Batf Promotes Growth Arrest and Terminal Differentiation of Mouse Myeloid Leukemia Cells

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Abstract

Batf is a basic leucine zipper transcription factor belonging to the activator protein-1 superfamily. Batf expression is regulated following stimulation of both lymphoid and myeloid cells. When treated with leukemia inhibitory factor, mouse M1 myeloid leukemia cells commit to a macrophage differentiation program that is dependent on Stat3 and involves the induction of Batf gene transcription via the binding of Stat3 to the Batf promoter. RNA interference was employed to block Batf induction in this system and the cells failed to growth arrest or to terminally differentiate. Restoring Batf expression not only reversed the differentiation-defective phenotype but also caused the cells to display signs of spontaneous differentiation in the absence of stimulation. Efforts to define genetic targets of the Batf transcription factor in M1 cells led to the identification of c-myb, a proto-oncogene known to promote blood cell proliferation and to inhibit the differentiation of M1 cells. These results provide strong evidence that Batf mediates the differentiation-inducing effects of Stat3 signaling in M1 cells and suggest that Batf may play a similar role in other blood cell lineages where alterations to the Jak–Stat pathway are hallmarks of disrupted development and disease. Mol Cancer Res; 9(3); 1–14. ©2011 AACR

Introduction

The activator protein-1 (AP-1) transcription factor is comprised of dimerizing basic leucine zipper (bZIP) proteins of the widely expressed Jun, Fos, and Atf protein families and of the more tissue-restricted Maf and Batf families (1, 2). Batf, the founding member of the Batf protein family (3), is expressed at low levels in both myeloid and lymphoid cells and is induced by a variety of stimuli (4–9). Batf proteins preferentially form heterodimers with the Jun proteins and while Batf/Jun dimers bind equally well as Fos/Jun dimers to AP-1 consensus DNA, they possess a reduced ability to transactivate gene expression (3, 5, 10, 11). Consistent with this role as an AP-1 inhibitor, overexpression of Batf effectively blocks the ability of AP-1-associated signaling pathways to stimulate cell growth (5, 6, 12, 13). On the other hand, lymphocytes from Batf knock-out mice do not display the reciprocal phenotype of increased proliferation, but instead display defects in the differentiation of specific T helper cell subsets, in cytokine gene expression and in the responsiveness of B cells to antigen presentation (14, 15). This apparent contradiction in behavior supports evidence that AP-1 functions in cellular processes as diverse as cellular transformation, differentiation and apoptosis and that the role of a cell-specific AP-1 component, such as Batf, will depend on the cellular context in which it is expressed (1).

In an effort to learn more about the impact of Batf on the growth and differentiation properties of individual blood cell types, we chose to study the mouse M1 myeloid leukemia cell line (16). M1 cells proliferate as a transformed blast cell population until exposed to a member of the IL-6/LIF family of cytokines (17–19), at which point the cells exit the cell cycle and initiate a program duplicating many aspects of monocyte to macrophage differentiation (20). Binding of the appropriate cytokine to the gp130 receptor complex and subsequent activation of Jak–Stat3 signaling are critical to this developmental program (21, 22). M1 cells have been exploited to identify myeloid differentiation (MyD) primary response genes as well as genes encoding key cell cycle regulators, transcription factors, and structural proteins that are necessary to convert blast cells to terminally differentiated macrophages (20, 23). Interestingly, the list of genes that fit the profile of MyD genes in this system includes c-Jun, JunB, JunD, and Batf, but not c-Fos (7, 24). Thus, M1 cells provide an opportunity to examine the impact of Batf/Jun heterodimers on a well-defined program of myeloid lineage differentiation.

In this study, we investigated the mechanisms underlying the rapid induction of Batf following exposure of M1 cells and primary bone marrow cells to differentiation-inducing cytokines and have defined the Batf gene as a direct target of regulation by Stat3. Utilizing a gene knockdown approach...
in M1 cells, we demonstrated that Stat3-induced Batf expression is essential for the leukemia inhibitory factor (LIF)-induced growth arrest that precedes the conversion of these cells to macrophages. Furthermore, forced expression of Batf drives M1 cells to spontaneously adopt differentiated characteristics, even in the absence of LIF. A comparative analysis of gene expression in wild type M1 and Batf-deficient M1 cells revealed a correlation between the induction of Batf and the repression of c-myc, a gene with a well-documented role in myeloid leukemia (25) and a known inhibitor of the macrophage differentiation program of M1 cells (26–28). These studies serve to classify Batf as a MyD gene and support a role for Batf as an AP-1 component functioning to restrict M1 cell growth and negatively regulate at least one target gene whose repression is required for the differentiation of these cells.

Materials and Methods

Cell culture

Mouse M1 myeloid leukemia cells (ATCC, TIB-192), A20 B lymphoma cells (ATCC, TIB-208), and primary mouse bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco/Invitrogen), penicillin (100 U/mL) and streptomycin (100 µg/mL). Primary mouse bone marrow cells were isolated from 8-week-old C57BL/6 mice (Harlan Laboratories). Briefly, both femurs were dissected and marrow removed by forcing RPMI-1640 growth medium through the bone cavity with a 26-gauge needle. Cells were pelleted by centrifugation at 1,000 rpm for 5 minutes at 4°C, resuspended in RPMI-1640 growth medium, counted and plated.

