Role of the p38 Mitogen-Activated Protein Kinase Pathway in Cytokine-Mediated Hematopoietic Suppression in Myelodysplastic Syndromes

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Abstract
The p38 mitogen-activated protein kinase (MAPK) pathway is activated by IFNs and other cytokines to mediate signals for important cellular functions, including transcriptional regulation and apoptosis. We examined the role of the p38 pathway in the generation of the effects of myelosuppressive cytokines on human hematopoiesis. Pharmacologic inhibition of p38 using BIX-01208 resulted in reversal of IFN-α, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β)–mediated suppression of human erythroid (blast-forming unit-erythroid) and myeloid (granulocyte-macrophage colony-forming unit) colony formation, consistent with a key role for p38 in the generation of myelosuppressive signals by different cytokines. Similarly, the myelosuppressive effects of TNF-α and TGF-β were reversed by small interfering RNAs targeting p38α expression, further establishing the requirement of this kinase in the induction of myelosuppressive responses. As TNF overproduction has been implicated in the pathophysiology of bone marrow failure states, we determined whether pharmacologic inhibition of p38 reverses the hematopoietic defects seen in bone marrows from patients with myelodysplastic syndromes (MDS) and the anemia of chronic disease. Addition of pharmacologic inhibitors of p38 on such bone marrows resulted in increased numbers of erythroid and myeloid progenitors. Similarly, inhibition of the activity of the downstream effectors of p38, MAPK activated protein kinase-2, and mitogen and stress activated kinase 1 partially restored the hematopoietic defect seen in these bone marrows. Taken altogether, our data implicate the p38 MAPK in the pathophysiology of myelodysplasias and suggest that p38 pharmacologic inhibitors may have therapeutic applications in the treatment of MDS.

Introduction
The p38 mitogen-activated protein kinase (MAPK) pathway is activated in response to a variety of stress stimuli and mediates induction of important cellular responses, such as apoptosis, activation of transcription factors and transcriptional regulation, cytokine production, and cell cycle progression (reviewed in refs. 1–4). In addition to its engagement by stress stimuli, p38 is activated by several cytokines and growth factors, including IFNs (5, 6), tumor necrosis factor-α (TNF-α; reviewed in ref. 7), and transforming growth factor-β (TGF-β; ref. 8). Previous work has shown that the p38 MAPK pathway is activated by these cytokines in primitive human hematopoietic progenitors, suggesting its involvement in the regulatory activities of these cytokines on hematopoiesis (9, 10). Moreover, it has been shown previously that pharmacologic inhibition of p38 reverses the suppressive effects of IFN-α on leukemic progenitors from chronic myelogenous leukemia (CML) patients (11). BCR-ABL has been shown to suppress p38 activation (12), whereas blocking BCR-ABL activity with imatinib mesylate (STI571) reverses such inhibition (13), implicating the p38 pathway in the regulation of leukemic hematopoiesis in the context of BCR-ABL transformation.

Among the various cytokines that are engaged in the regulation of normal hematopoiesis, some play positive roles on hematopoietic progenitor cell growth, whereas others exhibit negative regulatory effects (4). An appropriate balance between hematopoietic growth factor signals and signals generated by myelosuppressive cytokines seems to be necessary for optimal production of hematopoietic cells. Type I (α and β) and type II (γ) IFNs are well-known hematopoietic suppressors in vitro and in vivo (reviewed in ref. 14) and exhibit negative regulatory effects on progenitor cells of all hematopoietic lineages, including early [colony-forming unit-erythroid (CFU-E)] and late [blast-forming unit-erythroid (BFU-E)] erythroid precursors, myeloid progenitors [granulocyte-macrophage colony-forming unit (CFU-GM)], and megakaryocytic progenitors (colony-forming unit-megakaryocyte; ref. 14). Other cytokines that are known to exhibit negative regulatory roles on normal hematopoiesis include TGF-β and TNF-α (15, 16).

