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# p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells

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**Abstract** **Glucocorticoids (GC) are common components in chemotherapeutic protocols for lymphoid malignancies. GC-induced apoptosis requires the intrinsic, BCL-2 family-regulated pathway. Treatment of CCRF-CEM (T cell acute lymphoblastic leukemia) cells with the GC, dexamethasone (Dex), activates p38-mitogen activated protein kinase (p38-MAPK) and then induces mRNA transcription and synthesis levels of BIM, a BH3-only pro-apoptotic BCL-2 family member. Dex-induced apoptosis is dramatically inhibited by downregulation of BIM by shRNA or by pretreatment with a p38-MAPK inhibitor, SB203580, which also reduces BIM induction. These findings indicate that BIM induction through p38-MAPK activation is a critical pathway in GC-induced cell death.**


*Keywords:* Apoptosis; BIM; Acute lymphoblastic leukemia; Dexamethasone; p38-MAPK

## 1. Introduction

Programmed cell death (apoptosis) is a well-conserved genetic pathway with basic tenets that appear to be common to all metazoans. Defective control of this pathway is implicated in disorders ranging from cancer and autoimmune diseases to degenerative syndromes. Specific defects in the capacity to undergo apoptosis, or in the upstream signal transduction pathways not only provide cancer cells with an intrinsic survival advantage, but may also confer inherent resistance to chemotherapeutic drugs. Many chemotherapeutic drugs kill target cells via the mitochondria-dependent, BCL-2 family-regulated pathway. The BCL-2 family is characterized by the presence of structural homology, termed BCL-2 homology (BH) domains. The "multi-domain" BCL-2 family members either suppress apoptosis (e.g., MCL-1, BCL-2, BCL-$X_L$) or promote apoptosis (e.g., BAX, BAK), whereas the "BH3-only" subfamily members identified to date (e.g., BAD, BID, BIM, PUMA) function to promote cell death [1,2]. The BH3-only proteins act by antagonizing the function of anti-apoptotic BCL-2 family members and also by directly activating BAX/BAK function. It has become clear that BH3-only proteins are essential initiators of programmed cell death and may be pivotal in certain types of cancer cell killing.

BIM (BCL-2-interacting mediator of cell death), a BH3-only protein, can be modulated at the transcriptional and post-translational levels (reviewed by [3,4]). Transcriptional control of BIM can involve contributions from Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K), and extracellular signal-regulated kinase (ERK) pathways. It has been reported that phosphorylation and subsequent stability changes followed by ubiquitination can regulate BIM's protein level. We have shown that ERK-dependent phosphorylation of BIM in response to survival factor inhibits BIM–BAX interaction and consequently promotes survival [5]. Studies of BIM-deficient mice/cells indicate important roles of BIM in hematopoietic cell homeostasis [6] and response to a variety of chemotherapeutic drugs. It has been recently demonstrated that loss of BIM facilitates myc-induced tumorigenesis in B cells [7,8]. In humans, *Bim* is a candidate tumor suppressor gene in mantle cell lymphoma [9]. BIM is required for apoptosis induced by Taxol, dexamethasone, Gleevec, and histone deacetylase inhibitors [4,10,11], indicating that this protein has a role in anticancer therapy against human malignancies. However, the molecular mechanisms of both BIM regulation and BIM's role in downstream cell death machinery are not entirely clear.

Glucocorticoids (GC) are effective agents for the treatment of leukemia/lymphoma, including acute lymphoblastic leukemia (ALL), multiple myeloma, chronic lymphocytic leukemia, and non-Hodgkin's lymphoma [12–15]. GC-induced apoptosis requires the BCL-2 family-regulated pathway, but the upstream signaling pathways and participation and interaction of downstream target molecules remain largely undefined. Studies of BIM- or PUMA (p53 upregulated modulator of apoptosis)-deficient mice/cells suggest that these BH3-only proteins play important roles in dexamethasone (Dex)-induced cell death in normal lymphocytes [6,16,17].