Recombinant mouse interleukin-6 (IL-6) (Gibco/Invitrogen) was used at a concentration of 50 ng/mL and human leukemia inhibitory factor (LIF1005, Chemicon International) was used at a concentration of 5 ng/mL. AG490 (Calbiochem) was stored at −20°C as a 17 mmol/L stock solution in dimethyl sulfoxide (DMSO). To generate Batf-RNAi and Neg-RNAi cells, 1 × 10^7 M1 cells in 250 µL of PBS containing 50 µg of plasmid DNA were electroporated at 310 V, 950 µF and allowed to recover for 24 hours in RPMI-1640 growth medium prior to selection with 200 µg/mL G418 (Cellgro). Batf-RNAi clone 11 cells were isolated from the stable Batf-RNAi cell pool by limiting dilution. To generate the Batf RNAi + BATF and Batf RNAi + EGFP cells, Batf-RNAi cells were exposed overnight to virus supernatants from 293FT cells (gift from S. F. Konieczny) expressing the appropriate retroviral vector. Infected cells were transferred to RPMI-1640 growth medium for 24 hours prior to selection using both G418 and 1 µg/mL puromycin (Sigma).

RNA and protein analysis

Total RNA was isolated from bone marrow cells or M1 cell lines treated with IL-6, LIF, AG490, or vehicle control (times and concentrations are indicated in Figure legends) using Trizol Reagent (Invitrogen). Real-time, quantitative reverse transcription PCR (RT-qPCR) was performed as described (15) using an Applied Biosystems 7300 thermocycler and the FastStart Universal SYBR Green PCR system (Roche) with the following primers for Batf (f 5' GAG-GAA-GATCGCATCGCTGC 3'; r 5' GTTCTGGTCTTCTGC-AGTTCC 3'); c-myc (QT00123340, Qiagen), and β-actin (QT00095242, Qiagen) as a control. Rapid amplification of 5' cDNA ends was performed using the 5' RACE System (Gibco/Invitrogen). For the analysis of protein expression, 50 µg of total cell extract or 100 µg of nuclear extract was prepared, resolved by SDS—PAGE, and immunoblotted as described (29, 30). Primary antibodies were used at a 1:1,000 dilution and were purchased from Santa Cruz (anti-Stat3, H-190; anti-Hsp-60, N-20; anti-Hsp-90α/β, H-114), Millipore (anti-phospho-Stat3, clone 9E12), Roche (anti-HA, clone 3F10), or ICN Pharmaceutical (anti-Actin, clone C4). The rabbit polyclonal anti-Batf antisemur was described previously (9). The appropriate, secondary HRP-conjugated antibodies were purchased from Santa Cruz and used at a 1:5,000 dilution. Antibody complexes were visualized using SuperSignal West Dura Extended Duration Substrate Kit (Pierce) and X-ray film (Kodak).

Plasmid DNAs

The −1000 B-Luc reporter gene was constructed by inserting an EcoRV/KpnI fragment of the genomic, human BATF DNA (4) into Small/KpnI linearized, pGL2-basic (Promega). The −273 B-Luc and −123 B-Luc reporters were generated using the Erase-a-Base Kit (Promega). The −50 B-Luc reporter was generated by PCR amplification and cloning into pGL2-basic as above. Quick Change Site-Directed Mutagenesis (Stratagene) was used to alter the Stat3C site at position −69/−60 in −1000 B-Luc from (5′-TACCAGAAAA-3′) to (5′-GGCCAGAAGACATCCAGAG-3′) to generate StatC MutB-Luc. pSBatf-RNAi and pSNeg-RNAi were generated by ligating a mouse Batf-specific anti-sense double-stranded DNA oligomer (5′ GATCCCCGAGCCGCA- CAGAGACAGACATTCAAGAGATGTCTGTCTCTG-TCGGCTCTTTTGAAA 3′) or a control nonsense oligomer (5′ GATCCCCGGCAGCTTCTTGATAGTATGCTGCTGC- GCAGCAGAATCTTACAAAGCGCCGTGTCTG- CGCTTCTTTTGGAA 3′), respectively, to the pSUPER.neo + gfp vector (OligoEngine, Inc.) linearized with BglII/HindIII. Retroviral vectors to express human hemagglutinin-tagged (HA)-BATF or control EGFP were constructed by inserting a BamHI fragment and a BamHI/EcoRI fragment, respectively, into the pBABE-puro vector (29).

Luciferase assays

Reporter gene expression was monitored using the Dual Luciferase Assay system (Promega). 5 × 10^6 M1 cells per 60 mm dish were transfected using the SuperFect protocol (Qiagen), 5 µg of the indicated luciferase reporter gene and 0.1 µg of Renilla luciferase gene as a normalization control. After 24 hours, LIF was added for 6 hours and cell extracts were assayed for luciferase activity as described previously (31).
Electrophoretic mobility shift assays

The procedures for the preparation of nuclear extracts, radio labeling of probe DNA, and performing electrophoretic mobility shift assays (EMSA) have been described previously (13). Oligonucleotide probes for mouse (M) and human (H) Stat A, B, and C are indicated in Figure 3A. Competitor oligonucleotides were as follows: Stat3 (sc2571, Santa Cruz), mutStat3 (sc2572, Santa Cruz), mutC (5’-TCCAGGCT-GAGGGAGGACAAA-3’), ABC (a 173 bp DNA fragment spanning all 3 Stat3 sites), and AB mutC (an ABC variant with mutC). These oligos were added in 100-fold molar excess directly to the binding reactions. DNA probes for the putative AP-1 binding sites in the mouse c-myb gene are:

A (5’ TAAAATATGACTATTTTCAGTAAG 3’), 
B (5’ GCTACTTTATGAATCATATAATGTAGAG 3’), 
C (5’ AAAAAAGTAATCCAGAGTGACACAG 3’), 
D (5’ GAGAGGATGTTGTCAGACCCCA-3’), 
E (5’ CCTTGAACCTTGCAGGAGGAGG 3’), 
F (5’ GTCAAGGAATATGCTGTGACCCCT 3’), 
G (5’ GTGCTTTAGTTACTCAGAGACAGCA 3’).