There is strong evidence that myelosuppressive cytokines, particularly TNF-α and type II IFN (IFN-γ), are involved in the pathogenesis of bone marrow failure syndromes in humans, including aplastic anemia (17–19) and Fanconi anemia (20, 21), although TNF-α is also implicated in the pathogenesis of the anemia of chronic disease (ACD; refs. 22, 23). Moreover, in addition to classic cytokine-mediated bone marrow failure syndromes, TNF-α, TGF-β, and IFN-γ have all been implicated in the pathophysiology of certain subtypes of myelodysplastic syndromes (MDS), especially those with hypoplastic features (24–27). As the p38 pathway is a common element in the signaling cascades of several myelosuppressive cytokines, we examined whether pharmacologic inhibition of its activation reverses the hematopoietic defects seen in MDS and ACD. Our data establish
that pharmacologic inhibition of p38 activity and/or small interfering RNA (siRNA)–mediated p38x knockdown reverse the suppressive effects of TNF-α, TGF-β, and IFNs on normal hematopoietic progenitors, showing a role for this signaling cascade in the negative regulation of human hematopoiesis. Importantly, our data also show that inhibitors of p38 partially reverse the hematopoietic suppression seen in the bone marrows of patients with MDS or patients suffering from ACD.

Materials and Methods

Cell lines and reagents. The CML-derived KT-1 cell line was grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Human recombinant IFN-α2 and IFN-γ were provided by Hoffmann-La Roche (Nutley, NJ). Human recombinant TNF-α was obtained from R&D Systems (Minneapolis, MN). The TNF-α monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and was used to neutralize TNF-α activity (27). Boehringer-Ingelheim (Ridgefield, CT) provided the p38 MAPK inhibitor BIX-01208. The p38 MAPK inhibitors SB203580 and SB202190, the inactive structural homologue SB202474, and the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor PD98059 were purchased from Calbiochem (La Jolla, CA). The H89 inhibitor, which inhibits mitogen and stress activated kinase 1 (MSK1) kinase, was purchased from Alexis Biochemicals (San Diego, CA). The MAPK activated protein kinase-2 (MAPKAPK-2) synthetic inhibitor (KKKKNBQLGVA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Epigallocatechin 3-gallate (EGCG) was obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against the phosphorylated forms of p38 and MSK1 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against p38, MSK1, and MAPKAPK-2 were purchased from Santa Cruz Biotechnology. An anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Chemicon International, Inc. (Temecula, CA). Hsp25 was obtained from StressGen Biotechnologies (San Diego, CA). The Akt/SGK peptide, which was used as a substrate for MSK1, and Hsp27, which was used as a substrate for MAPKAPK-2, were obtained from Upstate Biotechnology.

Cell lysis, immunoblotting, and in vitro kinase assays. Cells were lysed in phosphorylation lysis buffer as described previously (28, 29). In the experiments in which the effects of BIX-01208, SB203580, SB202190, SB202474, and PD98059 were studied, DMSO (diluent)–treated cells were used as control. Immunoprecipitations, immunoblotting, and in vitro kinase assays were done as described previously (28, 29). Human CD34+ cells were isolated from normal bone marrows or CML patients after obtaining informed consent approved by the institutional review board of Northwestern University (Chicago, IL). Erythroid progenitors at the CFU-E level of differentiation were enriched using the methodologies described in our previous studies (9, 10, 30). Each immunoblot or kinase assay shown is representative of at least two independent experiments (five experiments for Fig. 1A, two for Fig. 1B, three for Fig. 3A, two for Fig. 3B, four for Fig. 3C, three for Fig. 3D, two for Fig. 4F, and two for Fig. 4I), and all were highly reproducible.

Cell proliferation assays. Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methodology as described previously (11).

Transfections of small interfering RNAs. siRNA duplexes (siRNAs) were synthesized and purified by Qiagen, Inc. (Valencia, CA). The p38x target sequence was 5′-AAGGCCCATACCTCTCCTGTTT-3′. CD34+ cells were transfected with either p38x-specific siRNA duplexes or the control scrambled siRNA using the RNAiFect transfection system (Qiagen) before performing hematopoietic progenitor cell assays. For the knockdown of MSK1, a prevalidated siRNA mix from New England Biolabs, Inc. (Beverly, MA) was used. In some experiments, hematopoietic progenitors at the CFU-E level of differentiation were enriched from normal bone marrows (9, 10, 30), and after transfection with p38x-specific siRNA duplexes or control scrambled siRNA, the cells were lysed and cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

Hematopoietic progenitor cell assays. The effects of cytokines on hematopoietic cell progenitor colony formation were determined by clonogenic assays in methylcellulose as in our previous studies (9–11, 13). All participants in the study obtained informed consent approved by the institutional review board of Northwestern University. CFU-GM and BFU-E from bone marrow samples were scored on day 14 of culture. Some patient samples were obtained from the Pathology Core Facility/Hematologic Malignancies Repository of the Robert H. Lurie Comprehensive Cancer Center of Northwestern University. The doses of IFN-α2 and IFN-γ used in the methylcellulose assays were 1,000 IU/mL, whereas the dose of TNF-α was 10 ng/mL. SB203580, SB202190, SB202474, EGCG,