In this report, we used a T cell-ALL (T-ALL) cell line, CCRF-CEM to study the molecular mechanisms of Dex-induced cell death in malignant cells. We demonstrate that Dex induced apoptosis is critically dependent upon upregulation of BIM, which is primarily regulated at RNA level and dependent upon p38-MAPK activation.

## 2. Materials and methods

### 2.1. Cell line and culture

The human T-ALL cell line CCRF-CEM was purchased from the American Tissue Culture Collection. The cells were cultured with RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, streptomycin, and penicillin G at 37 °C in a 5% $CO_2$ incubator.

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*2.2. Chemicals and antibodies*

Dexamethasone, U0126, LY294002 and SB203580 were purchased from Sigma. SP600125 was purchased from Calbiochem. Antibodies were purchased as follows: BIM (202000) from Calbiochem, PUMA, BAX, BAK, BCL-$X_L$, β-tubulin, phospho-JNK, ERK, JNK, and AKT from Santa Cruz Biotechnology, BCL-2 from Pharmingen, MCL-1 from Stressgen, phospho-p38-MAPK, phospho-ERK, phospho-AKT, and p38-MAPK from Cell Signaling Technology.

*2.3. Western blot analyses*

Whole cell lysates were prepared with lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), 1% CHAPS, 20 mM NaF, 10 mM β-glycerophosphate, and a protease inhibitor cocktail (Sigma)]. Equal amounts of proteins were loaded on SDS–PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblotting.

*2.4. Cell viability assay*

Cell death was quantified by Annexin-V-FITC (Becton Dickinson)-propidium iodide (PI, Sigma) staining according to the manufacturer's protocol, followed by flow cytometric analysis using FACScan (Becton Dickinson).

*2.5. Real-time PCR*

Total RNAs were prepared using Trisol reagent (Invitrogen). Residual DNAs in the samples were removed by treatment with RNase-free DNase I (Ambion) according to the manufacture's protocol. Experiments were performed in the Virginia Commonwealth University Nucleic Acid Research Facility with the ABI Prism® 7900 Sequence Detection System (Applied Biosystems) using the TaqMan® One Step PCR Master Mix Reagents Kit. 18S rRNA was quantified as endogenous control. A probe and primer set for BIM was purchased from Applied Biosystems (Hs001982_m1).

*2.6. Plasmid construction and transfection*

pSR-BIM was constructed by inserting the target sequence for human BIM (GenBank AF032457, nucleotide 37–56; GACCGAGAAGGTAGACAATT) into pSUPER.retro.puro (Oligoengine) according to the manufacture's protocol. As a control, a scrambled, non-specific sequence (AATTCTCCGAACGTGTCACGT) was inserted into the same vector (pSR-con). Transfection was performed by electroporation using a Bio-Rad electroporator. The cells were suspended in RPMI 1640 ($4 \times 10^6$/400 μl) with 10 μg of DNA and electroporated in 0.4 cm cuvettes at 300 V, 500 μF. Puromycin (2 μg/ml) selection to establish stable clones began 24 h after electroporation.