Consensus AP-1, SP-1, and CRE oligonucleotide probes were purchased from Promega. For protein detection, 0.4 μg of a rabbit polyclonal anti-Stat3 antibody (sc482, Santa Cruz), 0.4 μg anti-estrogen receptor (ER) antibody (sc542, Santa Cruz), 5 μL of Batf antiserum (9), and 5 μL Mst-l antisemur (32) were added to the binding reactions.

Chromatin immunoprecipitation

For the indicated times, 1.5 × 10^7 M1 cells per 100 mm dish were incubated +/− LIF and protein-DNA complexes crosslinked with 270 μL of 37% formaldehyde for 10 minutes prior to the addition of 1.25 mL of 1 mol/L glycine for 5 minutes to stop the reaction. Cells were lysed for 10 minutes in 400 μL of 50 mmol/L PIPES pH 8.0, 85 mmol/L KCl, 0.5% NP40. Nuclei were pelleted and lysed in 100 μL of 50 mmol/L Tris-HCL pH 8.1, 10 mmol/L EDTA, 1% SDS. Nuclear lysates were sonicated on ice to fragment the DNA to an average size greater than 200 bp but less than 1,000 bp. Each sample was diluted 1:10 with IP buffer [0.01% SDS, 1.1% Triton X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCL pH 8.1, 167 mmol/L NaCl], precleared with 40 μL of protein G agarose beads for 30 minutes and incubated overnight at 4°C with 2.5 μL of anti-Stat3 antibody or rabbit anti-IgG antibody (Santa Cruz), or 20 μL of anti-Batf antiserum (9) or polyclonal rabbit anti-IgG antibody (Santa Cruz). Samples were incubated for 1 hour at 4°C with 40 μL of blocked protein G agarose beads, after which the chromatin was pelleted and washed for 10 minutes at RT with 1 mL of the following buffers in sequence: low salt [0.1% SDS, 1% Triton X 100, 2mmol/L EDTA, 20 mmol/L Tris pH 8.1, 150 mmol/L NaCl], high salt [0.1% SDS, 1% Triton X 100, 2mmol/L EDTA, 20 mmol/L Tris pH 8.1, 500 mmol/L NaCl], LiCl [0.25 mol/L LiCl, 1% NP40, 1% deoxycholate, 1mmol/L EDTA, 10 mmol/L Tris pH 8.1] and 1 X TE, 2 times. Chromatin was eluted in 100 mmol/L NaHCO3 and 1% SDS and the crosslinking was reversed by incubation at 65°C for a minimum of 5 hours in the presence of 0.3 mol/L NaCl. RNaseA (0.2 mg/mL) was added for 30 minutes at 37°C and 4 μL of 0.5 mol/L EDTA, 8 μL of 1 mol/L Tris pH 6.8, and 1 μL of 10 mg/mL protease K were added and the mixture incubated for an additional 2 hours. DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen). Semiquantitative real-time PCR (Fig. 3E) or quantitative real-time PCR (Fig. 7B) was performed using 10 or 2 μL of eluted DNA, respectively. Ten percent of sheared chromatin was used as input. Primers to amplify a 175 bp region of the Batf promoter are: (f 5’ TCTTGTGTCTTTTCCCCAG 3’; r 5’ CTCT-TCTGGGTGCAGAGG 3’). Amplified DNA was resolved using 1.5% agarose gels and visualized by EtBr staining. For the c-myb promoter, the primers used are:

A (f 5’ GGAGCATCTGTTTTCAAAACAGCA 3’; r 5’ GTTCAACATCAGTCGAGAGG 3’),
B (f 5’ GAATTTTCTCTGTTGTTGGC 3’; r 5’ GCTGGACGTGTTATCGACGCT 3’),
C (f 5’ GTCAAGAATACCTGAGGACG 3’; r 5’ CTACTGGATCCCAAAACGAC 3’),
D (f 5’ CCTCTACACTCTACTGACT 3’; r 5’ GCCTCTCTCCGTAGGCTC 3’),
E (f 5’ CTCCGAGGTTGAGCCCGAG 3’; f 5’ GTTAAATATCCCTGGCGAG 3’),
F (f 5’ CGAGCCCGTTTCTTGTACG 3’; r 5’ GACC GGAGAAGGTGCTG 3’),
G (f 5’ CATGCACTCAGAGACAGACG 3’; r 5’ GCAAATCATTCAACGCCGCG 3’).

Primers for region N, located 6.5 kbp to c-myb exon 1 and not containing an AP-1 DNA site, were used as a control (f 5’ GCTGGAGGGATGCTGTAAG 3’; r 5’ GCCATTTTGCTCACAACC 3’).

Analysis of growth and macrophage differentiation in M1 cells

For growth curves, M1 cells were seeded in growth medium at 1 × 10^3 cells per 60 mm dish and treated +/− LIF on day 0. On days 1 through 7, three plates of 1 × 10^6 cells in 100 mm dishes were treated +/− LIF on day 0. On days 1, 2, and 3, cells were harvested and counted. On days 1, 2, and 3, cells were harvested and counted. 1 × 10^6 cells were washed 4 times with cold PBS and suspended in 500 μL of PBS. The side- and forward-light scatter profiles of individual cells in each culture were measured using a Beckman Coulter FC500 Flow Cytometer. To measure phagocytic activity in the cultures, on day 0, 1 × 10^6 M1 cells per 100 mm dish were seeded and after treating +/− LIF, 2.0 μm Nile Red, carboxylate-modified Fluosphere microspheres (Molecular Probes, Inc.) were added at a density of 150 microspheres per cell. On day 3, 1 × 10^6 cells were removed from each culture, washed 4 times with...
cold PBS, resuspended in 500 µL of PBS and analyzed by flow cytometry for fluorescence intensity. The remaining cells were cultured until day 5 at which time they were washed as above, viewed with an Olympus BX51 microscope, and photographed at 400× magnification. The same plating, treatment, and washing conditions were used to process cells for the analysis of macrophage markers. In this case, 1 × 10⁶ cells were resuspended in 100 µL of FBS stain buffer supplemented with 2 µL CD16/32 Fc block (BD Pharmingen). After 15 minutes on ice, cells were stained with PE-Cy5 anti-mouse F4/80 (eBiosciences) for 30 minutes on ice in the dark. Stained cells were washed with cold PBS and analyzed by flow cytometry as detailed above. A minimum of 30,000 cells was counted for each experiment. Data were analyzed using FCSExpress3 (DeNovo Software).