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Type I IFN-dependent phosphorylation/activation of the p38 MAPK and its downstream effector MAPKAPK-2 in primary leukemic cells and its inhibition by BIX-01208. A, CD34+ cells isolated from a CML bone marrow were induced to differentiate to CFU-E hematopoietic progenitors. The cells were subsequently treated with IFN-α for the indicated times in the presence or absence of the p38 inhibitor BIX-01208 (1 μmol/L). Total cell lysates were resolved using SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of p38. The same blot was stripped and reprobed with an anti-p38 antibody to control for protein loading. B, KT-1 cells were treated with IFN-α for 60 minutes in the presence or absence of the p38 inhibitor BIX-01208 (1 μmol/L) as indicated. Cell lysates were immunoprecipitated with an anti-MAPKAPK-2 antibody or control nonimmune rabbit IgG, and in vitro kinase assays were carried out in the immunoprecipitates using Hsp25 as an exogenous substrate. Phosphorylated Hsp25 was detected by autoradiography. C, KT-1 cells were incubated with the different doses of IFN-α in the presence or absence of the indicated concentrations (in μmol/L) of BIX-01208 for 7 days. Cell proliferation was assessed using MTT assays. Points, mean of two independent experiments; bars, SE. bars, untreated; –, BIX 1; –, BIX 5.
and H89 were all used at a final concentration of 10 μmol/L unless otherwise indicated. BIX-01208 was added to the cultures at a final concentration of 1 or 5 μmol/L as indicated. PD98059 was added to the cultures at a final concentration of 2 μmol/L.

Results

We initially examined the induction of IFN-dependent phosphorylation and activation of the p38 MAPK in primary hematopoietic progenitors and the effects of the p38 pharmacologic inhibitor BIX-01208 on such activation. BIX-01208 is a compound in the BIRB796 class of p38 inhibitors, which have been extensively characterized in previous studies (31, 32). CFU-E cells, expanded from CD34+ hematopoietic progenitors from a patient with CML, were incubated for different times with IFN-α and total lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p38 antibody. IFN-α treatment resulted in strong phosphorylation of p38, and such phosphorylation was inhibited when cells were pretreated with BIX-01208 (Fig. 1A). Pretreatment of cells with BIX-01208 also blocked the IFN-α-dependent activation of the kinase MAPKAPK-2 in the CML-derived KT-1 lymphoblastoid cell line (Fig. 1B) and reversed the growth-inhibitory effects of IFN-α on KT-1 cells (Fig. 1C), showing that such inhibition of p38 activation was functionally relevant.

In subsequent studies, we examined whether inhibition of the p38 MAPK pathway using BIX-01208 abrogates the induction of the antileukemic effects of IFN-α on primitive CML progenitors. Bone marrow mononuclear cells from four different patients with CML were studied for that purpose. Treatment of cells with the p38 inhibitor alone had no significant effects on the formation of leukemic CFU-GM colonies from such patients (Fig. 2). As expected, addition of IFN-α to the cultures resulted in potent inhibition of leukemic CFU-GM colony growth (Fig. 2). However, in the cultures in which BIX-01208 was added concomitantly with IFN-α, there was reversal of the growth-inhibitory effects of IFN-α (Fig. 2). Such reversal of the effects of IFN-α was complete or near complete in three of four cases (Fig. 2A, B, and D) and partial in one case (Fig. 2C). Paired t test analysis to compare the effects of IFN-α alone versus IFN-α plus BIX-01208 showed that the reversal of the effects of IFN-α was statistically significant (two-tailed P = 0.049). Altogether, these studies showed that the p38 MAPK pathway is essential for the generation of the growth-inhibitory effects of IFN-α on leukemic progenitors, consistent with our previous observations in studies using SB203580 (11).