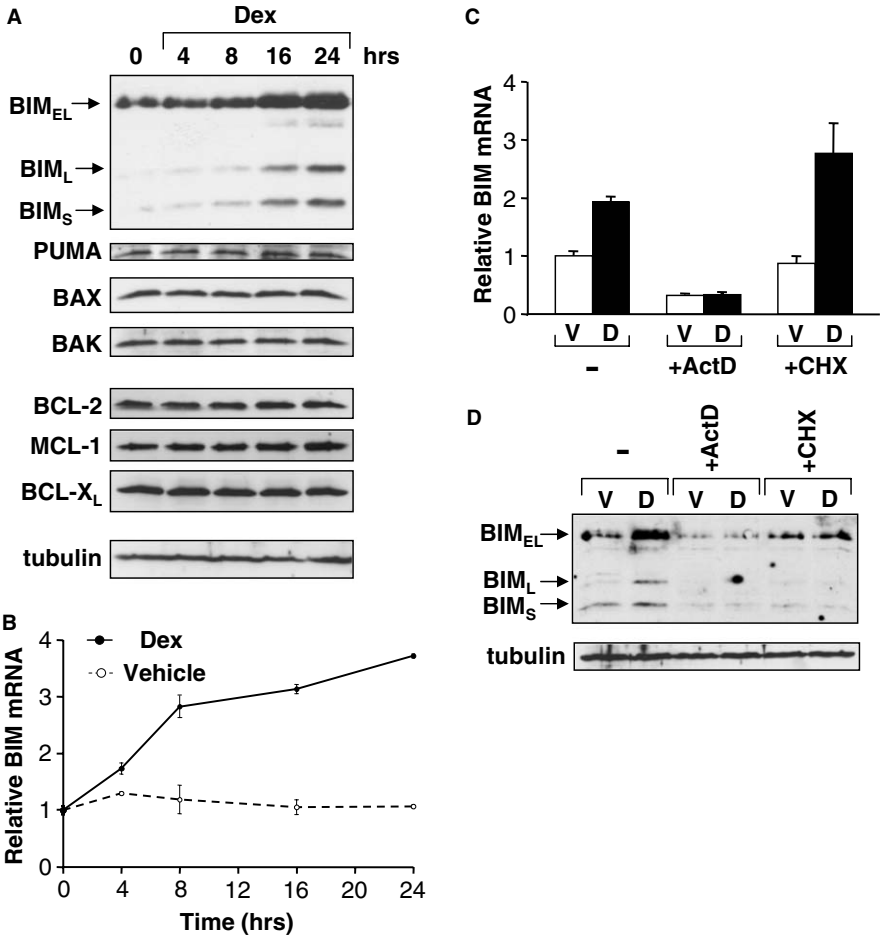


Fig. 1. The expression of the BCL-2 family members with Dex treatment in CEM cells. (A) CEM cells were treated with dexamethasone (Dex, 0.3 μM) for the indicated times. Equal amounts of total cell extracts were subjected to Western blots with the indicated antibodies. (B) CEM cells were treated with Dex (0.3 μM) or vehicle (0.1%DMSO) for the indicated times. Total RNAs were subjected to real-time PCR. Each value was normalized with the value of 18S RNA at the same time point. The results are the mean ± S.D. of triplicates. (C) CEM cells were pretreated with actinomycin D (Act D, 1 μg/ml) or cycloheximide (CHX, 10 μg/ml) for 30 min, and then treated with vehicle (V) or Dex (D, 0.3 μM) for 4 h. Total RNAs were subjected to real-time PCR as (B). The results are the mean ± S.D. of triplicates. (D) CEM cells were pretreated as (C), and then treated with vehicle (V) or Dex (D, 0.3 μM) for 16 h. Equal amounts of total cell extracts were subjected to Western blots with anti-BIM and anti-β-tubulin antibodies.

## 3. Results and discussion

### 3.1. BIM is induced by dexamethasone treatment in CCRF-CEM cells prior to the onset of apoptosis

Dex is an effective drug against ALL and BIM is induced by Dex (0.3 μM) treatment in the Dex-sensitive T-ALL cell line, CCRF-CEM (CEM) (Fig. 1A). We examined the expression of the BCL-2 family proteins before the onset of apoptosis, which begins in this cell line at 24 h after treatment. Eight to sixteen hours after treatment, all isoforms of BIM ($BIM_{EL}$, $BIM_L$, $BIM_S$) are strongly induced and continue to increase to 24 h (Fig. 1A). Non-malignant thymocytes from PUMA (another BH3-only member)-deficient mice are resistant to Dex-induced apoptosis [16,17], but the expression of PUMA is not much changed, suggesting this protein may not be essential for Dex-induced apoptosis in CEM cells. The overall expression of the multi-domain pro-apoptotic effectors, BAX and BAK, are constant. We also examined the expression of anti-apoptotic members, BCL-2, MCL-1 and BCL-$X_L$. All these anti-apoptotic BCL-2 family members also show constant expression (Fig. 1A). Upregulation of BIM and constant expression of BAX/BAK and anti-apoptotic BCL-2, MCL-1, and BCL-$X_L$ are also observed in a Dex-sensitive multiple myeloma cell line, MM.1S (data not shown). It has also been shown that BIM is induced by Dex in CEM cells, murine T-cell lymphoma cell lines, and human pre-B acute lymphoblastic leukemia cells [18–21]. These results suggest that BIM induction is critical for apoptosis in leukemia and myeloma cells that are sensitive to Dex treatment.

