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The Novel Primary Response Gene *MyD118* and the Proto-oncogenes *myb*, *myc*, and *bcl-2* Modulate Transforming Growth Factor β1-Induced Apoptosis of Myeloid Leukemia Cells

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Cell numbers are regulated by a balance among proliferation, growth arrest, and programmed cell death. A profound example of cell homeostasis, controlled throughout life, is the complex process of blood cell development, yet little is understood about the intracellular mechanisms that regulate blood cell growth arrest and programmed cell death. In this work, using transforming growth factor β1 (TGFβ1)-treated M1 myeloid leukemia cells and genetically engineered M1 cell variants, the regulation of growth arrest and apoptosis was dissected. Blocking of early expression of *MyD118*, a novel differentiation primary response gene also shown to be a primary response gene induced by TGFβ1, delayed TGFβ1-induced apoptosis, demonstrating that MyD118 is a positive modulator of TGFβ1-mediated cell death. Elevated expression of *bcl-2* blocked the TGFβ1-induced apoptotic pathway but not growth arrest induced by TGFβ1. Deregulated expression of either c-*myc* or c-*myb* inhibited growth arrest and accelerated apoptosis, demonstrating for the first time that c-*myb* plays a role in regulating apoptosis. In all cases, the apoptotic response was correlated with the level of *MyD118* expression. Taken together, these findings demonstrate that the primary response gene *MyD118* and the c-*myc*, c-*myb*, and *bcl-2* proto-oncogenes interact to modulate growth arrest and apoptosis of myeloid cells.

Cell numbers are regulated by a balance among proliferation, growth arrest, and programmed cell death (apoptosis) (15, 42, 64). Until recently, studies of oncogenesis have focused on regulation of cell proliferation (61). The recognition that negative growth control, including growth arrest and programmed cell death, must be understood to comprehend how appropriate cell numbers are maintained and how alterations in any part of the equation can contribute to malignancy has led to a burst of work in this field (42, 57, 61, 62, 64). Among the genes induced by various growth arrest and apoptotic stimuli are the tumor suppressor gene *p53* (8, 32, 41), myeloid differentiation primary response (MyD) genes (1, 2, 38–40), growth arrest and DNA damage (GADD) genes (17–19), growth arrest-specific (gas) genes (56), and small proline-rich (spr) genes (31). It is known that apoptosis is an active process requiring protein synthesis and specific endonucleolytic digestion of cellular DNA in many cases (65); however, little is understood about the intracellular mechanisms that lead to growth arrest and programmed cell death.

A profound example of cell homeostasis which is regulated throughout life is the complex process of blood cell formation, whereby a hierarchy of hematopoietic progenitor cells in the bone marrow proliferate and terminally differentiate along multiple, distinct cell lineages. This includes the proliferation and differentiation of myeloid precursor cells into mature granulocytes and macrophages (44, 51, 55). As a first step towards dissection of the regulation of terminal differentiation, we have isolated MyD genes, induced in the absence of de novo protein synthesis following induction of differentiation of autonomously proliferating M1 myeloid leukemia cells, by using mouse lung conditioned medium (LCM), which contains interleukin-6 and leukemia-inhibitory factor (1, 2, 27, 38–40). Since growth arrest and apoptosis are part of the myeloid developmental program (58), it was expected that some of the MyD genes would play a role in these processes. Consistent with this expectation, it has been shown that *MyD32*, which encodes interferon regulatory factor 1, participates in the suppression of growth during the myeloid differentiation program (2). The novel genes *MyD116* and *MyD118* code for proteins that are strikingly similar to proteins encoded by two novel genes, *gadd34* and *gadd45*, coordinately activated in hamster cells by GADD stimuli, including alkylating agents and irradiation (1, 17, 19, 40). The observed homologies led to the conclusion that *MyD116* and *gadd34* are murine-hamster homologs of the same gene, whereas *MyD118* and *gadd45* represent two separate but closely related genes, defining a new family of growth arrest and DNA damage-inducing genes (17, 19, 33). These homologies led us to surmise that *MyD116* and *MyD118* are involved in the growth arrest and apoptosis associated with the myeloid developmental program.

To molecularly dissect growth arrest and apoptosis, the myeloid leukemia M1 cell line was analyzed following treatment with transforming growth factor β1 (TGFβ1), which induces rapid growth arrest and apoptosis in M1 cells. TGFβ, a family of closely related peptides important in growth control, development, and differentiation, is a group of strong inhibitors of proliferation of most epithelial, endothelial, and hematopoietic cells (43, 48), and in some cell types growth arrest is reversible (9, 35) but in others it is accompanied by programmed cell death (49, 53). TGFβ1 has been implicated in

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the regulation of hematopoietic cell development (29, 54). Although little is known about the mechanism of growth arrest by TGFβ1, a role for c-*myc* suppression has been suggested (48, 50).

The putative players we chose to investigate for roles in growth arrest and apoptosis induced by TGFβ1 were (i) the proto-oncogene c-*myc*, which has been implicated in the control of cell proliferation and differentiation (3, 11, 14, 24, 27, 36), in which its suppression was suggested to play a pivotal role in TGFβ1-induced growth inhibition of various cell types (48, 50) and its deregulated expression in cells deprived of growth factors (5, 15) or exposed to reversible growth-blocking conditions (6, 15) has been shown to stimulate apoptosis; (ii) the proto-oncogene c-*myb*, which, like c-*myc*, plays a role in the regulation of proliferation and differentiation (4, 20, 57), but for which a role in apoptosis has not been reported; (iii) *bcl-2*, which has been shown to promote cell survival and inhibit apoptosis induced by many types of stimuli in certain cells (46, 47, 62, 66); and (iv) the novel MyD genes *MyD116* and *MyD118*, which are described above.