Figure 2. Requirement of the p38 MAPK pathway in the generation of the growth-inhibitory effects of IFN-α on leukemic CFU-GM hematopoietic progenitors. Bone marrow mononuclear cells from four individual patients with CML (A-D) were plated in methylcellulose in the absence or presence of IFN-α, BIX-01208 (1 μmol/L), or the combination of IFN-α and BIX-01208 as indicated. Data are expressed as percent control of CFU-GM or BFU-E colony numbers for untreated cells. E, columns, mean of the values from the experiments using different patient samples (A-D); bars, SE. *, P = 0.049, paired t test analysis.
Phosphorylated Hsp25 was detected by autoradiography. Antibody or control nonimmune rabbit IgG as indicated, and for protein loading, the blot was then stripped and reprobed with an anti-ERK antibody. Treatment of KT-1 cells with TNF-α resulted in phosphorylation/activation of p38, and such phosphorylation was blocked by pretreatment with BIX-01208 (Fig. 3A). TNF-α treatment of KT-1 cells also resulted in activation of MAPKAPK-2 in a p38-dependent manner (Fig. 3D). Such activation of MAPKAPK-2 by TNF-α was inhibited by pretreatment of the cells with BIX-01208 or the p38 MAPK inhibitor SB203580 (10 μmol/L) but not the structural homologue SB202474, establishing that it occurs downstream of p38 (Fig. 3C).

In subsequent studies, we sought to identify other downstream effectors of the TNF-α-activated p38 pathway in cells of hematopoietic origin. Previous work from our group has implicated the nucleosomal kinase MSK1 in the generation of type I IFN signals in hematopoietic cells (33). MSK1 is a kinase known to control serine phosphorylation of histone H3 and high mobility group-14 and to regulate induction of transcription of immediately early genes (34–36). Treatment of KT-1 cells with TNF-α resulted in phosphorylation of MSK1 on Ser376 (Fig. 3D) and activation of its kinase domain (Fig. 3E). Such phosphorylation/activation of MSK1 was blocked by pharmacologic inhibition of p38 MAPK (Fig. 3D and E). Thus, as in the case of the type I IFN receptor (33), engagement of the TNF-α receptor in hematopoietic cells results in p38 MAPK–mediated activation of the MSK1, suggesting that this kinase may be participating in the induction of the myelosuppressive effects of TNF-α.

In previous studies, we have shown that the SB203580 pyridinyl imidazole compound reverses the suppressive effects of multiple cytokines, including IFNs, TNF-α, and TGF-β, on normal bone marrow colony formation (9, 10). Several of these cytokines have been implicated in the pathophysiology of MDS and other bone marrow failure disorders, including ACD (22–27). Therefore, we considered the possibility that pharmacologic inhibitors of the p38 MAPK pathway may reverse the suppressive effects of these cytokines in the bone marrow of patients with MDS and ACD, in which bone marrows there is abnormal cytokine overproduction. Before examining the effects of p38 MAPK inhibitors on colony formation from MDS bone marrows, studies were done to evaluate whether the p38 inhibitor BIX-01208 abrogates the effects of TNF-α, TGF-β, and IFN-γ on normal hematopoietic colony formation. Bone marrow mononuclear cells from normal donors were cultured in methylcellulose in the presence or absence of the various cytokines and BIX-01208. As expected, all cytokines strongly suppressed the growth of normal myeloid (CFU-GM) and erythroid (BFU-E) hematopoietic progenitors (Fig. 4A-C).

