Bcl-xL Expression Correlates with Primary Macrophage Differentiation, Activation of Functional Competence, and Survival and Results from Synergistic Transcriptional Activation by Ets2 and PU.1*

Lidia Sevilla‡, Arnaud Zaldumbide§, Francoise Carlotti§, Manal Abdel Dayem, Philippe Pognonec, and Kim E. Boulukos¶

From the Institute of Signalisation, Developmental Biology and Cancer, INSERM 470, Centre de Biochimie, Université de Nice, Faculté des Sciences, 06108 Nice, France

Depriving primary bone marrow-derived macrophages of colony-stimulating factor-1 (CSF-1) induces programmed cell death by apoptosis. We show that cell death is accompanied by decreases in the expression of anti-apoptotic Bcl-xL protein and the Ets2 and PU.1 proteins of the Ets transcription factor family. Macrophages require both priming and triggering signals independent of CSF-1 to kill neoplastic cells or microorganisms, and this activation of macrophage competence is accompanied by increased expression of bcl-xL, ets2, and PU.1. Furthermore, we show that only Ets2 and PU.1, but not Ets1, function in a synergistic manner to transactivate the bcl-x promoter. The synergy observed between PU.1 and Ets2 is dependent on the transactivation domains of both proteins. Although other transcription factors like Fos, c-Jun, Myc, STAT5, and STAT5a are implicated in the activation of macrophage competence or in CSF-1 signaling, no synergy was observed between Ets2 and these transcription factors on the bcl-x promoter. We demonstrate that the exogenous expression of both Ets2 and PU.1 in macrophages increases the number of viable cells upon CSF-1 depletion and that Ets2 and PU.1 can functionally replace Bcl-xL in inhibiting Bax-induced apoptosis. Together, these results demonstrate that PU.1 and Ets2 dramatically increase bcl-xL activation, which is necessary for the cytocidal function and survival of macrophages.

The Ets family of transcription factors consists of ~30 members conserved from sea urchins to man. Ets members contain a conserved DNA-binding domain of ~85 amino acids known as the Ets domain (reviewed in Ref. 1). Ets1, the progenitor of v-Ets found in the E26 retrovirus, and Ets2 are 97% conserved in the Ets domain, whereas PU.1/Spi.1 is highly divergent in this domain (37% identity) (2). These differences in sequence identity of the Ets domain allow Ets1, Ets2, and PU.1 to bind to common as well as distinct optimal DNA target sequences. Ets proteins bind to DNA as monomers to activate transcription alone or in conjunction with other transcription factors binding to their respective sites (reviewed in Ref. 1).

PU.1 is expressed in early progenitor cells as well as in fully differentiated B cells, neutrophils, and macrophages. The importance of PU.1 in hematopoietic development has been confirmed by PU.1 gene disruption studies showing that PU.1-deficient mice lack mature B cells, neutrophils, and macrophages (3, 4). ets2 null mice die early during embryonic development (5). However, transgenic studies using a dominant-negative form of Ets2 under the control of a monocyte/macrophage-specific promoter have provided insight into the role of Ets2 in macrophages (6). Abnormal macrophage development occurs in these transgenic mice during the first 40 days following birth, and peritoneal macrophages obtained from these transgenic animals do not have the characteristic macrophage morphology when cultivated in vitro with CSF-1 (1). These results imply the importance of Ets2 in the development of macrophages, yet the molecular mechanisms by which Ets2 functions had not been elucidated in these studies.

The level of PU.1 is highly abundant in immature myeloid progenitor cells, and this level of expression remains high throughout macrophage differentiation (7, 8). In contrast, Ets2 is not expressed in early myeloid progenitors, but later in more mature myeloid cells (9–11), and Ets2 become rapidly upregulated upon induction of macrophage differentiation or activation of primary macrophages (10).

Bcl-xL is believed to be the key anti-apoptotic protein expressed in myeloid precursors and macrophages (12–17). In contrast, Bcl-2 is down-regulated in these systems. Recently, we showed that the induction of bcl-xL results from an increase in bcl-xL promoter activity and that de novo protein synthesis is required for this activation of bcl-xL transcription (17). The human bcl-xL promoter contains nine potential EBS. The capacities of PU.1 and Ets2 to individually transactivate the bcl-xL promoter led us to ask whether PU.1 and Ets2 could compete or act in synergy to transactivate this promoter. In parallel, we wanted to determine the biological relevance of the coexpression of PU.1, ets2 and bcl-xL in primary macrophages upon induction of proliferation and differentiation and activation of

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

‡ Supported by European Communities Grant ERBFMBICT972684 and by the Foundation pour la Recherche Medicale.