By employing M1 cells and genetically engineered M1 cell variants to dissect the growth arrest and apoptosis induced by TGFβ1, we demonstrated for the first time that (i) *MyD118* is a primary response gene induced by TGFβ1 which plays a positive role in the modulation of apoptosis; (ii) *bcl-2* expression is down-regulated by TGFβ1, in which elevated expression of *bcl-2* suppresses the TGFβ1-induced apoptotic pathway; (iii) proto-oncogenes c-*myc* and c-*myb* stimulate TGFβ1-mediated apoptosis, implicating c-*myb* for the first time in the regulation of apoptosis; and (iv) in all cases, the TGFβ1-induced apoptotic response is correlated with the level of *MyD118* expression. Taken together, these findings delineate a molecular-cellular network of interactions in which the differentiation-TGFβ1 primary response gene *MyD118* and proto-oncogenes participate to control growth arrest and apoptosis of hematopoietic cells.

MATERIALS AND METHODS

Cells and cell culture. The differentiation-competent murine M1 myeloid leukemic cell line and the M1 variant M1myc and M1myb cell lines have been described previously (27, 57, 58). The data presented were obtained by using the M1myc2 and M1myb11 cell lines; however, for each cell variant, three independent cell lines were examined (M1myc2, M1myc5, and M1myc12 and M1myb3, M1myb7, and M1myb11), and the results were similar to the data presented. Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) and 10% horse serum at 37°C in a humidified atmosphere with 10% CO_2. Cells were seeded at 0.15×10^6/ml with or without appropriate treatments. For RNA extractions at early times following various treatments, cell concentrations were adjusted to give a final density of $>0.25 \times 10^6$/ml at the time of extraction. Viable cell numbers were determined by trypan blue dye exclusion and counting in a hemocytometer. EL-4 and Mv1Lu were obtained from the American Type Culture Collection.

β-Estradiol was obtained from Sigma and used at 2 μM. Porcine TGFβ1 (R & D Systems) was used at a concentration of 10 ng/ml. Cycloheximide (Sigma) was used at 10 μg/ml.

DNA transfections. M1 cells were transfected via electroporation (Bio-Rad Gene Pulser) with pMLV or pMLVASMyD 118 linearized by digestion with *Eco*RI, and transfectants were selected for resistance to Geneticin (G418 sulfate; 400 μg/ml; GIBCO) as previously described (27, 57).

Retroviral infections. Recombinant plasmid pZip-bcl-2 and parental plasmid pZip-neo were constructed and packaged as infectious amphotropic retroviruses as described previously (46, 47). Supernatants of clones of PA317 exhibiting the highest-titer virus production were used to infect M1 cells as previously described (45). Selection for infected cells by resistance to G418 was the same as for transfectants.

Assays for growth arrest and apoptosis. Viable cell numbers were determined by trypan blue dye exclusion and counting in a hemocytometer. DNA synthesis was measured by incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material, as previously described (28), when cells were pulse-labeled for 2 h. DNA fragmentation, indicative of apoptosis, was determined by a modification of a procedure previously described (59), in which, after lysis of cells and treatment with proteinase K, samples were treated with RNase A and 5 μg of the purified sample was fractionated on a 2% agarose gel. Cytofluorometric analysis was performed as previously described (27). Viable cells were stained with propidium iodide for analysis. DNA fragmentation and cytofluorometric analysis were done at least two, and sometimes three, times.

General recombinant DNA techniques, expression vectors, and DNA probes. Plasmid preparations, restriction enzyme digestions, DNA fragment preparations, and agarose gel electrophoresis were done as described before (27, 38). Probes for murine c-*myc*, c-*myb* (murine), c-*myb* (human), *MyD116*, and *MyD118* were the same as those used previously (1, 27, 40, 57); *gadd45* was obtained from A. Fornace (17). DNA for probes was labeled by random priming to a specific activity equal to or greater than 10^9 cpm/μg (16). The pMLV expression vector was constructed by digesting pHb-APr-1-neo (23) with *Eco*RI and *Sal*I to excise the β-actin promoter and substituting the murine leukemia virus promoter (1.1-kb *Spe* fragment from pBC14) (63) by blunt-end ligation. To obtain pMLVASMyD 118, *MyD118* cDNA containing the complete coding sequence (1) was blunt end ligated into the *Hin*dIII site of pMLV, and the antisense orientation was ascertained by appropriate restriction enzyme digestions.

RNA extraction, Northern (RNA) blotting, and hybridization. RNA was extracted by the method of Chomczynski and Sacchi (7), with guanidinium thiocyanate. Total RNA (5 μg per lane; the presence of equal amounts of RNA in all lanes was confirmed by the equal intensities of ethidium bromide staining of rRNA bands) was electrophoresed on 1% agarose–formaldehyde gels. Northern blots were prepared by using Duralon-UV membranes (Stratagene), and UV cross-linked (Stratalinker; Stratagene) prior to baking. The hybridization and washing conditions used and the method used to strip blots of the probe for rehybridization were described previously (38, 57).

Reverse transcription (RT)-PCR. To assay for the presence of sense transcripts in M1AS118 cell lines, RT of total RNA with *MyD118*-specific 3′ primers was carried out and followed by amplification as described by Kawasaki et al. (34). Total RNA (1.5 μg) in a final volume of 20 μl (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate, 1 U of RNasin [Promega, Madison, Wis.] per μl, 10 pmol of a *MyD118*-specific 3′ PCR primer) was incubated at 95°C for 5 min and cooled to 42°C for annealing of the primer to its target sequence. Complementary DNA was synthesized by adding 1 μl (200 U) of Moloney MLV reverse transcriptase (GIBCO BRL), incubating the reaction mixture at 42°C for 60 min, and then subjecting the Moloney murine leukemia virus reverse transcriptase to heat inactivation at 95°C for 10 min. The reaction mixture was diluted with 80 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1 mM $MgCl_2$) containing 40 pmol of the 3′ primer, 50 pmol of