May-Grünwald/Giemsa staining
2 × 10⁶ M1 cells were immobilized on glass slides by cytospin and air-dried. May-Grünwald solution (Electron Microscopy Sciences) was applied to the slides for 5 minutes, followed by 50% May-Grünwald/water solution for 10 minutes, a water rinse and 20% Giemsa/Wright solution (EM Diagnostic Systems) for 20 minutes. Stained slides were rinsed in water, air-dried, viewed, and photographed under bright field conditions at 600× using an Olympus BX51 microscope and QIMAGING Microimager camera.

Results
Batf mRNA is induced following treatment of M1 cells or primary bone marrow cells with Stat3-activating cytokines
Previous studies have shown that IL-6/LIF-induced macrophage differentiation in mouse M1 myeloid leukemia cells is Stat3-dependent and correlates with an up-regulation of Batf mRNA and protein expression (7). To examine temporal and quantitative aspects of Batf gene induction in this system, RNA was prepared from M1 cells treated with 5 ng/mL of LIF for various times. RT-qPCR was used to measure levels of Batf mRNA. Results showed that Batf mRNA is induced more than 5-fold within 2 hours following LIF-stimulation and declines gradually thereafter (Fig. 1A).

The rapid induction of Batf mRNA in LIF-treated M1 cells occurs in the absence of de novo protein synthesis and is blocked by expression of a dominant-negative Stat3 protein (Y705F) or the Janus kinase (Jak) inhibitor, Socs1/Jab (7). This implies that Batf is a direct target of the Stat3 transcription factor. As a further demonstration that Jak–Stat signaling is important for the induction of Batf mRNA in this system, we employed AG490, a potent and specific inhibitor of the Jak2 and Jak3 tyrosine kinases (33, 34). M1 cells were treated for 16 hours with increasing concentrations of AG490 and then exposed to LIF for 2 hours to stimulate Jak/Stat3 signaling downstream of the gp130 receptor complex. RNA was isolated and Batf transcripts evaluated by RT-qPCR. Results showed that LIF-induced expression of Batf mRNA is reduced as AG490 concentration in the medium is increased (Fig. 1B). Cell extracts from AG490-treated or vehicle-treated cells were immunoblotted using an anti-phospho-Stat3 antibody to establish that the relatively high level of AG490 employed for these studies was functioning as expected to inhibit LIF-induced, Stat3 phosphorylation (Fig. 1C).

To demonstrate that the induction of Batf in M1 cells reflects a response of primary hematopoietic cells to the same stimulus, bone marrow cells isolated from mice were...
rested for 4 hours and then stimulated for 2 hours with medium alone or medium plus 50 ng/mL IL-6 or 5 ng/mL LIF. RT-qPCR was performed on RNA prepared from the cultures and the results demonstrated a stimulation-dependent, 2- to 3-fold increase in Batf mRNA expression (Fig. 2A). This experiment was repeated including an overnight incubation with AG490 prior to stimulation with IL-6 and results showed a significant reduction in Batf mRNA when Jak/Stat3 signaling was inhibited (Fig. 2B).

Again, as in M1 cells, a relatively high concentration of AG490 was necessary to block phosphorylation of Stat3 in these cultures (Fig. 2C) and the observation that Batf mRNA levels are not reduced to the same extent as in AG490 treated M1 cells is most likely a reflection of the cellular heterogeneity of the bone marrow cultures. These data from primary cells validate the use of M1 myeloid leukemia cells as a model system to further dissect the role of Stat3 induced Batf expression in the molecular decisions controlling myeloid cell growth and terminal differentiation.

Identification of a LIF-responsive DNA element within the Batf promoter that binds Stat3

To investigate the direct regulation of Batf gene expression by Stat3, it was necessary to first locate the region of the Batf gene containing the proximal promoter. 5′ rapid amplification of cDNA ends was performed on RNA isolated from control M1 cells and from M1 cells treated with LIF for 2 hours. Sequence analysis of 5 independent DNA clones derived from control cell RNA revealed four different 5′ termini (located at −53, −33, −28, and −24) while all 5 clones analyzed from the LIF-treated sample terminated with the same 5′ adenosine residue. This base was designated as (+1) and maps 190 bp 5′ to the translational start site of the Batf gene in exon 1 (Fig. 3A). Although this amplification technique cannot distinguish a transcription start from a reverse transcriptase pause, these results define a proximal promoter region and a starting point for identifying the Stat3 binding sites important for Batf gene expression.

To identify potential LIF-responsive motifs within the Batf promoter, 1.0 kbp regions of both the mouse and human Batf genes were compared using VISTA (http://genome.lbl.gov/VISTA/index.shtml) and TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html). Neither Batf gene possesses consensus TATA or CAAT box motifs that map within the promoter, but both contain 3 conserved DNA elements for Stat3 binding (designated A, B, and C) (Fig. 3A). The 1.0 kbp promoter of human BATF was linked to a luciferase reporter and a series of 5′ deletion variants were generated to test the contribution of the Stat3A, B, and C sites to LIF-induced luciferase expression in M1 cells. As shown in Figure 3B, the −123 B-Luc construct, which retains only the Stat3C site, is responsive to LIF, while a deletion to −50, or a site-directed mutation that alters Stat3C in the context of the full 1.0 kbp promoter, displays minimal activity. These results implicate Stat3C as the primary DNA element modulating the responsiveness of BATF to LIF.