### 3.2. Dexamethasone-mediated BIM induction is transcriptionally regulated

Accumulating evidence indicates that various external stimuli regulate BIM in several possible ways, both transcriptionally and post-translationally, e.g. by phosphorylation. We first examined *Bim* mRNA expression and found that induction starts 4 h after Dex treatment and continues to increase to 16–24 h (Fig. 1B). *Bim* mRNA induction is completely inhibited by a transcription inhibitor, actinomycin D. However, treatment with a protein synthesis inhibitor, cycloheximide, augments Dex-induced *Bim* mRNA accumulation (super-induction) (Fig. 1C), suggesting that *de novo* protein synthesis is not required for the induction of *Bim* mRNA. BIM protein induction is inhibited by actinomycin D or cycloheximide (Fig. 1D), thus new BIM protein synthesis is required for its accumulation. Similar observations have been reported in previous studies [18,21]. These data indicate that Dex treatment increases BIM levels mainly by new mRNA synthesis.

### 3.3. BIM is required for dexamethasone-induced cell death in CEM cells

To confirm the significance of BIM in Dex-induced apoptosis, we introduced a shBIM (short hairpin BIM) construct with pSUPER.retro (pSR) vector into CEM cells to reduce the expression of endogenous BIM. As a control, a scrambled, non-specific shRNA construct was transfected. The expression of BIM is strongly inhibited by shBIM (Fig. 2A, upper panel), although both control and shBIM-expressing cells show similar p38-MAPK activation (Fig. 2A, lower panel), indicating that GC signaling is still intact (see below in detail). Cell death in response to Dex treatment is also strongly reduced by the expression of shBIM (Fig. 2B). These data indicate that BIM is an essential molecule in Dex-induced apoptosis in CEM cells.

### 3.4. The activation of p38-MAPK is required for dexamethasone-induced apoptosis and BIM induction

It has been reported that transcriptional regulation of BIM can involve contributions from JNK PI3K/AKT pathways [3,4]. Phosphorylation and activity of BIM is directly regulated by ERK and JNK. The phosphorylation status of BIM is not altered after Dex treatment, judged by unchanged band-shift on SDS–PAGE (Fig. 1A) [5]. The expression level

