apoptosis even in the presence of exogenous TNF-α, in disagreement with a report by others that TNF-α blockade interfered with ATO cytotoxicity. In purified CD34+ cells from primary marrow samples, the rate of ATO-induced apoptosis was higher than in unfractionated BMMCs (Figure 5B). Furthermore, ATO inhibited proliferation to a greater extent in CD34+ cells from MDS marrow than in normal marrow CD34+ cells; there was a suggestion that the addition of etanercept further decreased proliferation in MDS, but not in normal CD34+ cells (Figure 5C).

**Figure 4. Inhibitory effects of ATO and etanercept (Enbrel) on NF-κB.** A. 5 × 10⁶ ML1 cells (A). BMMCs from a patient with RAEB (tested were marrows from patients 14, 15, 19, and 20; shown are results from patient 14) (B), and a patient with CMMML (tested were patients 22 and 24; shown is patient 22) (C), and from a healthy donor (tested were 3) (D) were exposed to ATO in the presence of etanercept for 1 hour, after which TNF-α was added at the indicated concentration for 20 hours. Nuclear extracts were isolated, and NF-κB gel shift was performed. A 50-fold molar excess of unlabeled probe (Cold) was used for competitive inhibition. (E) ML1 cells were exposed to ATO, etanercept, and BMS345541 (in this example at concentrations of 10 µM, 5 µg/mL, and 5 µM, respectively), for 1 hour, after which TNF-α (20 ng/mL) was added; cells were harvested 1 hour later. Phosphorylated IkBα and Bcl-XL were determined by Western blot (shown are results from 1 of 3 independent experiments). Actin served as protein control load. BMS345541, a specific inhibitor of IkBα kinase, was used here for comparison with the inhibitory effect of ATO on NF-κB. (F) ML1 cells were exposed to ATO (shown are results at 5 µM) for various time periods (4-24 hours). Results in untreated cells and cells exposed to TNF-α (patient 14) (B), and a patient with CMML (tested were patients 22 and 24; shown is patient 22) (C), and from a healthy donor (tested were 3)

**Figure 5. Effects of ATO, etanercept, and TNF-α on apoptosis in normal marrow, MDS marrow, and ML1 cells.** 1 × 10⁶ mononuclear cells/mL were exposed to ATO in the presence of etanercept for 1 hour, and TNF-α was added at the indicated concentrations for 20 hours. Results shown here were obtained with ATO at 5µM, etanercept at 5µg/mL, and TNF-α at 20 ng/mL. Apoptosis was determined by Annexin V and propidium iodide staining. Values are corrected for background apoptosis as described in “Materials and methods.” (A) Results represent the mean plus or minus SE of 3 independent experiments, each carried out in duplicates. Differences between normal and MDS marrows were significant (P < .01, **P < .05, ***P < .002, unpaired t-test). (B) CD34+ cells from a healthy control and 2 MDS patients (CMMML, RA) were exposed to ATO as described for panel A (without etanercept and TNF-α). (C) CD34+ cells from healthy controls (normal bone marrow [NBM]) and MDS patients (RAEB) were exposed to ATO for 20 hours, and thymidine incorporation was determined to evaluate cell proliferation. As expected with CD34+ cells, spontaneous thymidine uptake was low, but it was further reduced by treatment with ATO or ATO + etanercept.
down-regulated FLIP, but FLIPLong overexpression was associated with increased NF-
levels of FLIPLong increased and levels of FLIPShort decreased with
FLIPShort splice variants is dysregulated in MDS. Specifically,
/H9260 B activity (Table 2).
overexpressed FLIP Long in ML1 cells using a lentiviral vector
/H9251 MDS categories
in BMMCs and corresponding NF-
and the FLIP Long/FLIPShort ratio directly correlated with the levels
splice variant was involved in ATO-induced apoptosis, while
FLIP is an antiapoptotic molecule, which exists in the form of
several splice variants. It interferes with receptor-mediated activation
of caspase-8, induced by TNF-
or other proapoptotic ligands.
FLIP transcription is regulated by NF-kB, and FLIP participates in
an auto-amplification loop via interactions with RIP and
TRAF2, mRNA levels of both FLIPLong and FLIPShort splice
variants declined in ML1 cells treated with the IкB kinase inhibitor, BMS543541, in agreement with the control of FLIP by
NF-kB. Similar effects were observed for FLIPLong when cells
were exposed to ATO, suggesting that down-regulation of the FLIPLong
splice variant was involved in ATO-induced apoptosis, while
FLIPShort control may have different kinetics (Figure 6A).

We showed previously that the expression of FLIPLong and
FLIPShort splice variants is dysregulated in MDS. Specifically,
levels of FLIPLong increased and levels of FLIPShort decreased with progression of MDS from RA to RAEB (or more advanced
disease), leading to an increase in the FLIPLong/FLIPShort ratio.10
Determination of mRNA levels for FLIPLong and FLIPShort in 3
samples in the present study from which high-quality RNA was
available, showed a pattern identical to that in our original report,10
and the FLIPLong/FLIPShort ratio directly correlated with the levels
of NF-kB activity (Table 2).