EMSA were performed to investigate the direct interaction of LIF-induced Stat3 activity from M1 cells with double-stranded oligonucleotides representing the Stat3A, B, or C sites from the human BATF and mouse Batf promoters. In agreement with the results of the luciferase assays, only the C oligos from both genes were bound by proteins in nuclear extracts from LIF-treated cells (Fig. 3C). The participation of Stat3 in the complex bound to oligo C

Figure 2. Batf mRNA expression is induced by IL-6 and LIF in mouse primary bone marrow cells and requires Stat3. A, RT-qPCR was performed on RNA isolated from mouse bone marrow cells 2 hours following treatment with IL-6, LIF, or PBS as control (−) to quantify Batf mRNA induction. Results were normalized to β-actin. Batf expression in the absence of treatment (−) was set to 1.0. The average Batf mRNA expression of duplicate reactions performed with RNA obtained from the cells of 3 different mice (n = 3) is shown. Bars indicate S. E. B, RNA prepared from mouse bone marrow cells treated with the indicated concentrations of AG490 or DMSO as vehicle control (0) and then with IL-6 for 2 hours was analyzed as in (A) for Batf transcripts. Batf expression with IL-6 and no AG490 was set to 100%. The average Batf mRNA expression of duplicate reactions performed with RNA obtained from the cells of 3 different mice (n = 3) is shown. Bars indicate S. E. C, protein lysates were prepared from mouse bone marrow cells treated with the indicated concentration of AG490 or DMSO as vehicle control (0) and then with IL-6 for 20 minutes. Representative immunoblot detecting phosphorylated(p) Stat3 (top), total Stat3 (middle), and control Hsp60 protein (bottom) in these cells are shown.
in the presence of LIF was confirmed by the loss of the shift following preincubation with anti-Stat3 antibody (Fig. 3C, lanes 8 and 10; Fig. 3D, lane 3) but not with a control antibody (Fig. 3C, lanes 7 and 9; Fig. 3D, lane 4).

Specificity was demonstrated further by additional reactions in which excess, unlabeled, consensus Stat3 DNA, oligo C or DNA spanning all three Stat3 sites (ABC) competed binding (Fig. 3D, lanes 5, 7, and 9), whereas mutant Stat3

Figure 3. Phosphorylated Stat3 binds directly to the Batf promoter. A, schematic of the Batf promoter showing sequences of the A, B, and C probes containing Stat3 motifs (underlined) and the DNA sequence surrounding the base identified as a potential start of Stat3-induced transcription from the mouse (M) and human (H) Batf genes. Dashes represent bases conserved between M and H. B, the indicated reporter genes were assayed for activity in M1 cells +/−LIF for 6 hours. The shaded box indicates a mutated C site. The average luciferase activities for 3 independent experiments performed in duplicate are shown and expressed relative to the activity of −1000 B-Luc without LIF which is set to 10. Bars indicate S.E. C, representative EMSA using nuclear extracts from LIF-treated M1 cells (30 minutes) and the human (H) or mouse (M) A, B, or C oligo probes. Anti (α)-Stat3 antibody (+) or control IgG antibody (−) was added where indicated to detect Stat3. D, representative EMSA using nuclear extracts from M1 cells treated with LIF (+) or PBS (−) and HC as the probe. Anti (α)-Stat3 or control α-ER antibody was added to lanes 3 and 4, respectively, to detect Stat3. A 100-fold molar excess of the indicated unlabeled competitor DNA was used in lanes 5 to 10. E, ChIP was performed to isolate Stat3 bound chromatin from M1 cells treated with LIF for the indicated times. Semiquantitative PCR was used to amplify the Stat3C sequence (top). DNA prepared without the ChIP step (input, middle) or from samples precipitated with IgG (bottom) served as controls. This experiment was repeated twice with the same result.
DNA, mutant oligo C or ABC DNA containing a mutant C site did not compete (Fig. 3D, lanes 6, 8, and 10). Collectively, these EMSA provide strong evidence for a Stat3 complex binding to the Stat3C site of the Batf promoter in the presence of LIF. As a demonstration of the temporal aspects of Stat3 binding to the endogenous mouse Batf promoter, M1 cells were treated with LIF for various times and chromatin immunoprecipitation (ChIP) performed using an anti-Stat3 antibody or an anti-IgG antibody as a control. DNA fragments from the precipitates were incubated with PCR primers designed to amplify a 175 bp region of the mouse promoter (+21 to −154) spanning the Stat3B and C sites. Results showed the presence of a strong PCR product within 20 minutes of LIF stimulation and a gradual decrease in product thereafter (Fig. 3E). This profile is consistent with the observed accumulation of Batf mRNA (Fig. 1) and protein (7) in LIF-treated M1 cells.

**Batf knockdown impairs the differentiation program of M1 cells**

The immediate, yet transient, induction of Batf gene expression initiated by LIF and dependent on Stat3 activation suggests that Batf regulates important aspects of the early differentiation program of M1 cells. The documented role of Batf as an AP-1 inhibitor and negative growth regulator (5, 6, 13) suggests that Batf may promote the growth arrest that precedes the conversion of M1 cells to macrophages. In support of this hypothesis, forced expression of BATF in unstimulated M1 cells leads to an increase in the percentage of cells in G1 phase of the cell cycle (7).