**Figure 3.** TNF-α-dependent phosphorylation/activation of the p38 MAPK and its downstream effectors MAPKAPK-2 and MSK1. A, KT-1 cells were treated for the indicated times with TNF-α in the presence or absence of BIX-01208 (1 μmol/L). Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p38. The same blot was stripped and reprobed with an anti-p38 antibody to control for protein loading. B, KT-1 cells were treated for the indicated times with TNF-α in the presence or absence of BIX-01208 (5 μmol/L). Cell lysates were immunoblotted with an anti-phospho-ERK antibody. To control for protein loading, the blot was then stripped and reprobed with an anti-ERK antibody. C, KT-1 cells were treated with TNF-α for 60 minutes in the presence or absence of BIX-01208 (1 μmol/L), SB203580 (10 μmol/L), or SB202474 (10 μmol/L) as indicated. Cell lysates were immunoprecipitated with an anti-MAPKAPK-2 antibody or control nonimmune rabbit IgG as indicated, and in vitro kinase assays were carried out on the immunoprecipitates using Hsp25 as an exogenous substrate. Phosphorylated Hsp25 was detected by autoradiography. D, KT-1 cells were treated for the indicated times with TNF-α in the presence or absence of BIX-01208 (1 μmol/L). Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of MSK1 on Ser376. The same blot was stripped and reprobed with an anti-tubulin antibody to control for protein loading. E, KT-1 cells treated with TNF-α for 60 minutes in the presence or absence of the p38 inhibitor BIX-01208 (1 μmol/L). Cell lysates were immunoprecipitated with an anti-MSK1 antibody and in vitro kinase assays were carried out in the immunoprecipitates. The amounts of phosphorylated substrate were detected using a scintillation counter. Columns, mean of three independent experiments; bars, SE. *, P = 0.035, paired two-tailed t test analysis comparing MSK1 kinase activity in cells treated with TNF or TNF + BIX.
Addition of BIX-01208 alone to the cultures had no significant effects on normal CFU-GM or BFU-E colony formation, indicating that the p38 pathway does not mediate signals required for growth or differentiation of hematopoietic progenitors. However, concomitant treatment of cells with the different myelosuppressive cytokines and BIX-01208 resulted in reversal of the growth-inhibitory effects of TNF-\(\alpha\) (Fig. 4A). These effects were statistically significant for both BFU-E (two-tailed \(P = 0.0128\)) and CFU-GM (two-tailed \(P = 0.0004\)) progenitors. Similarly, BIX-01208 reversed the suppressive effects of TGF-\(\beta\) (Fig. 4B; \(P = 0.0153\) for BFU-E and \(P = 0.0262\) for CFU-GM) as well as IFN-\(\gamma\) (Fig. 4C; \(P = 0.0115\) for BFU-E and \(P = 0.0012\) for CFU-GM) and TNF-\(\alpha\) (Fig. 4C; \(P = 0.0371\) for BFU-E and \(P = 0.0134\) for CFU-GM) on hematopoietic progenitor growth.

In further studies, we sought to determine whether inhibition of the kinase domains of downstream effectors of p38 also reverses the myelosuppressive effects of TNF-\(\alpha\) on hematopoietic progenitors. Concomitant treatment of hematopoietic progenitor cells with H89, a pharmacologic inhibitor known to inhibit MSK1 (36, 37), resulted in partial reversal of the suppressive effects of TNF-\(\alpha\) (Fig. 4D). Similarly, concomitant treatment with a synthetic peptide inhibitor of MAPKAPK-2 also reversed partially the suppressive effects of TNF-\(\alpha\) (Fig. 4E), suggesting that this kinase also participates in the generation of the myelosuppressive effects of TNF-\(\alpha\).

To definitively establish the functional relevance of p38 in the generation of myelosuppressive responses, we sought to examine whether inhibition of p38 protein expression in human hematopoietic progenitors reverses the growth-inhibitory effects of TNF-\(\alpha\) on normal BFU-E and CFU-GM colony formation. As shown in Fig. 4F, siRNA duplexes specifically targeting p38a inhibited the expression of the protein in primitive hematopoietic precursors. Such inhibition of p38a expression reversed the suppressive effects of TNF-\(\alpha\) (Fig. 4G; \(P = 0.0168\) for BFU-E and \(P = 0.0124\) for CFU-GM) or TGF-\(\beta\) (Fig. 4H; \(P = 0.0044\) for BFU-E and \(P = 0.0362\) for CFU-GM) on normal hematopoietic progenitors, establishing that p38 MAPK is required for induction of such responses. As our data using the H89 inhibitor suggested a potential role for the MSK1 kinase in the generation of the suppressive effects of TNF-\(\alpha\) on hematopoiesis, we sought to definitively establish the role of the MSK1 kinase in hematopoietic suppression by using the siRNA methodology to knockdown MSK1 (Fig. 4I). MSK1 knockdown alone did not alter the growth of BFU-E or CFU-GM colonies (Fig. 4J). However, the transfection of CD34+ hematopoietic progenitors with the MSK1 siRNA clearly reversed the myelosuppressive effects of TNF-\(\alpha\) on BFU-E and CFU-GM colony formation (Fig. 4J). Such effects were statistically significant (\(P = 0.0107\) for BFU-E and \(P = 0.0234\) for CFU-GM), underlining the significance of the described findings. Taken altogether, these results strongly suggest that the p38 MAPK pathway is a common signaling mediator for different myelosuppressive cytokines and plays a key role in the generation of signals for inhibition of normal bone marrow–derived hematopoietic progenitor cell growth.