To further characterize the role of FLIP in this model, we
overexpressed FLIPLong in ML1 cells using a lentiviral vector
(Figure 6C). As shown in Figure 6B, following treatment with ATO
(2-10 μM), FLIPLong-overexpressing ML1 cells exhibited significantly
lower rates of apoptosis than did cells transduced with a control vector, supporting a role for FLIP in the regulation of responses to ATO. FLIPLong-overexpressing ML1 cells showed
higher levels of NF-kB activity than GFP control cells, as
determined by EMSA (Figure 6D). Further, treatment of FLIPLong-
overexpressing ML1 cells with ATO, followed by exposure to
TNF-α, was still associated with IкBα phosphorylation (and, hence, NF-kB activation) rather than the expected NF-kB blockade
seen in control cells (Figure 6E). These observations are consistent
with the concept that the resistance to apoptosis mediated by
FLIPLong overexpression was due not solely to its direct antiapoptotic
to function, but also an autocrine amplification loop, which
contributes to further NF-kB activation.

Effect of ATO on cellular GSH content
Other mechanisms proposed for ATO-mediated cytotoxicity are the
insertion of sulfhydryl groups into the mitochondrial membrane,
and production of reactive oxygen species. Glutathione regulates
intracellular redox status and provides antioxidant activity by
cycling between its reduced (GSH) and oxidized (GSSG [oxidized
GSH]) forms. GSH content in ML1 cells was measured after
exposure to ATO. There was no significant change in GSH levels
after 6 hours (30.5 ± 1.5 vs 34.5 ± 0.5 nmol/mg protein untreated
and ATO-exposed, respectively), and 24 hours (33.1 ± 5.6 vs
34.1 ± 5.9 nmol/mg protein untreated and ATO-exposed, respectively).
Also, there was no significant difference in GSH levels between wild-type, GFP control and ML1 cells overexpressing
FLIPLong, that would have explained differences in apoptosis
sensitivity (Figure 7A, insert), as shown by others.47,48 We depleted
GSH from the cell lines by exposure to L-buthionine sulfoximine
(BSO; 10-200 μM). A plateau of 50% to 60% GSH depletion was
achieved at a BSO concentration of 50-100 μM (Figure 7A).

Table 2. Quantitative FLIPLong and FLIPShort mRNA expression in BMMCs and corresponding NF-kB activity in different MDS categories

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>MDS classification</th>
<th>NF-kB level</th>
<th>FLIPLong ± 10^-3</th>
<th>FLIPShort ± 10^-3</th>
<th>FLIPLong/FLIPShort ± 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>RA</td>
<td>0.9</td>
<td>0.2</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>RAEB</td>
<td>6.79</td>
<td>0.28</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>RAEB</td>
<td>5.86</td>
<td>0.44</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

Relative levels of FLIPLong and FLIPShort expression in bone marrow mononuclear cells using quantitative real-time PCR.

Values for each gene were normalized to expression levels of β2-microglobulin.

*As defined in Table 1.
TNF-\(\alpha\) and other proapoptotic cytokines play an important role in the pathophysiology of MDS.\(^5,7,29\) As MDS progresses, the rate of apoptosis in clonal cells declines. The mechanism responsible for disease progression is not known. Some reports have shown increased expression of antiapoptotic proteins of the Bcl-2 family with more advanced disease.\(^5\) More recently, Yamamoto et al. evaluated the IAP family proteins in MDS and suggested a role for XIAP in the transforming process to overt leukemia.\(^51\) We have shown previously that FLIP is dysregulated in MDS. FLIP\(_{\text{Long}}\) levels correlated negatively with apoptosis, and while FLIP\(_{\text{Long}}\) protein levels were readily detectable in advanced disease, only low levels were present in early-stage MDS.\(^10\) As these antiapoptotic proteins are under NF-\(\kappa\)B control, we hypothesized that this transcription factor may be a key regulator for disease progression. The status of NF-\(\kappa\)B at a given disease stage in the marrow of patients with MDS, and its functional relevance are not well characterized.\(^52\) Bueso-Ramos et al. studied NF-\(\kappa\)B levels in mononuclear cells in a series of myeloid malignancies and showed low levels in 5 MDS cases studied, while high activity was detected in 47% of AML cases.\(^53\)