To test if Batf induction is necessary for the LIF-induced differentiation of M1 cells, M1 cells were electroporated to introduce a pSUPER vector expressing Batf-specific RNAi (Batf-RNAi) or control RNAi (Neg-RNAi). Clones were selected using G418 and both individual colonies and a resistant pool of cells were assayed for Batf expression following treatment of LIF. Batf-RNAi cells showed reduced levels of Batf mRNA (Fig. 4A) and a level of protein that was below the limits of detection (Fig. 4B).

The differentiation of M1 cells to macrophages involves growth arrest, morphological changes and the acquisition of macrophage markers and phagocytic activity (16, 26). To assess the ability of M1 cells expressing Batf-RNAi to arrest growth, M1 cells (WT), Batf-RNAi cells (both pool and clone 11) and control RNAi cells (Neg-RNAi) were plated on day 0 and monitored for growth over the next 7 days in the presence or absence of LIF (Fig. 5A). Trypan blue exclusion was used to insure counting of viable cells. In the absence of stimulation, all cells proliferated as expected, reaching a saturation density 5 to 7 days after plating. When maintained in LIF, however, the growth of WT and Neg-RNAi cells was noticeably slower, with the cells arresting growth well before reaching saturation density. In contrast, LIF-treated Batf-RNAi cells displayed a growth profile similar to untreated controls, suggesting that the inability to express normal levels of Batf prevented LIF-induced signaling from promoting growth arrest and differentiation.

A second established feature of macrophage differentiation that is displayed by M1 cells is a morphological change manifest by an increase in cell size, an accumulation of cytoplasmic vacuoles and the acquisition of cell adherence properties (16, 26). These changes become apparent in M1 cells 2 to 3 days after LIF stimulation and are detected easily using flow cytometry to measure light scatter (35). WT, Neg-RNAi, and Batf-RNAi cells were treated with LIF and analyzed by flow cytometry after 3 days. Vehicle-treated cells served as controls. Representative flow plots of WT and Batf-RNAi pool cells are shown in Figure 5B; cumulative data from 3 independent experiments are tabulated in Figure 5C. Results showed an average 5-fold increase in the number of control cells (WT and Neg-RNAi) that acquire an altered morphology when treated with LIF. In contrast, only a minor population of LIF-treated Batf-RNAi pool or clone 11 cells displayed a similar morphological change.

To assess if LIF-treated cultures display the phagocytic activity typical of mature macrophages, Batf-RNAi, and Neg-RNAi cells were seeded in the presence of fluorescent-
Figure 5. Differentiation-defective phenotype of M1 cells expressing Batf-RNAi. A, growth of WT, Neg-RNAi, and Batf-RNAi (pool and clone 11) cells in the presence of LIF or PBS as a control. Plotted are the average cell numbers for days 1 to 7, determined from counting triplicate plates. Bars indicate S.E. This experiment was repeated once with similar results. B and C, flow cytometry was used to obtain light scatter profiles of WT, Neg-RNAi, and Batf-RNAi cells (pool and clone 11) maintained for 3 days with LIF or PBS. Representative flow plots are shown in (B) where circles mark differentiated cells which were quantified as a percentage of the total cells counted. The average percentages from a minimum of 3 independent experiments per cell type are presented in C. Bars indicate S.E. D and E, phagocytic activity of control Neg-RNAi cells /+LIF and Batf-RNAi cell (pool and clone 11) /+LIF was assessed by flow cytometry and visual inspection as described in Materials and Methods section. Representative flow plots showing the percentage of highly fluorescent cells in each group, along with phase contrast and fluorescent images of each cell type, are shown in (D). Arrows point to examples of bead-positive or -negative cells in the images. The percentages averaged from 3 independent flow cytometry experiments are presented in (E). Bars indicate S.E.
tagged microspheres and treated with or without LIF for 3 days. Cells were collected, washed, and analyzed for fluorescent content by flow cytometry. For each group, parallel cultures were maintained for an additional 2 days and photographed. Representative plots and images of each experimental group are presented in Figure 5D. The cumulative data from 3 independent experiments are tabulated in Figure 5E. While over 40% of control cells demonstrate substantial phagocytic activity, neither the Batf-RNAi pool nor the clone 11 cells efficiently take up the microspheres. Collectively, the data presented in Figure 5 support the hypothesis that Batf plays a pivotal role in the LIF-induced growth arrest that is a prerequisite for the differentiation of M1 cells to functional macrophages in culture.

Expression of human BATF rescues the differentiation-defective phenotype of Batf-RNAi cells

Batf-RNAi silences mouse Batf expression and should have no effect on the expression of human BATF. Therefore, to restore Batf expression in Batf-RNAi cells and test if the differentiation-defective phenotype is reversible, a retrovirus expressing human BATF, tagged at the N-terminus with HA, was used to infect Batf-RNAi cells (both pool and clone 11 cells). A new pool of cells, maintaining expression of Batf-RNAi and expressing exogenous HA-BATF, was selected using G418 and puromycin. Infection of Batf-RNAi cells with a retrovirus expressing EGFP was used as a control. The expression of EGFP in the control culture was monitored by flow cytometry (data not shown) and the presence of Batf protein in the rescued culture was monitored by immunoblotting. The anti-HA antibody detected robust expression of human BATF in the rescued cells, as did the cross-reactive, polyclonal anti-Batf antiserum, while only a trace of endogenous Batf was detected in the Batf-RNAi + EGFP cells (Fig. 6A). These cells and WT M1 cells were treated with LIF and the morphological differentiation of the cultures was assessed by flow cytometry (Fig. 6B). As expected, LIF induced a dramatic increase in the differentiation of WT cells, but had minimal effect on the Batf-RNAi + EGFP cells. In contrast, after 3 days in LIF, differentiation of the rescued cells closely approached the level of the WT culture. Interestingly, expression of HA-BATF appeared to drive over 10% of the cells to adopt an altered morphology in the absence of LIF. This rescue experiment also was performed with Batf-RNAi clone 11 cells and produced similar results (data not shown).