Overproduction of myelosuppressive cytokines has been implicated in the pathogenesis of ACD and cytopenias seen in MDS. This prompted us to examine whether pharmacologic inhibitors of p38 can restore normal hematopoietic progenitor colony formation from bone marrows of patients suffering from ACD or MDS. Three patients with ACD (Table 1, ACD 1-3) and five patients with MDS (Table 1, MDS 1-5) were studied. The clinical characteristics of these patients at the time of the studies are summarized in Table 1. Bone marrow mononuclear cells were isolated and cultured in methylcellulose in the presence or absence of the p38 MAPK–specific inhibitors SB203580, SB202190, or BIX-01208. As controls, the structural inactive homologue of SB203580, SB202474, or the MEK kinase (MEKK) inhibitor PD98059 were used. In all three cases of ACD studied, there was an increase in the number of erythroid colonies (BFU-E) in response to either SB203580 or BIX-01208, although the effect of SB203580 was more pronounced (Table 1, ACD 1-3). In two cases (ACD 2 and 3), in which the effects of SB202190 were studied, we observed that this p38 MAPK inhibitor also enhances BFU-E and CFU-GM colony formation to similar degrees to that of SB203580. The effects of EGCG (38), a nonspecific kinase inhibitor and green tea derivative, which we have also found to inhibit phosphorylation of p38 but not ERK (data not shown), were also studied in two cases. EGCG enhanced BFU-E colony formation to a slightly lower degree to what seen in response to the p38 MAPK inhibitors (Table 1, ACD 1-3). Addition of a neutralizing anti-TNF-\(\alpha\) monoclonal antibody in the bone marrow cultures in one case (ACD 3*) resulted in enhancement of hematopoietic colony formation, suggesting that TNF-\(\alpha\) is a major mediator of hematopoietic suppression in such cases. However, concomitant addition of anti-TNF-\(\alpha\) monoclonal antibody and p38 MAPK inhibitors resulted in further increase, especially for CFU-GM progenitors, indicating involvement of other cytokines as well, likely IFNs and/or TGF-\(\beta\).

Five patients with MDS were also examined (Table 1, MDS 1-5). In all cases, addition of SB203580, SB202190, or BIX-01208 resulted in enhancement of colony formation for either erythroid of myeloid progenitors, whereas PD98059 or SB202474 had no significant effects. In four cases, in which the effects of EGCG were also examined (MDS 1 and 3-5), we also found some enhancement of myeloid and erythroid colony formation. Similarly to what seen in the ACD bone marrows, addition of an anti-TNF-\(\alpha\) monoclonal antibody to the cultures clearly enhanced hematopoietic colony formation (Table 1, MDS 5*).

Statistical analysis of the effects of the various inhibitors on progenitor colony formation from all patients (MDS and ACD) was done. As expected, an inactive analogue to SB203580, SB202474, did not increase BFU-E or CFU-GM colony numbers (two-tailed \(P > 0.35\) for both BFU-E and CFU-GM colony formation, \(n = 5\)). In contrast, treatment with the p38 MAPK inhibitor BIX-01208 resulted in clear increases of BFU-E and CFU-GM colony formation (Table 1). There were statistically significant increases seen when BIX-01208 was used at concentrations of either 1 \(\mu\)mol/L (two-tailed \(P\) for BFU-E = 0.0364 and for CFU-GM = 0.0110, \(n = 7\)) or 5 \(\mu\)mol/L (two-tailed \(P\) for BFU-E = 0.0207 and for CFU-GM = 0.0258, \(n = 6\)). Similarly, treatment of bone marrow progenitors with SB203580 strongly enhanced colony formation for BFU-E (two-tailed \(P = 0.0096\), \(n = 8\)) and CFU-GM (two-tailed \(P = 0.0075\), \(n = 7\)), whereas the effects of EGCG were less pronounced (two-tailed \(P\) for BFU-E = 0.0632 and for CFU-GM = 0.013595, \(n = 7\)). Figure 5A and B shows the data expressed as means ± SD of percent control colony formation for BFU-E and CFU-GM from the different ACD and MDS patient samples studied with the indicated pharmacologic inhibitors. Altogether, these data establish that pharmacologic inhibitors of the p38 MAPK pathway by either the pyridinyl imidazole class of compounds (SB203580) or the pyrazole aryl urea class of compounds (BIX-BIRR) significantly enhance hematopoietic progenitor cell growth from MDS or ACD bone marrows, implicating abnormal activation of p38 MAPK in the pathophysiology of these syndromes.
Discussion