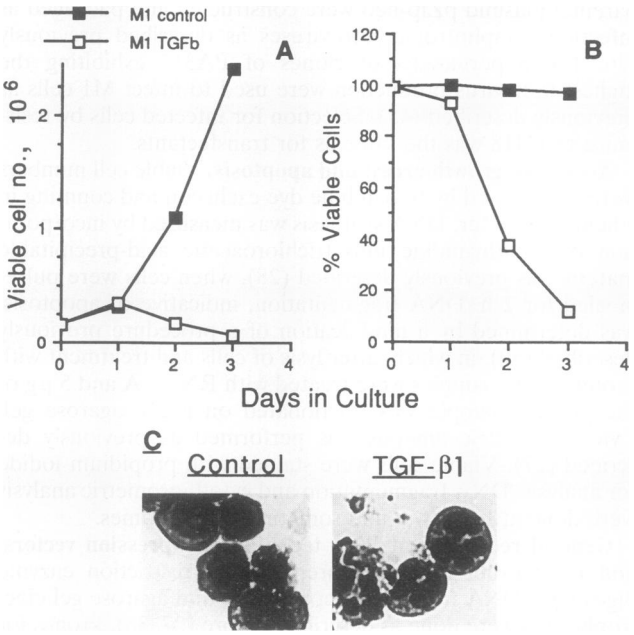

FIG. 1. Induction of growth arrest and apoptosis in M1 cells by TGFβ1. (A and B) Viability of M1 cells treated with TGFβ1. M1 cells (0.15×10^6/ml) were treated with TGFβ1 (10 ng/ml), and at indicated times the concentration of viable cells was determined by trypan blue exclusion. (C) Representative photomicrographs of May-Grunwald-Giemsa-stained cytospin smears of M1 cells untreated and treated with TGFβ1 for 36 h (magnification, ×350).

the 5′ primer, and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus); covered with mineral oil; heated at 95°C for 30 s, and subjected to 20 or 22 cycles of PCR in a Perkin-Elmer Thermal Cycler with 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of polymerization at 72°C. Aliquots (10 μl) were electrophoresed, blotted, and hybridized with a *MyD118* probe. Control samples not reverse transcribed were used to monitor for possible contamination with genomic DNA. DNase treatment of RNA and RNase treatment following RT had no effect on the outcome.

Immunoblotting. To detect the c-Myc and c-Myb proteins, cell extracts were prepared, cell lysates were normalized for protein content, and 100 μg of protein per lane was fractionated on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels, blotted, probed, and washed as described by Selvakumaran et al. (57, 58). The probes used were murine anti-c-Myc antibodies (OM-11-904; Cambridge Research Biochemicals) and sheep anti-c-Myb antibodies (OA-11-843; Cambridge Research Biochemicals). Bcl-2 protein was detected by using rabbit antiserum specific for the human Bcl-2 protein as described by Reed et al. (52). Rabbit antiserum specific for the murine Bcl-2 protein was raised against a synthetic peptide corresponding to amino acids 68 to 86 of the murine Bcl-2 protein essentially by using methods described previously (52).

RESULTS

Induction of growth arrest and apoptosis in M1 cells by TGFβ1. M1 cells treated with TGFβ1 underwent growth arrest and apoptosis (Fig. 1 and Table 1). Between 1 and 2 days following TGFβ1 treatment, the cells ceased to proliferate (Fig. 1A); this was accompanied by inhibition of DNA synthesis and a block in cell cycle progression at G_0-G_1 (Table 1). In addition to growth arrest, TGFβ1-treated M1 cells lost viability, as determined by failure to exclude trypan blue (Fig. 1B), and underwent programmed cell death, as seen by distinct cell morphology, including chromatin condensation and cytoplasmic blebbing (Fig. 1C), and DNA fragmentation (Fig. 2) resulting from cleavage of nuclear DNA in internucleosomal regions, a characteristic of apoptotic cells (65).

TABLE 1. Growth arrest properties induced by TGFβ1 in M1 and M1 variant cell lines

Cell line[a]	DNA synthesis[b] on day 1	Cells in G_0/G_1[c] (%) on:			c-*myc* expression[d] on day 1
		Day 0	Day 1	Day 2	
M1	0.41	46	56	74	–
M1AS118	0.39	45	58	80	–
M1bcl-2	0.43	49	60	76	–
M1myc	0.93	41	43		+
M1myb	0.96	42	40		–

[a] Each cell line was seeded at 0.15×10^6/ml with or without 10 ng of TGFβ1 per ml.
[b] DNA synthesis was measured as described in Materials and Methods. Relative DNA synthesis was calculated as counts per minute obtained with TGFβ1-treated cells divided by counts per minute obtained with untreated cells; cell numbers were not affected for the first 24 h after TGFβ1 treatment.
[c] Cells in G_0/G_1 were determined as described in Materials and Methods.
[d] c-*myc* expression was determined by Northern blot analysis.