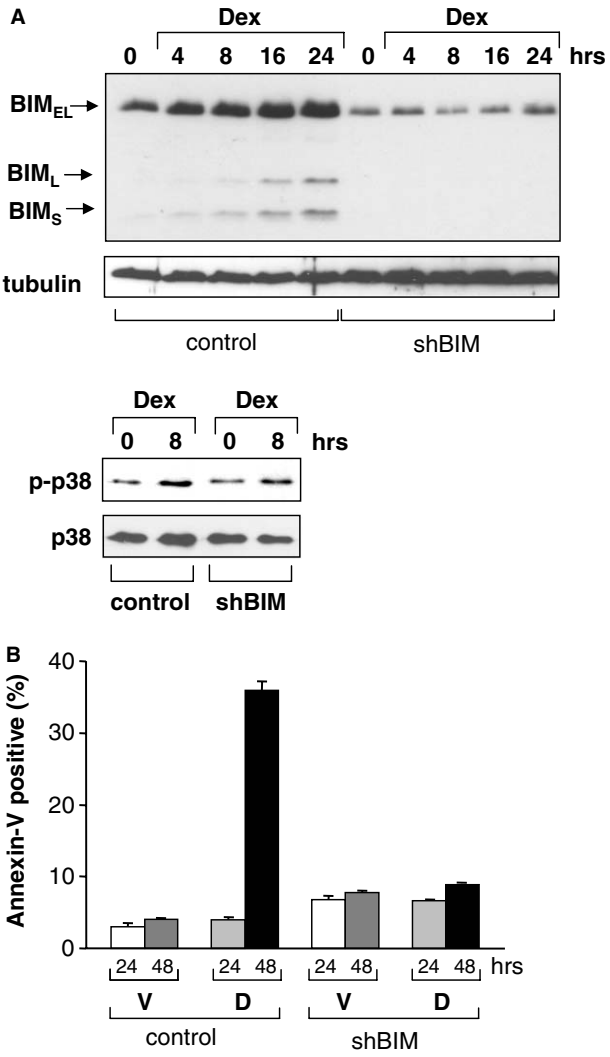


Fig. 2. BIM is required for Dex-induced apoptosis in CEM cells. (A) Upper panel: CEM cells were transfected with either pSR-BIM (shBIM) or pSR-con (control). Puromycin-resistant clones were established from each transfection. Cells were treated with Dex as Fig. 1A. Equal amounts of total cell extracts were subjected to Western blots with anti-BIM and anti-β-tubulin antibodies. Lower panel: equal amounts of the same total cell extracts at 0 and 8 h after Dex treatment used above were subjected to Western blots with anti-phospho-p38-MAPK and anti-p38-MAPK antibodies. (B) The cells in Fig. 2A were treated with vehicle (V) or Dex (D, 0.3 μM) and cell viabilities were determined at the indicated time points by Annexin V-PI staining followed by FACS analysis. Values represent the mean ± S.D. of duplicates. We analyzed three independent clones and the results are reproducible.

and phosphorylation status of ERK, JNK and AKT all show little change throughout the time course (Fig. 3A). In contrast, phosphorylation of p38-MAPK starts to increase at 4–8 h after Dex treatment compared to control (vehicle only), although there is no significant difference in p38-MAPK total protein levels (Fig. 3A). Treatment with a p38-MAPK inhibitor, SB203580, dramatically reduces Dex-induced apoptosis (Fig. 3B). The activation of p38-MAPK and the inhibition of apoptosis with SB203580 are also observed in MM.1S, a multiple myeloma cell line (data not shown). However, a PI3K inhibitor, LY294002 does not affect cell death by Dex (Fig. 3B). Treatment with a JNK inhibitor, SP600125 kills CEM cells even without Dex treatment. Treatment with either U0126 (a MEK inhibitor) or SP600125 plus Dex enhances Dex-induced apoptosis (Fig. 3B). We determined the effect of the kinase inhibitors that we used on p38-MAPK phosphorylation. LY294002 shows no effect on p38-MAPK phosphorylation induced by Dex (Fig. 3C, lanes 2 and 4), U0126 slightly reduces the p38-MAPK phosphorylation, but the phosphorylation is still induced by Dex treatment (Fig. 3C, lanes 2 and 6), and the amount of cell death parallels BIM induction (Fig. 3B and C). SP600125 alone strongly induces p38-MAPK phosphorylation in the absence of Dex (Fig. 3C, lane 7) and phosphorylation does not increase with Dex treatment (Fig. 3C, lane 8). BIM levels decrease with co-treatment of SP600125 plus Dex, but Dex-induced apoptosis