§ Supported by Ministère de L’Education Nationale de La Recherche et de la Technologie.

¶ Supported by Association pour la Recherche contre le Cancer Grant 9691. To whom correspondence should be addressed. Tel. and Fax: 33-4-92-07-64-13; E-mail: boulukos@unice.fr.

1 The abbreviations used are: CSF-1, colony-stimulating factor-1; CSF-1R, colony-stimulating factor-1 receptor; PBS, phosphate-buffered saline; IFN-γ, interferon-γ; LPS, lipopolysaccharide; HA, hemagglutinin; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; FACS, fluorescence-activated cell sorting; BMM, bone-derived macrophages; USF-1, upstream stimulatory factor-1; FIP, Fos-interacting protein; GAS, interferon-α activation sequence; EBS, Ets-binding sites.
macrophage competence and during programmed cell death by apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293 cells, NIH3T3 cells, and NIH3T3 cells exogenously expressing the CSF-1R (NIH3T3-cfms) (18) were maintained in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum. Primary bone marrow–derived cells were isolated from femurs of 2–3-month-old male C57BL/6 mice. Femurs were flushed with PBS to recover cells. After several washes in PBS, cells were cultured in Dulbecco’s modified Eagle’s medium, 20% fetal calf serum, and 3% conditioned medium from L cells as a source of CSF-1 (19) or in Dulbecco’s modified Eagle’s medium and different concentrations of human recombinant CSF-1. After 4–5 days, fully differentiated macrophages were obtained. When recombinant CSF-1 was used, the concentrations are indicated below. IFN-γ (100 units/ml) or LPS (10 μg/ml) was added to macrophages for 4 h.

The BAC1.2F5 and BACets2.1D macrophage cell lines have been previously described (17). For transfection studies, BAC1.2F5 macrophages were electroporated either with 5 μg of pRK-ets2 or 5 μg of pRK-PU.1 or with 5 μg of pRK-APU.1 and 5 μg of pRK-Dets2 (for description of plasmids, see below) as previously described (7). BAC1.2F5 cells constitutively expressing Ets2 (BACets2.1D) were electroporated with 10 μg of pRKS or pRK-APU.1. After electroporation, the cells were plated with CSF-1 in duplicate dishes until they became adherent. Dishes were washed two times in PBS, and then the cells were cultivated in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum with or without 20% conditioned medium as a source of CSF-1. The number of viable cells was determined by trypan blue exclusion.

**Transactivation Studies**—The cloning of the 5′-regulatory sequences of the bcl-x gene upstream of the luciferase gene has been described previously (17). Ets2, a dominant-negative mutant of Ets2 (∆1–238Ets2), Ets1, and PU.1/Spl were cloned into pKn5 (20) to generate pRK-ets2, pRK1–238Ets2, pRKets1, and pRK-PU.1, respectively, as previously described (7, 17). A hemagglutinin (HA) epitope tag was inserted upstream of the first ATG codon of Ets2, ∆Ets2, Ets1, or PU.1. APU.1 was constructed by deleting sequences corresponding to the transactivation domains found in the first 144 amino acids. An HA tag was inserted upstream of the newly created ATG codon. 293 cells were transfected by the calcium phosphate coprecipitation method in 96-well dishes by adding cells in suspension to pXP-Bcl-xPr (45 ng) and to different concentrations of pRK-ets2, pRK-PU.1, or pRK-APU.1 (4–256 ng) or of pRKets2 or pRKets1–238Ets2 in these experiments, 5 ng of pCMV-βgal was used as an internal control for transfection efficiency. For experiments performed in 12-well dishes, 293 cells or NIH3T3 cells were transfected by the calcium phosphate coprecipitation method or with LipofectAMINE Plus (Life Technologies, Inc.), respectively, using 200 ng of the reporter construct in the presence of varying concentrations of pRK5, pRKets2, or pRK-PU.1 as indicated in the figure legends with 20 ng of pCMV-βgal as an internal control for transfection efficiency as described above. One-half of the lysate was used to quantify transacted protein levels by Western analysis, and the other half was used to measure luciferase and β-galactosidase activities. For transfections in 24-well dishes, 293 cells or NIH3T3 cells exogenously expressing the CSF-1R were transfected by the calcium phosphate coprecipitation method or with LipofectAMINE Plus, respectively, as described above for 12-well dishes.