Novel MyD gene *MyD118* also is a primary TGFβ1 response gene: effect of blocking of *MyD118* expression. MyD genes activated in the absence of de novo protein synthesis following treatment of M1 cells with a variety of differentiation inducers have been cloned in this laboratory (1, 2, 38–40). Recently cloned GADD genes coordinately activated in hamster cells by GADD stimuli have been shown to be related to two of our novel MyD genes; *MyD116* and *gadd34* are homologs, and *MyD118* and *gadd45* are members of the same gene family (17, 19). Since terminal myeloid differentiation is associated with growth arrest and programmed cell death (58) and *MyD116* and *MyD118* are related to GADD genes, expression of these two genes, as well as *gadd45*, was assessed in M1 cells during TGFβ1-induced growth arrest and apoptosis. *MyD116* and *gadd45* were not expressed (data not shown); however,

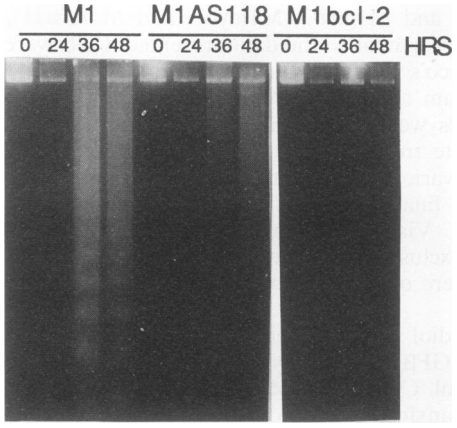

FIG. 2. Induction of DNA fragmentation by TGFβ1 in M1, M1AS118, and M1bcl-2 cells. Cells were seeded at 0.15×10^6/ml with TGFβ1 and harvested at the indicated times for DNA extraction. DNA extraction and electrophoresis were done as described in Materials and Methods.

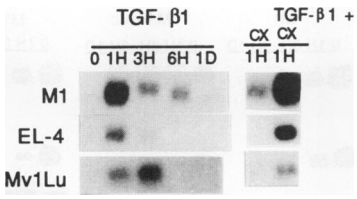

FIG. 3. *MyD118* gene expression following treatment of M1, EL-4, and Mv1Lu cells with TGFβ1. Expression was analyzed by hybridization to Northern blots with total RNA extracted from cells at the indicated times following treatment with TGFβ1 without or with cycloheximide (CX) or treatment with cycloheximide only. Equal amounts of RNA were loaded in all of the lanes, as confirmed by the equal intensities of ethidium bromide staining of rRNA bands.

MyD118 was rapidly induced and steady-state transcript levels peaked by 1 h (Fig. 3). *MyD118* also was expressed when M1 cells were concomitantly treated with TGFβ1 and cycloheximide, indicating that *MyD118* is a primary response gene induced by TGFβ1 (Fig. 3). As can be seen in Fig. 3, *MyD118* also is a primary response gene induced following TGFβ1-mediated growth suppression of lymphoid cell line EL-4 and mink lung epithelial cell line Mv1Lu. Thus, it can be concluded that *MyD118* is a primary response gene that is induced in the absence of de novo protein synthesis following induction of growth inhibition and apoptosis by TGFβ1 in M1 cells.

To determine whether a role in mediating the effects of TGFβ1 can be attributed to *MyD118*, M1 cell lines have been established which express antisense (AS) *MyD118* transcripts at elevated levels, thereby blocking early expression of the *MyD118* gene product (Fig. 4A and B). The pMLV expression vector, containing the *MyD118* cDNA cloned in the AS orientation, where it is under control of the murine leukemia virus promoter, was used; following transfection by electroporation, transfectants were selected by resistance to Geneticin (G418). Control M1 transfectants also were established by using the expression vector without the MyD cDNA. Three control cell lines analyzed behaved similarly to the parental M1 cell lines.

Of the many M1AS118 cell lines established, RNAs from three which expressed elevated levels of AS *MyD118* transcripts are shown in Fig. 4A. Southern blot analysis of genomic DNA digested with vector null cutters demonstrated that exogenous *MyD118* cDNA was integrated at different sites for each of these three transfectants, showing that each transfectant was an independent clone (data not shown). All experiments were carried out with these three M1AS118 cell lines, and similar data were obtained. The data presented were obtained with M1AS118/5.

To verify that no *MyD118* transcripts were present following treatment of M1AS118 cells with TGFβ1, RNA extracted from the cells at various times following treatment with TGFβ1 was reverse transcribed with a primer specific to the sense *MyD118* transcript. Following inactivation of the reverse transcriptase, the cDNA was subjected to amplification by PCR (RT-PCR). As seen in Fig. 4B, no sense *MyD118* transcripts were present in M1AS118 cells (subjected to 22 cycles of PCR) but they were present in M1 cells following treatment with TGFβ1 for 1 h (the sample displayed was subjected to 20 cycles of PCR). The increased sensitivity of detection provided by RT-PCR (subjected to 22 cycles of PCR) revealed *MyD118* transcripts at 1 and 2 days following TGFβ1 treatment for both the M1 and M1AS118 cell lines. Thus, it can be concluded that the AS *MyD118* transcripts effectively blocked early, but not later, expression of the *MyD118* gene product.