The effects of exogenous human BATF protein on the growth of M1 cells had been studied previously (7) and while it was noted that cells accumulated in G1 of the cell cycle, a full characterization of the ability of these cells to differentiate was not assessed. Therefore, control WT and Batf-RNAi + BATF cells were treated with LIF and differentiation of the cultures was quantified by flow cytometry on days 0, 1, 2, and 3. Results showed that WT cells displayed a linear increase in the number of altered cells over time, while the Batf-RNAi + BATF cultures contained a significant number of differentiated cells on day 0 and only a 1.5-fold increase by day 3 (Fig. 6C). Differentiation also was assessed using flow cytometry to detect F4-80, a well-characterized, surface marker of macrophages. Figure 6D shows that the rescued cells displayed a similar level of F4-80 in the absence of LIF as WT cells displayed in the presence of LIF. Lastly, May-Grünwald Giemsa staining was employed to visualize cells in the presence and absence of LIF. As expected, morphologically altered, macrophage-like cells were detected readily in the rescued culture, even in the absence of LIF (Fig. 6E), whereas the Batf-RNAi control cells retained a blast-like morphology even in the presence of LIF. These experiments demonstrate that forced expression of HA-BATF rescues the differentiation-defective phenotype of the Batf-RNAi cells and, in fact, propels a significant percentage of cells to enter the differentiation program spontaneously. This is further evidence to implicate Batf and its target genes as critical downstream effectors of the LIF-induced, Stat3 signaling pathway responsible for the conversion of M1 myeloid leukemia cells to macrophages.

Batf expression correlates with altered expression of c-myb, which is a known regulator of M1 cell differentiation and a potential Batf target gene

As a member of the AP-1 transcription factor superfamily, Batf is likely to exert its effects in M1 cells by influencing the expression of genes critical to the differentiation program. A microarray analysis was performed to identify transcripts with differential representation in Batf-RNAi versus Neg-RNAi cells following treatment with LIF. The data from this experiment are available at ArrayExpress under accession number E-MEXP-2995. Putative Batf target genes, based on the activity of Batf as an AP-1 inhibitor, were identified as those whose transcription was maintained at a high level in LIF-treated, Batf-RNAi cells. One of the genes meeting this criterion was c-myb. Since constitutive expression of the c-Myb transcription factor is known to be associated with myeloid leukemia (25) and does effectively block M1 cell differentiation (27, 28), the role of Batf in regulating c-myb gene expression was investigated further. RNA isolated from WT, Batf-RNAi + EGFP, and Batf-RNAi + BATF cells exposed to LIF for different times was analyzed for c-myb transcripts by RT-qPCR. As shown in Figure 7A, the c-myb mRNA level in WT cells was reduced upon LIF stimulation, whereas Batf-RNAi + EGFP cells maintained a high level of c-myb transcription in the presence of LIF. c-myb mRNA was low in Batf-RNAi + BATF cells and did not change following exposure to LIF. These observations are consistent with the behavior of these cells in the differentiation assays (Fig. 6).

c-Myb is important to the early development of blood cell lineages and the expression of the c-myb gene is highly regulated (36–38). There is limited evidence supporting a direct role for AP-1 in regulating c-myb transcription, although we and others have identified multiple AP-1 or AP-1-like DNA motifs within the 5′ promoter region of c-myb (39) and close to an intragenic attenuator element.
that controls \textit{c-myb} expression by blocking transcript elongation (40, 41) (see Materials and Methods section for details). To investigate if Batf associates with any of these AP-1 DNA motifs in \textit{c-myb}, M1 cells were treated with or without LIF for 5 hours and ChIP performed using anti-Batf antiserum or anti-IgG antibodies as a normalization control. PCR analysis of the DNA purified from the precipitates revealed a 3-fold enrichment of DNA representing the \textit{c-myb} A site (Fig. 7B). Although all of the \textit{c-myb} AP-1 motifs showed some enrichment compared to the non-AP-1 DNA control (N), amplification of the B through G AP-1 motifs was not increased significantly in the presence of LIF.

The formation of LIF-induced protein complexes on AP-1 sites within the \textit{c-myb} gene was examined further by EMSA. A standard AP-1 oligo was used to demonstrate the dramatic increase in overall AP-1 DNA binding generated by LIF signaling (Fig. 7C, compare lanes 1 and 2). Additional reactions using nuclear extract from LIF-treated M1 cells and each of the \textit{c-myb} AP-1 sites demonstrated that a shift similar in mobility and intensity to the control AP-1 shift appears only with the \textit{c-myb} A site.
and B probes (Fig. 7C, lanes 4 and 5). Consequently, a more comprehensive analysis of the protein/DNA complexes formed with probes A and B was performed. For the A probe, protein binding was enhanced by LIF and was effectively competed using unlabeled A DNA (self), AP-1 DNA and, to a lesser degree, by the related CRE consensus DNA (Fig. 7D, top, lanes 5–7). Incubation with excess SP-1 DNA had no effect on A binding and the addition of anti-Batf antiserum, not control serum (anti-Mist1), to the reaction resulted in a partial supershift (Fig. 7D, top, lanes 8–10). For the B probe, overall protein binding was enhanced minimally by LIF. Furthermore, while incubation with DNA competitors suggested that some of the proteins bound in the presence of LIF have affinity for AP-1 DNA (Fig. 7D, bottom, lane 6), the lack of any supershift following incubation with anti-Batf antiserum, coupled with the notable reduction in binding following incubation with antiserum to a pancreas-specific, bHLH protein, Mist1 (Fig. 7D, bottom, lanes 9 and 10), demonstrated that the B complex does not contain Batf.
there are several complexes in LIF-treated M1 cells that associate with the A region of the *c-myb* promoter, we have established that Batf is a component of those complexes under conditions where *c-myb* expression is repressed. Whether Batf, the *c-myb* A motif and/or the other nuclear factors that bind to the A motif are necessary for *c-myb* repression remains to be determined.