Overproduction of myelosuppressive cytokines has been implicated in the pathogenesis of a variety of bone marrow failure states, including aplastic anemia and isolated cytopenias of the erythroid and myeloid lineages (17–19). In addition, there is evidence that some myelosuppressive cytokines, including TNF-α, TGF-β, and IFN-γ, play important roles in the pathogenesis of MDS and ACD (22–26). Previous studies have shown that neutralizing antibodies against TNF-α partially reverse the hematopoietic defects seen in bone marrow cells from patients with MDS (27) or ACD (22) in vitro. Such observations have led to clinical trials in which antibodies against TNF-α or soluble TNF receptors have been used for the treatment of MDS. These studies have provided evidence that inhibition of TNF activity induces clinical responses in some patients with MDS, underscoring the importance of this cytokine in the pathogenesis of myelodysplasia (44). In addition, other pharmacologic agents that have shown efficacy in the treatment of MDS may act in part by inhibition of cytokine production (45). Thus, inhibition of cytokine overproduction in the bone marrows of MDS patients...
of TNF-α, bone marrow mononuclear cells from normal donors were plated in a methylcellulose culture assay system in the presence or absence of IFN-γ MAPK inhibitor BIX-01208 (1 μM/L) as indicated. Data are expressed as percent control of CFU-GM or BFU-E colony numbers for untreated cells. Columns, mean of four independent experiments for each condition; bars, SE. *, P = 0.0115, paired two-tailed t test analysis for BFU-E; **, P = 0.0012, paired two-tailed t test analysis of IFN-γ versus TNF-α/BIX-01208 for BFU-E; ***, P = 0.0014, paired two-tailed t test analysis of TNF-α versus TNF-α/BIX-01208 for BFU-E. E, Bone marrow mononuclear cells from normal donors were plated in a methylcellulose culture assay system in the presence or absence of TNF-α and/or the p38 MAPK inhibitor BIX-01208 as indicated. Data are expressed as percent control of CFU-GM or BFU-E colony numbers for untreated cells. Columns, mean of four independent experiments for each condition; bars, SE. *, P = 0.0115, paired two-tailed t test analysis of IFN-γ versus TNF-α/BIX-01208 for BFU-E; **, P = 0.0012, paired two-tailed t test analysis of IFN-γ versus IFN-γ/BIX-01208 for CFU-GM; *, P = 0.0371, paired two-tailed t test analysis of TNF-α versus TNF-α/BIX-01208 for BFU-E; ***, P = 0.0134, paired two-tailed t test analysis of TNF-α versus TNF-α/BIX-01208 for BFU-GM. D, Bone marrow mononuclear cells from normal donors were plated in a methylcellulose culture assay system in the presence or absence of TNF-α and/or the MAPKAPK-2 inhibitor peptide. Data are expressed as percent control of CFU-GM or BFU-E colony numbers for untreated cells. Columns, mean of four independent experiments for each condition; bars, SE. *, P = 0.0329, paired two-tailed t test analysis of TNF-α versus TNF-α/H89 for BFU-E; **, P = 0.01212, paired two-tailed t test analysis of TNF-α versus TNF-α/H89 for CFU-GM. E, Bone marrow mononuclear cells from normal donors were plated in a methylcellulose culture assay system in the presence or absence of TNF-α and/or a MAPKAPK-2 inhibitor peptide. Data are expressed as percent control of CFU-GM or BFU-E colony numbers for untreated cells. Columns, mean of four independent experiments for each condition; bars, SE. *, P = 0.0182, paired two-tailed t test analysis for BFU-E; **, P = 0.0119 paired two-tailed t test analysis for CFU-GM. F, Enriched CFU-E hematopoietic progenitors were transfected with either p38α-specific siRNA or control scrambled siRNA (Scr). The cells were lysed in phosphorilation lysis buffer and cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against p38α. The same blot was stripped and reprobed with an anti-tubulin antibody to control for protein loading. G, CD34+ cells from the bone marrows of normal donors were transfected with either p38α-specific siRNA or control scrambled siRNA. Cells were subsequently plated in a methylcellulose culture assay system in the presence or absence of TNF-α as indicated. Erythroid (BFU-E) and myeloid (CFU-GM) progenitor colonies were scored on day 14 of culture. Data are expressed as percent control colony numbers for untreated cells. Columns, mean of three independent experiments; bars, SE. *, P = 0.0168, paired two-tailed t test analysis for BFU-E; **, P = 0.01243 paired two-tailed t test analysis for CFU-GM. H, CD34+ cells from the bone marrows of normal donors were transfected with p38α siRNA or control scrambled siRNA. Cells were then plated in a methylcellulose in the presence or absence of TGF-β1. BFU-E and CFU-GM progenitor colonies were scored on day 14 of culture. Data are expressed as percent control colony numbers for untreated cells. Columns, mean of two independent experiments; bars, SE. *, P = 0.0044, paired two-tailed t test analysis for BFU-E; **, P = 0.0362 paired two-tailed t test analysis for CFU-GM. I, KT-1 cells were transfected with MSK1 siRNA or control nontargeting siRNA (NS-Si). The cells were lysed in phosphorilation lysis buffer and cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against MSK1. To confirm equal protein loading, the same blot was stripped and reprobed with an anti-GAPDH antibody. J, CD34+ cells from the bone marrows of normal donors were transfected with either MSK1-specific siRNA or control nontargeting siRNA. Cells were subsequently plated in a methylcellulose culture assay system in the presence or absence of TNF-α. Erythroid (BFU-E) and myeloid (CFU-GM) progenitor colonies were scored on day 14 of culture. Data are expressed as percent control colony numbers for untreated cells. Columns, mean of three experiments; bars, SE. *, P = 0.0107, paired two-tailed t test analysis for BFU-E; **, P = 0.0234 paired two-tailed t test analysis for CFU-GM.
examined whether p38 plays a role in hematopoietic failure in MDS and ACD. Our data establish that in addition to the specific p38 inhibitors SB203580 (9) the inhibition of growth of normal bone marrow–derived hematopoietic progenitors also reverses such myelosuppressive effects, strongly implicating p38 MAPK as a physiologic regulator of normal hematopoiesis by cytokines. Our findings also show that pharmacologic inhibition of p38 enhances hematopoietic colony formation for both myeloid and erythroid progenitors from patients with MDS and ACD. This effect was seen in eight of eight abnormal bone marrows studied. In the cases in which the effects of a neutralizing antibody against TNF-α were analyzed in parallel, we found reversal of hematopoietic suppression by the anti-TNF-α antibody, confirming that TNF-α overproduction plays a major role in the abnormal activation of the p38 MAPK in the bone marrows of such patients.