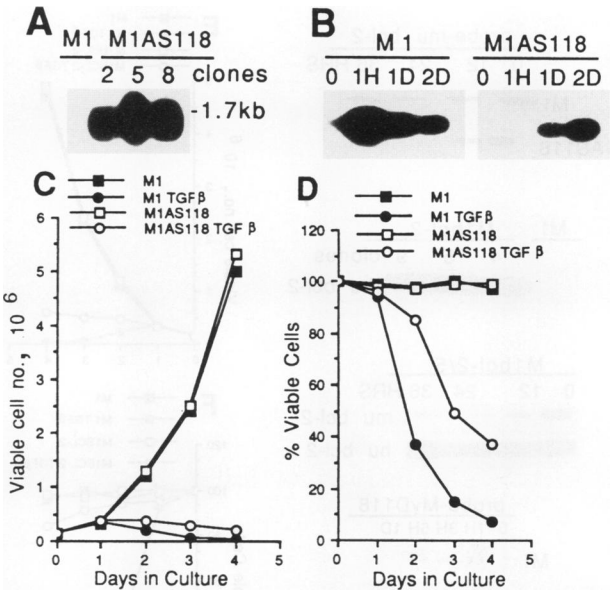

FIG. 4. Establishment of M1AS118 cell lines and analysis following treatment with TGFβ1. (A) Northern blot analysis demonstrating that M1AS118 transfectants express *MyD118* hybridizing transcripts. M1 cells were transfected with expression vector pMLV-AS118 as described in Materials and Methods. Shown are RNAs from three independently isolated M1AS118 clones. (B) Analysis of *MyD118* sense transcripts in M1 and M1AS118 cells following treatment with TGFβ1. RNAs extracted from M1 and M1AS118 cells at various times following treatment with TGFβ1 were reverse transcribed by using a primer specific to *MyD118* sense transcripts. The cDNA was subjected to amplification by PCR as described in Materials and Methods and analyzed by agarose gel electrophoresis, Southern blotting, and hybridization to a *MyD118* probe. Shown are results obtained with M1 and M1AS118 clone 5, for which samples from M1 treated with TGFβ1 for 1 h were subjected to 20 cycles of PCR and all of the other samples were subjected to 22 cycles of PCR. (C and D) Viability of M1 and M1AS118/5 cells treated with TGFβ1. M1 and M1AS118/5 cells (0.15×10^6/ml) were treated with TGFβ1 (10 ng/ml), and at the indicated times the concentration of viable cells was determined by trypan blue exclusion.

M1AS118 cell lines were compared with parental M1 cells following treatment with TGFβ1. M1AS118 cell lines underwent TGFβ1-induced growth arrest and apoptosis; however, loss of cell viability and apoptosis were delayed relative to the parental M1 cell line, as determined by the percentage of cells which excluded trypan blue, cell morphology, and DNA fragmentation (Fig. 2 and 4C and D and Table 1). Following 2 days of treatment with TGFβ1, 88% of M1AS118 cells were viable, compared with 37% of M1 cells. These data demonstrate a positive role for the TGFβ1-induced primary response gene *MyD118* in regulating programmed cell death and suggest that failure to express *MyD118* at early times has no effect on growth arrest.

The effect of elevated expression of *bcl-2* on TGFβ1-mediated growth suppression and apoptosis of M1 cells. Elevated expression of the proto-oncogene *bcl-2* has been shown to play a role in the promotion of cell survival by blocking programmed cell death induced by a wide variety of stimuli in certain types of cells (46, 47, 62, 66). *bcl-2* was expressed in M1 cells and downregulated following treatment with TGFβ1 (Fig. 5A). It was therefore of interest to examine the effects of elevated *bcl-2* expression on TGFβ1-mediated growth arrest and apoptosis of M1 cells. M1bcl-2 cell lines that constitutively

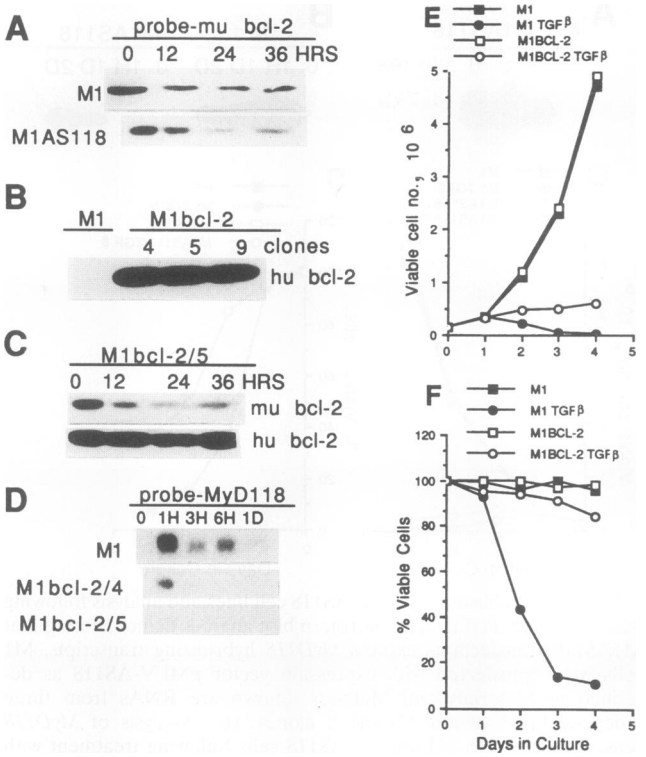

FIG. 5. Establishment of M1bcl-2 cell lines and analysis following treatment with TGFβ1. (A) Bcl-2 protein in the M1 and M1AS118 cell lines at the indicated times following treatment with TGFβ1. Antibody specific for the murine Bcl-2 protein was used. (B) Human (hu) *bcl-2* transgene expression in the M1 and M1bcl-2 cell lines. Establishment of M1bcl-2 cell lines with retroviral vector pZip-bcl-2, containing the human *bcl-2* gene, is described in Materials and Methods. (C) Expression of endogenous (murine [mu]) and exogenous (human) Bcl-2 proteins in M1bcl-2/5 at the indicated times following treatment with TGFβ1. For panels A, B, and C, 100 μg of protein per lane was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and analyzed with appropriate antibodies. (D) *MyD118* gene expression in the M1 and M1bcl-2 cell lines at the indicated times following treatment with TGFβ1. Expression was determined by Northern blot analysis, for which equal amounts of RNA were loaded in all of the lanes, as confirmed by the equal intensities of ethidium bromide staining of rRNA bands. (E and F) Viability of M1 and M1bcl-2/5 cells treated with TGFβ1. M1 and M1bcl-2/5 cells (0.15×10^6/ml) were treated with TGFβ1 (10 ng/ml), and at the indicated times the concentration of viable cells was determined by trypan blue exclusion.