The present results show an increase in NF-\(\kappa\)B activation that correlated with MDS disease stage (ie, cells from patients with more advanced MDS had higher NF-\(\kappa\)B activity). While in marrow from healthy donors NF-\(\kappa\)B activity was comparable in CD34\(^+\) and CD34\(^-\) subsets, significant changes were noted in MDS marrow. In early-stage MDS, NF-\(\kappa\)B activity was lower in CD34\(^+\) than in CD34\(^-\) cells, consistent with a high rate of apoptosis in early (CD34\(^+\)) precursors at that disease stage. In more advanced MDS, however, NF-\(\kappa\)B activity was higher in CD34\(^+\) than in CD34\(^-\) cells. Such a pattern would be consistent with the up-regulation of FLIP, and other NF-\(\kappa\)B–dependent antiapoptotic regulators (bcl-XL, bcl-2, XIAP), and resistance to apoptosis (in clonal MDS cells), as also observed in other disease states.\(^16,18,21\) While we were unable to show a strict positive correlation between NF-\(\kappa\)B and mRNA of the 2 FLIP splice variants, the FLIP\(_{\text{Long}}/\text{FLIP}_{\text{Short}}\) ratio was low in marrows from early-stage MDS and increased with more advanced disease, in excellent agreement with the potent antiapoptotic activity of FLIP\(_{\text{Long}}\). Although FLIP has been shown to be under NF-\(\kappa\)B control, FLIP\(_{\text{Long}}\) and FLIP\(_{\text{Short}}\) expression is differentially regulated and exhibits different kinetics,\(^12\) which may explain the differences at the mRNA and protein levels that we observed previously.\(^10\)

The mechanisms involved in the activation of NF-\(\kappa\)B in MDS are not well defined. Levels of TNF-\(\alpha\), a potent activator of NF-\(\kappa\)B, are frequently up-regulated in MDS but do not strictly correlate with NF-\(\kappa\)B activity.\(^5,54\) Further, TNF-\(\alpha\) levels are more commonly elevated in early stage disease, where NF-\(\kappa\)B activity is not enhanced, suggesting that an autocrine TNF-\(\alpha\) stimulus is probably not the sole mechanism involved in the constitutive NF-\(\kappa\)B activation.

Based on observations by Swanabori et al., who showed that the ratio of TNF R1 and R2 mRNA shifts in favor of R2 with more advance disease, we hypothesized that the R1/R2 ratio may be relevant for apoptosis (or resistance to it).\(^9\) Our data on TNF receptor R1 and R2 expression indeed corroborate Swanabori’s findings at the protein level in both BMMCs and CD34\(^+\) MDS cells. Therefore, shifting the balance from R1 to R2 expression would activate NF-\(\kappa\)B and downstream survival signals.

Since the regulation of apoptosis appears to be a central event in the pathophysiology of MDS, and since ATO, which inhibits NF-\(\kappa\)B, has been shown to offer therapeutic benefit particularly in patients with early-stage MDS, we attempted to further characterize the effects of ATO on MDS marrow cells. Our data show that ATO treatment resulted in NF-\(\kappa\)B inhibition and apoptosis, not only in ML1 cells, but also in normal and MDS-derived marrow cells. We had previously observed that blockade of TNF-\(\alpha\), which is up-regulated in many patients with MDS, improves hemopoiesis from MDS marrow,\(^1,5\) apparently by reducing apoptosis. Both ATO and TNF-\(\alpha\) affect NF-\(\kappa\)B activity, and the present data show that the addition of etanercept to neutralize TNF-\(\alpha\) (originally with the intent of protecting nonclonal cells)\(^7\) did not interfere with ATO-induced apoptosis and did not enhance proliferation in CD34\(^+\) MDS cells (Figure 5).

Associated with ATO-mediated NF-\(\kappa\)B inhibition and induction of apoptosis was the down-regulation of NF-\(\kappa\)B–dependent antiapoptotic genes, including FLIP\(_{\text{Long}}\), Bcl-XL, Bcl-2, and XIAP. As these molecules are dysregulated in MDS and up-regulation may contribute to MDS progression by mediating apoptosis resistance,\(^56\) these data suggest that ATO should be useful in the treatment of patients with MDS. However, studies on FLIP\(_{\text{Long}}\) overexpression in ML1 cells showed a significant decrease in
apoptosis in response to ATO, adding a note of caution. FLIP serves as a central regulator of apoptosis and exerts its antiapoptosis effects not only by inhibiting caspase-8, but also by modulating the NF-κB pathway via an autoamplification loop as shown here (Figure 6D) and in other reports. Thus, in patients with more advanced MDS and high levels of FLIP, ATO very likely would have to be combined with other agents capable of interfering with FLIP amplification in order to be therapeutically effective. Attempts to use GSH depletion as a way of enhancing ATO toxicity, as has been shown in monocytic and promyelocytic leukemic cell lines, were not successful in our model, suggesting cell type–dependent differential responses.

In summary, the present study showed that NF-κB activity was correlated with MDS disease stage and flow scores, which have been shown to be of prognostic relevance. ATO induced apoptosis in hemopoietic cells in general, and in marrow cells from patients with MDS in particular, by interfering with NF-κB and the transcription of NF-κB–dependent antiapoptotic proteins. However, constitutive NF-κB activity and dysregulated FLIP levels may confer resistance to ATO in advanced-stage MDS, and combined approaches are likely to be necessary to enhance responses to ATO.

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NF-κB and FLIP in arsenic trioxide (ATO)-induced apoptosis in myelodysplastic syndromes (MDSs)

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