In other studies, we provide the first evidence that the downstream effector kinase of p38, MSK1, is activated during engagement of the TNF-α receptor in cells of hematopoietic origin, whereas in previous studies we have established a similar activation of this kinase in response to IFN treatment (33). Engagement of MSK1 suggests a potential TNF-dependent transcriptional regulatory mechanism for early-response genes in hematopoietic progenitors, as this kinase has been implicated in the induction of histone phosphorylation and transcription of early-response genes in response to stress (35–37, 46, 47). It is therefore possible that MSK1 regulates TNF- and IFN-inducible immediate gene transcription in hematopoietic cells, but this remains to be directly established in future studies.

Independently of the precise mechanism involved, our studies that show inhibition of MSK1 expression using siRNA mimics the effects seen with p38 MAPK inhibitors or p38 MAPK knockdown, suggesting that MSK1 plays a key role in mediating suppression of hematopoiesis. Similarly, inhibition of another p38 effector kinase, MAPKAPK-2, also partially reverses the myelosuppressive effects of TNF-α, suggesting that the activities of more than one p38-dependent kinase may participate in the generation of such effects. Independently of the precise mechanisms involved, our findings provide strong evidence that pharmacologic inhibitors of p38 reverse the hematopoietic defects present in bone marrows from patients with MDS and ACD in vitro. This raises the possibility that p38 MAPK inhibitors may also reverse such defects in vivo in patients suffering from these syndromes. Pharmacologic inhibitors of p38 MAPK are currently in development for the treatment of rheumatoid arthritis, bronchial asthma, and other inflammatory diseases (48–50) based on their ability to decrease production of proinflammatory cytokines. Based on our findings, clinical studies in patients with MDS and ACD are also warranted and may lead to the development of new effective approaches for the treatment of these conditions.

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**Figure 5.** Pharmacologic inhibition of p38 MAPK with SB203580 (10 μmol/L) or BIX-01208 (1 or 5 μmol/L, as indicated) increases hematopoietic progenitor colony formation from bone marrows of patients with ACD and MDS. Columns, mean of BFU-E (A) and CFU-GM (B) of percent control colony formation for the indicated conditions; bars, SE. Percent control changes were extrapolated from the colony number values shown in Table 1. □ BFU-E; ○, CFU-GM.


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Role of the p38 Mitogen-Activated Protein Kinase Pathway in Cytokine-Mediated Hematopoietic Suppression in Myelodysplastic Syndromes

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