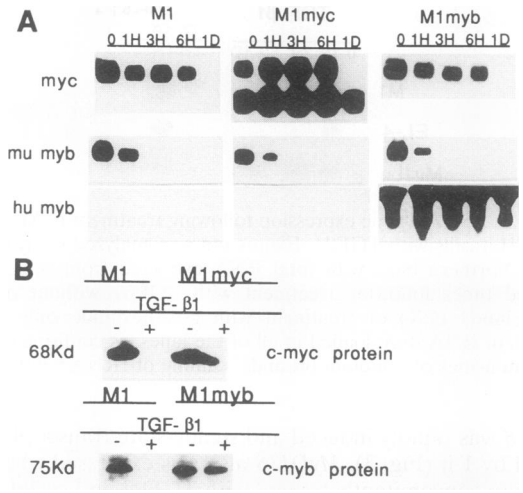

FIG. 6. c-*myc* and c-*myb* expression in M1, M1myc, and M1myb cells following treatment with TGFβ1. (A) Analysis of RNA expression with Northern blots. RNA was extracted at the indicated times following treatment with TGFβ1. The probes used were murine (mu) c-*myc* (for expression of both endogenous and exogenous c-*myc*), murine c-*myb* (for expression of endogenous c-*myb*), and human (hu) c-*myb* (for expression of exogenous c-*myb*). Equal amounts of RNA were loaded in all of the lanes, as confirmed by the equal intensities of ethidium bromide staining of rRNA bands. (B) Analysis of c-Myc and c-Myb proteins. Extracts were prepared from untreated cells (−) or 1 day following TGFβ1 treatment (+) and analyzed by immunoblotting with the probes described in Materials and Methods.

express elevated levels of *bcl-2* were established following retroviral infection of M1 with pZip-bcl-2 (Fig. 5B and C). Bcl-2 protein from three of the many M1bcl-2 cell lines established is shown in Fig. 5B. Southern blot analysis of each of these clones showed that each was an independent clone (data not shown). All of the experiments were carried out with these three M1bcl-2 cell lines, and similar data were obtained. The data presented were obtained with M1bcl-2/5, unless otherwise indicated. M1 cells were also infected with pZipneo, and the three control cell lines analyzed responded to TGFβ1 essentially like parental M1 cells.

Following treatment with TGFβ1, M1bcl-2 cell lines, like M1 cells, underwent growth arrest, including failure to proliferate, inhibition of DNA synthesis, and exiting from the cell cycle into a G_0-G_1 state (Fig. 5E and Table 1). In contrast to M1 cells, M1bcl-2 cells neither lost viability nor underwent programmed cell death, as determined by exclusion of trypan blue, cell morphology, and DNA fragmentation (Fig. 2 and 5F). Even as late as 6 days, M1bcl-2 cells appeared to be viable. Thus, overexpression of *bcl-2* protected M1 cells from TGFβ1-induced apoptosis. These results also demonstrated that TGFβ1 induces growth arrest independently of apoptosis in M1 cells.

Since *MyD118* has been shown to be a positive modulator of TGFβ1-induced apoptosis and overexpression of *bcl-2* blocks apoptosis, it was of interest to ascertain whether expression of *MyD118* is affected by elevated expression of *bcl-2*. As can be seen in Fig. 5D, no or extremely reduced levels of *MyD118* transcripts were apparent in M1bcl-2 cells following treatment with TGFβ1. These data suggest some interaction between *bcl-2* and regulation of expression of *MyD118*, as well as the possibility that the effects of overexpression of *bcl-2* are mediated, at least partially, through *MyD118*. In addition, our previous suggestion that *MyD118* is not necessary for TGFβ1-induced growth arrest is confirmed, and the notion of a positive role for *MyD118* in TGFβ1-induced apoptosis is reinforced.

Also, it can be asked whether the increased survival of TGFβ1-treated M1AS118 cells is mediated via increased levels of *bcl-2*. *bcl-2* expression was suppressed in M1AS118 cells to the same degree as in M1 cells following treatment with TGFβ1 (Fig. 5A), indicating that *bcl-2* plays no role in the enhanced survival of M1AS118 cells.

Effect of deregulated expression of proto-oncogenes c-*myc* and c-*myb* on TGFβ1-mediated growth arrest and apoptosis of M1 cells. It has been strongly suggested that suppression of the proto-oncogene c-*myc* plays a pivotal role in TGFβ1-induced growth inhibition of various cell types (48, 50). As shown in Fig. 6 and Table 1, c-*myc* mRNA levels rapidly declined following TGFβ1 treatment of M1, M1AS118, and M1bcl-2

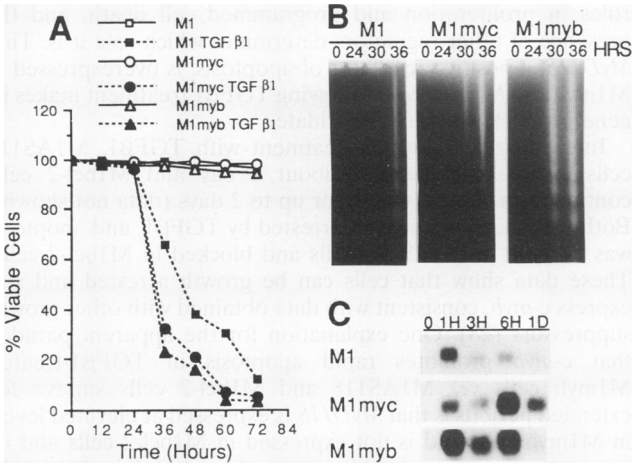

FIG. 7. Effect of deregulated expression of c-*myc* and c-*myb* on TGFβ1-induced loss of viability, apoptosis, and expression of *MyD118*. (A) Percent viable cells at the indicated times following TGFβ1 treatment of M1, M1myc, and M1myb cells (seeded at 0.15×10^6/ml). Viable cells were determined by trypan blue exclusion. (B) Induction of DNA fragmentation by TGFβ1 in M1, M1myc, and M1myb cells. Cells seeded at 0.15×10^6/ml with TGFβ1 were harvested at the indicated times for DNA extraction and analyzed as described in Materials and Methods. (C) Expression of *MyD118* at the indicated times following treatment with TGFβ1, as determined by Northern blot analysis. Equal amounts of RNA were loaded in all of the lanes, as confirmed by the equal intensities of ethidium bromide staining of rRNA bands.

cells, all of which became growth arrested. For all of these cell lines, in addition to loss of proliferative capability, there was a drop in the rate of DNA synthesis and withdrawal from the cell cycle (Table 1). Expression of the proto-oncogene c-*myb* was also rapidly suppressed by treatment of M1 with TGFβ1 (Fig. 6). The availability of the M1myc and M1myb cell lines, in which c-*myc* or c-*myb* expression is deregulated (27, 57), provided us with the opportunity to look at the antiproliferative and apoptotic effects of TGFβ1 in the setting of enforced production of c-*myc* or c-*myb*. Several independent lines of M1myc and M1myb were compared with parental M1 cells.