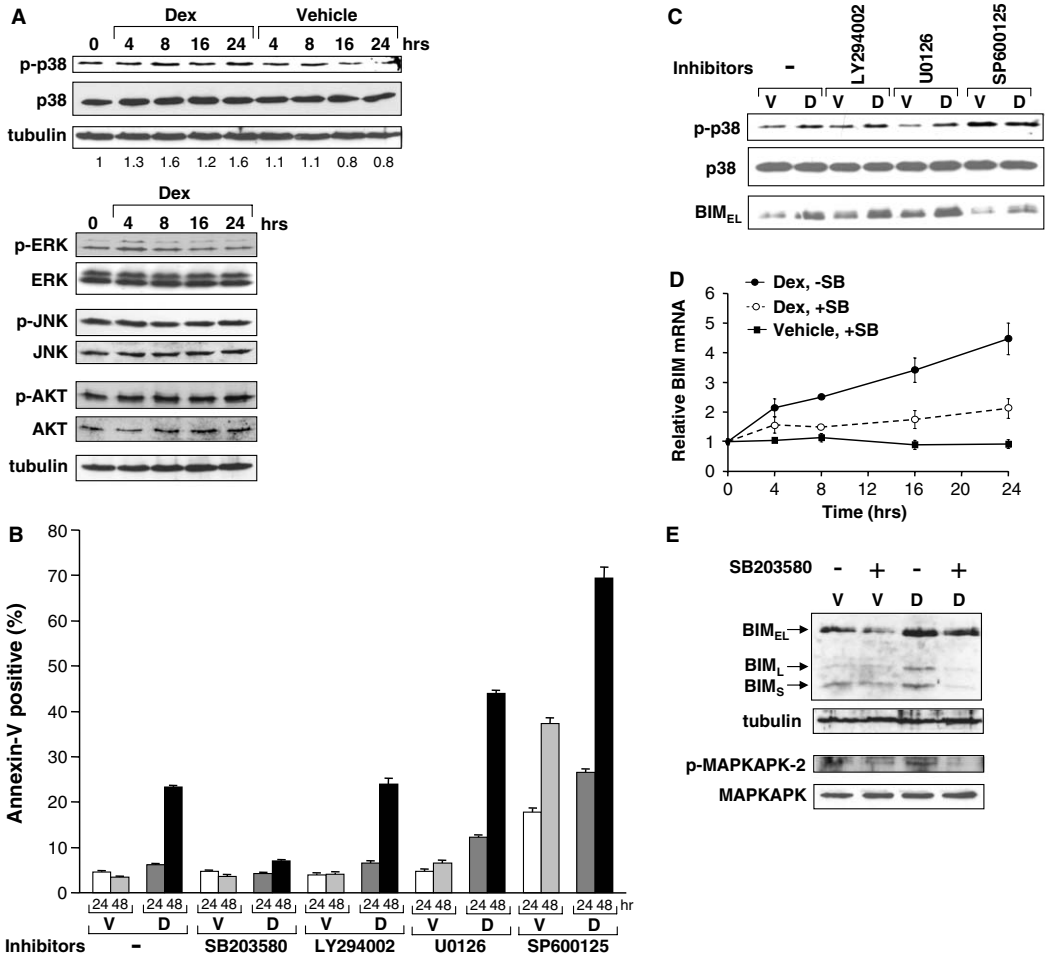


Fig. 3. The activation of p38-MAPK is required for Dex-induced apoptosis. (A) CEM cells were treated with vehicle or Dex (0.3 μM) for the indicated times. Equal amounts of total cell extracts were subjected to Western blots with the indicated antibodies. The relative amount of phosphorylated p38-MAPK in each band was determined by scanning densitometric analysis of the X-ray films using the NIH Image J program, and the relative value is shown at the bottom of the panel. The results are reproducible in three (p38-MAPK) and two (other kinases) separate experiments. (B) Kinase inhibitors [p38-MAPK inhibitor, SB203580 (10 μM), MEK inhibitor, U0126 (3 μM), JNK inhibitor, SP600125 (5 μM), and PI3K inhibitor, LY294002 (10 μM)] were added 30 min before Dex treatment. At 24 and 48 h after vehicle (V) or Dex (D, 0.3 μM) treatment, cell viability was determined with Annexin V-PI staining followed by FACS analysis. The result is the mean ± S.D. of duplicates. The results are reproducible in two independent experiments. (C) CEM cells were treated with kinase inhibitors as (B) followed by Dex treatment for 16 h. Equal amounts of total cell extracts were subjected to Western blots with the indicated antibodies. The results are reproducible in two independent experiments. (D) CEM cells were pretreated with SB203580 and were treated with vehicle or Dex (0.3 μM) for the indicated times. Total RNAs were subjected to real-time PCR. Each value was normalized with the value of 18S RNA at the same time point. The results are the mean ± S.D. of triplicates. (E) CEM cells were pretreated with SB203580, followed by vehicle (V) or Dex (D, 0.3 μM) treatment for 16 h (for BIM) or 8 h (for MAPKAPK-2). Equal amounts of total cell extracts were subjected to Western blots with the indicated antibodies. As a control, the phosphorylation of MAPKAPK-2, which is a direct substrate of p38-MAPK, was determined to see the effect of SB203580. The results are reproducible in two independent experiments.