Discussion

M1 myeloid leukemia cells are a classic experimental model system that has generated a wealth of information on signaling pathways and target genes that reverse leukemogenicity, cause cell cycle arrest and promote the differentiation of myeloblasts to the monococyte/macrophage lineage (16, 20). The ability of M1 cells to differentiate in response to treatment with the IL-6/LIF family of cytokines mimics what occurs when normal bone marrow myeloblasts respond to hematokines in vivo and, as a result, M1 cells have been exploited to identify physiologically relevant myeloid differentiation primary response genes (*MyD*) which include the genes encoding the Irf1 and Egr-1 transcription factors, as well as the genes encoding all 3 members of the Jun family of AP-1 proteins (c-Jun, JunB, and JunD) (20, 23, 42). Interestingly, c-Fos is not a *MyD* gene in M1 cells (24), but Batf is, since Batf mRNA and protein are induced rapidly by IL-6 or LIF in the absence of de novo proteins synthesis (7). Our current understanding of Batf function is that it competes with Fos for binding to Jun proteins and participates in Batf/Jun dimers that are less transcriptionally active than Jun/Jun or Fos/Jun dimers (3, 5). Based on that understanding, the co-induction of Batf and Jun as products of *MyD* genes suggests that the role of AP-1 activity in M1 cells is to restrict cell growth and promote differentiation. The results of our experiments with Batf in M1 cells support this statement.

The Batf gene is a direct target of the Stat3 transcription complex. Induction of Batf mRNA in response to LIF is rapid, but transient. Batf mRNA expression returns to a level slightly above basal status within 6 hours of exposure to LIF which points to an additional level of transcriptional control based perhaps on a feedback mechanism following the synthesis and accumulation of Batf protein in the cells. Blocking expression of Batf with Batf-RNAi generates M1 cells that are unable to arrest growth or to differentiate in response to LIF. This indicates that the role of Batf is to modulate the activity of the co-induced Jun proteins, whose dimerization in the absence of Batf may have no function or, alternatively, may provide M1 cells with an abnormal growth stimulus. The fact that we have observed elevated levels of *c-myb* mRNA expression in Batf-RNAi cells would support either of these alternatives. When Batf expression is restored in Batf-RNAi cells through the overexpression of human BATF, the differentiation block is reversed and the cells display macrophage-like characteristics even in the absence of LIF. The BATF rescued cells do not proliferate well in growth medium, are not viable long-term and do not recover from frozen storage. This has hampered our ability to comprehensively analyze the gene expression profiles of the rescued cells. To circumvent this problem, we are generating inducible cell lines to address questions regarding the importance of Batf expression at different times relative to the LIF-induced expression of other *MyD* genes. Interestingly, the nuclear translocation of an inducible c-Fos-ER protein in M1 cells (an AP-1 protein not present in M1 cells) results in apoptotic cell death in the absence of IL-6 and enhanced differentiation in the presence of IL-6 (43). In these experiments, the status of Batf was not examined. We plan to test our inducible Batf cells in combination with Fos to examine how these AP-1 competitors interact in stimulated M1 cells.

We have used Batf-RNAi cells to investigate how Batf/Jun heterodimers may function to direct the differentiation program of M1 cells. Microarray analysis was performed to identify transcripts that are not down regulated in LIF-treated Batf-RNAi cells as compared to LIF-treated Neg-RNAi cells (Array Express, MEXP-2995). Of the top 10 genes identified, the only one previously implicated as a critical regulator of M1 differentiation was c-myb. Upon further analysis, c-myb fit the profile of a Batf target gene based on its expression and on the observation that there are several AP-1 DNA motifs embedded within known regulatory regions of the c-myb gene (39, 41). Our work has demonstrated that the c-myb A site does bind Batf following LIF treatment. The next step is to demonstrate a role for the A site in regulating c-myb expression. These experiments will represent a challenge based on extensive literature showing that the murine c-myb gene is controlled by the complex interplay between transcription initiation (likely impacted by A site binding) and the binding of proteins to an intron I transcription attenuator sequence that functions to block elongation of c-myb transcripts (39, 40, 44). Simple reporter gene assays will not be informative here and, instead, nuclear run-on assays will be necessary to initially characterize the mechanism of c-myb mRNA silencing in the presence of LIF and then to examine how initiation and/or elongation is affected in the presence of LIF and Batf-RNAi.

The potential relationship between the expression of Batf as the product of a *MyD* gene and c-Myb as critical partner in the growth arrest and differentiation response of M1 cells is strikingly similar to what has been described recently for another *MyD* gene, Egr-1, and its relationship to overexpressed c-Myc or c-Myb in M1 cells (45–47). Although RNA interference has not been used to test how the absence of Egr-1 impacts M1 cells, forced expression of Egr-1, like Batf, leads to spontaneous differentiation (47). Additionally, although Egr-1 promotes differentiation and suppresses the leukemogenic phenotype of M1 cells constitutively expressing c-Myc, these cells do continue to proliferate (46). Egr-1 expression was less effective at abrogating the block to differentiation induced by overexpressed
c-Myb and had no effect on the in vivo tumorigenicity of M1 c-Myb cells (45). These results provided evidence that the differentiation response of M1 cells can be uncoupled from their growth arrest and suggest that the major genetic alterations associated with leukemia (in this case, deregulated c-Myc or c-Myb) are what will determine how effective a co-expressed protein (in this case, Egr-1) will be in suppressing the tumor phenotype. Future plans will utilize inducible Batf and inducible Batf-RNAI cells to test the potential of Batf to function in a similar way as a tumor suppressor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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