To ensure that the c-*myc* and c-*myb* transgenes were expressed in the TGFβ1-treated M1myc and M1myb cell lines, respectively, Northern blot and Western blot (immunoblot) analyses were performed. These analyses confirmed that exogenous c-*myc* mRNA and c-Myc protein continued to be present in M1myc cell and exogenous c-*myb* mRNA and c-Myb protein were present in M1myb cells following treatment with TGFβ1 (Fig. 6).

In contrast to M1 cells, in TGFβ1-treated M1myc and M1myb cell lines, the rate of DNA synthesis fell only minimally and the cells continued to traverse the cell cycle (Table 1). However, growth curves appeared very similar for the M1, M1myc, and M1myb lines, whose cells proliferated for up to 24 h (data not shown) and then underwent loss of viability (Fig. 7A). Interestingly, the M1myc and M1myb cell lines lost viability more rapidly than M1 cells, with M1myb cells responding even more rapidly than M1myc (Fig. 7A and B). The appearance of fragmented DNA in the different cell lines was consistent with accelerated apoptosis (Fig. 7B). Thus, failure to suppress either c-*myc* or c-*myb* expression in TGFβ1-treated M1 cells, although inhibiting growth arrest, accelerated programmed cell death.

Since *MyD118* plays a positive role in regulating TGFβ1-induced apoptosis, it was important to look at its expression in both M1myc and M1myb cells treated with TGFβ1. Both the kinetics and level of *MyD118* induction were altered in M1myc and M1myb compared with M1 cells (Fig. 7C). Elevated levels of *MyD118* transcripts were detected in M1myb cells by 1 h and continued to be present for at least 6 h. Expression of *MyD118* in M1myc cells mimicked parental M1 cells for up to 6 h following treatment with TGFβ1, but then in M1myc cells the *MyD118* levels increased further and continued to be expressed for up to 1 day, whereas in M1 cells, MyD118 mRNA levels had already declined. These observations suggest that the effects of continued expression of c-*myb* or c-*myc* on accelerated apoptosis is mediated, at least partially, through altered and increased expression of *MyD118*. These results, taken together with the data presented for the M1AS118 and M1bcl-2 cell lines, further demonstrate that the level of *MyD118* transcripts correlates with the apoptotic response to TGFβ1 (Fig. 4, 5, and 7C).

DISCUSSION

Using TGFβ1-treated M1 myeloid leukemia cells and genetically engineered M1 variants to dissect the regulation of growth arrest and apoptosis, in this study we showed for the first time that (i) *MyD118*, a differentiation primary response gene which has been shown also to be a primary response gene induced by TGFβ1, plays a positive role in the modulation of apoptosis; (ii) elevated expression of *bcl-2* blocks the apoptosis but not the growth arrest induced by TGFβ1; (iii) continuous expression of either c-*myc* or c-*myb* inhibits growth arrest and accelerates apoptosis, and this is the first demonstration that c-*myb* plays a role in regulating apoptosis; and (iv) in all of these cases, the level of *MyD118* expression correlated with the rapidity of the apoptotic response.

***MyD118* is a positive regulator and *bcl-2* is a negative regulator of apoptosis induced by TGFβ1.** Since terminal myeloid differentiation encompasses growth arrest and apoptosis (58), an overlap between the genetic responses to differentiation signals and TGFβ1 is expected. *MyD118*, a MyD gene, also is a member of a family of genes which includes *gadd45*, which was recently shown to be a downstream effector of *p53* required for cell cycle arrest following DNA damage (33). In this work, *MyD118* has been shown to be a primary TGFβ1 response gene in M1 cells, as well as in EL-4 and Mv1Lu cells, where it has been demonstrated that *MyD118* is a positive modulator of TGFβ1-induced apoptosis. Genes induced by TGFβ1 have been identified in various cells (21, 30), and a novel ryanodine receptor has been shown to be a primary response gene induced in Mv1Lu cells (21); however, no functional studies have been reported.

This report shows that inhibition of *MyD118* expression at early times in M1 cells following treatment with TGFβ1 delays the onset of apoptosis, demonstrating a positive role for the *MyD118* gene product in apoptosis. The increased sensitivity provided by RT-PCR has revealed that MyD118 transcripts are present at low levels in M1 cells for up to 2 days following treatment with TGFβ1 and that by 2 days M1AS118 cells express similarly low levels of MyD118 mRNA. It has not been possible to establish M1AS118 cell lines which completely block MyD118 mRNA expression. The breakthrough in MyD118 mRNA expression in M1AS118 cells is intriguing and should be clarified once the mode of MyD118 regulation, at the transcriptional and translational levels, and the long-term effects of AS RNA on this regulation are deciphered. Nevertheless, blocking of early MyD118 expression has a definitive effect on the TGFβ1-induced apoptotic response.