is increased approximately additively (Fig. 3B). Thus, we speculate that cell death with SP600125 is BIM-independent. The activation of p38-MAPK by Dex and similar kinase inhibitor studies have also been reported in Dex-sensitive CEM clones [22]. In conjunction with the inhibition of apoptosis, *Bim* mRNA and protein induction is partially reduced by SB203580 (Fig. 3D and E). As a control, SB203580 inhibits Dex-induced phosphorylation of MAPKAPK-2, a direct substrate of p38-MAPK (Fig. 3E). We also tested another p38-MAPK inhibitor, SB 202190, and the results are similar (data not shown). Taken together, the modulation of p38-MAPK activity correlates with BIM induction and apoptosis, indicating that p38-MAPK activation is required for BIM induction and consequent apoptosis with Dex treatment. However, cells expressing a shBIM construct are resistant to Dex-induced apoptosis, but still show Dex-induced p38-MAPK activation (Fig. 2A), suggesting that the activation of p38-MAPK alone is not sufficient to induce apoptosis by Dex treatment. Although the activation of p38-MAPK is reduced by SB 203580 to the basal level, *Bim* mRNA and protein induction are not completely inhibited (Fig. 3D and E). Thus, it is possible that a p38-MAPK-independent BIM induction pathway also contributes the induction of *Bim* mRNA and protein.

It has been recently shown that p38-MAPK phosphorylates GC receptor (GR) and contributes to Dex-induced apoptosis in CEM cells [22]. However, GR phosphorylation is observed 20 h after Dex treatment, which is well after the BIM induction (4 h). Clearly GR phosphorylation cannot cause BIM upregulation. Thus, we speculate that p38-MAPK first induces BIM expression, initiates apoptosis, and then phosphorylates GR to reactivate the cell death pathway. To date, several signaling pathways and transcription factors have been identified to regulate *Bim* mRNA, such as PI3K/Akt/FOXO (Forkhead box O), JNK/c-Jun, and c-myc [3,4,7,8]. However, we did not detect any changes in nuclear FOXO levels initiated by Dex (unpublished data). Although we do see increases in c-JUN levels, this induction appears to be independent of p38-MAPK activation (unpublished data). c-myc is known to be downregulated by Dex treatment in CEM cells [23]. Therefore, other transcription factors might be activated by p38-MAPK and contribute to *Bim* mRNA induction. This could be a novel mode of BIM transcriptional regulation.

In conclusion, we find a critical signaling pathway for GC-induced apoptosis, Dex-GR-p38-MAPK-BIM. It has recently been published that the *Bim* gene is induced in childhood ALL patients sensitive to Dex treatment [18]. Our results in CCRF-CEM model system also suggest that BIM may be a primary GC target molecule to induce cell death. GC resistant leukemia and myeloma are often associated with GC receptor defects. Precise delineation of the pathway downstream might enable bypassing the receptor to target downstream molecules that can initiate apoptosis in cells with non-functional GC receptors. Understanding the role of BIM in GC response could help develop new therapeutic strategies to combat GC resistant leukemia/myeloma.

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