Since antibodies to the *MyD118* gene product are not available, nothing is known about the stability of the MyD118 protein or at what level it is present in M1 and M1AS118 cells at later times. Although it is clear that the *MyD118* gene product plays a positive regulatory role in the TGFβ1-induced apoptotic pathway, the availability of antibodies and cell lines which completely block *MyD118* expression should enable better understanding of the role of *MyD118* in TGFβ1-induced apoptosis.

Additional evidence consistent with a positive role for *MyD118* in the promotion of apoptosis is the correlation between the level of *MyD118* expression and the rapidity of the TGFβ1-induced apoptotic response obtained with genetically engineered M1 cell lines M1myb, M1myc, M1AS118, and M1bcl-2. Attempts to obtain M1 cell lines that constitutively express *MyD118* have failed, consistent with the notion that *MyD118* expression is antagonistic to cell growth and/or survival. We are currently attempting to establish cell lines that express *MyD118* under control of an inducible promoter to overcome this difficulty.

bcl-2 can promote cell survival by blocking apoptosis induced by a wide variety of circumstances ranging from growth factor deprivation to treatment with gamma radiation and chemotherapeutic drugs (12, 25, 46, 47, 66). However, overexpression of *bcl-2* does not prevent apoptotic cell death in all circumstances (13), implying either that *bcl-2* alone can be insufficient or that *bcl-2*-independent pathways for programmed cell death exist. We found that TGFβ1-induced apoptosis was blocked by elevated *bcl-2* expression and *MyD118* was not induced, consistent with a positive role for *MyD118* in the apoptotic response. These observations suggest some interaction between *bcl-2* and regulation of *MyD118* induction, as well as the possibility that the effect of *bcl-2* is mediated via blocking of *MyD118* expression. Studies on the interaction between *bcl-2* and *MyD118* and the apoptotic response are under way.

Deregulated c-*myc* and c-*myb* accelerate apoptosis. Deregulated expression of either c-*myc* or c-*myb*, which is rapidly downregulated in M1 cells following treatment with TGFβ1, blocks TGFβ1-induced arrest of DNA synthesis and withdrawal from the cell cycle but accelerates TGFβ1-induced apoptosis. These findings are consistent with previous studies, in which a role for c-*myc* in the regulation of apoptosis was found in growth-arrested fibroblasts (15), factor-deprived interleukin-3-dependent 32D myeloid cells (5), and anti-T-cell-receptor antibody-stimulated T-cell hybridomas (60). Our findings obtained with TGFβ1 thus provide another indicator that continued expression of c-*myc* in the presence of growth arrest signals leads to apoptosis.

This work is the first reported demonstration that the proto-oncogene c-*myb* can regulate the apoptotic response. The accelerated apoptosis of TGFβ1-treated M1myb cells is not mediated via continued expression of c-*myc*, since c-*myc* is suppressed with the same kinetics in both TGFβ1-treated M1 and M1myb cells. It is intriguing that deregulated expression of c-*myb* in the myeloid M1myb cell line does not block TGFβ1-induced suppression of c-*myc*, given the findings that c-*myb* transactivates the c-*myc* promoter in various lymphoid and myeloid cell lines (10) and interleukin-6-treated M1myb cells continue to express c-*myc* (57). Unlike c-*myc*, which is expressed in almost all proliferating cell types (11), c-*myb* is expressed predominantly in hematopoietic cells (22). Thus, it will be interesting to ascertain whether c-*myb* can mediate apoptosis in other cell types, including those which do not normally express c-*myb*.

Apparently both proto-oncogenes c-*myb* and c-*myc* play roles in proliferation and programmed cell death, and the presence of other regulators determines which role it is. That *MyD118*, a positive regulator of apoptosis, is overexpressed in M1myc and M1myb cells following TGFβ1 treatment makes its gene product one likely candidate.

Interestingly, following treatment with TGFβ1, M1AS118 cells expressed c-*myb* for about 1 day and M1bcl-2 cells continued to express c-*myb* for up to 2 days (data not shown). Both cell lines were growth arrested by TGFβ1, and apoptosis was delayed in M1AS118 cells and blocked in M1bcl-2 cells. These data show that cells can be growth arrested and still express c-*myb*, consistent with data obtained with other growth suppressors (28). One explanation for the apparent paradox that c-*myb* promotes rapid apoptosis in TGFβ1-treated M1myb cells yet M1AS118 and M1bcl-2 cells survive for extended periods is that *MyD118* is expressed at elevated levels in M1myb cells and is not expressed in M1bcl-2 cells and its expression is reduced and extensively delayed in M1AS118 cells. These data further implicate *MyD118* in the accelerated apoptosis induced by TGFβ1 in M1myb cells and as a positive regulator of the apoptotic program induced by TGFβ1 in M1 cells.

Concluding remarks. The findings presented here, obtained with M1 cells and genetically engineered M1 cell variants as a model system for dissection of the growth arrest and apoptosis induced by TGFβ1, have delineated a molecular-cellular network of interactions in which differentiation-TGFβ1 primary response gene *MyD118* and proto-oncogenes c-*myc*, c-*myb*, and *bcl-2* participate to control growth arrest and apoptosis of myeloid cells.

During differentiation of primary cultures of myeloid precursor-enriched bone marrow cells, the patterns of expression of *MyD118*, c-*myc*, and c-*myb* closely resemble the situation in M1 cells (1, 37). In addition, high levels of *bcl-2* were observed in normal myeloid precursor cells and little or no expression was observed in terminally differentiated cells (26). TGFβ1, expressed in bone marrow, can modulate and regulate bone marrow-derived cells throughout their functional life span, and its pleiotropic effects depend on cell lineage, specific cellular phenotype, stage of differentiation, immediate microenvironment, other cytokines, and cellular function (29, 54). Taken together, the network of interactions elucidated in this work should help to provide insights into the complex controls that regulate myeloid cell homeostasis in vivo and the alterations in gene expression that play a role in the development of preleukemic myeloproliferative syndromes and the progression to leukemias.

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