AP-1 activity was also measured using the full-length pXP-Bcl-xPr reporter construct. pXP-Bcl-xPr was cotransfected in 24-well dishes with 200 ng of pKn5, pRK-fos, or pRK-jun (21) and 20 ng of pCMV-βgal as described (17). STAT activity was measured using the full-length pXP-Bcl-xPr reporter construct in the presence of STAT3 or STAT5a cloned into pKn5. For dimerization and activation of STAT proteins, NIH3T3-cfms cells were cotransfected with pXP-Bcl-xPr in 24-well dishes with varying amounts of STAT3, STAT5a, and Ets2. 12 h after transfection, the cells were placed in low serum conditions (0.5% fetal calf serum) for 24 h ad then stimulated with CSF-1 for 24 h.

Several independent experiments using the different cell types and different combinations were performed to validate the results. Cell lysates were prepared as previously described (22). Briefly, 48–72 h after transfections, cells lysates were prepared in 25 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, and 2 mM dithiothreitol and analyzed for luciferase (Promega) and β-galactosidase (Tropix Inc., Galactolight) activities as described by the manufacturers. For 96-well dishes, the luciferase and β-galactosidase activities were read on a MicrobetaTrilux 1450 luminescence counter (Wallace). All luciferase activities were corrected according to pCMV-βgal used as an internal control for transfection efficiency.

**Northern Hybridization Analysis**—Cells were washed twice in 1× PBS. Cells were then lysed in RNA InstaPure (Eurogentec) as described by the manufacturer. 5 μg of total RNA was loaded and electrophoresed on a 2.2% formaldehyde-containing 1% agarose gel and then transferred to a nylon membrane (Amerham Pharmacia Biotech) as described by the manufacturer. Purified et2, PU.1/ets1, bcl-xL, lysozyme M (lysM), and S26 as a control for loading. The sizes of the corresponding transcripts in kilobases (kb) are indicated.

**Western Analyses**—Cells were lysed in Laemmli buffer, and equal amounts of total protein from each lysate were electrophoresed on 10–15% polyacrylamide/bisacrylamide gels. Migrated proteins were transferred to a Polyfurscreen polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences) as described by the manufacturer; immunoblotted using anti-HA tag (12CA5), anti-Bcl-xL/S (S-18, Santa Cruz Biotechnology, Inc.), anti-PU.1 (8), anti-lysM (7), or anti- Mapk (gift of Jacques Pouyssegur) antibodies; and revealed by ECL (Amerham Pharmacia Biotech) as described by the manufacturer.

**Detection of Apoptotic Cells**—Apoptotic macrophage cells were detected with fluorescein isothiocyanate-conjugated annexin V (Roche Molecular Biochemicals). Interaction of annexin V with phosphatidylserines on the outer surface of cells was observed by the manufacturer with the following modifications. After incubating cells with annexin V and washing in binding buffer, they were fixed in PBS containing 3% paraformaldehyde for 15 min at 20 °C. Cells were washed in 1× PBS and incubated with a 1:5000 dilution of 4,6-diamidino-2-phenylindole for 5 min at 37 °C. Cells were then transferred to a nylon membrane (Amersham Pharmacia Biotech) and hybridized at 42 °C in a solution of 6× SSC, 5× Denhardt’s solution, 0.5% SDS, and 50% formamide containing 20 μg/ml denatured salmon sperm DNA. Normal stringency washes were performed at 50 °C using 0.1× SSC and 0.1% SDS. All mRNA transcripts were visualized after exposure to Biomax film (Eastman Kodak Co.) at 80 °C with Dupont Quantas Fast intensification screens.

**Results**

**FIG. 1. Proliferation and Northern analysis of BMM.** Primary macrophages were obtained by treating bone marrow–derived precursor cells with 12 or 120 ng/ml CSF-1 in the presence of 0.1 ng/ml leukemia inhibitory factor for 5 days. Viable cells were counted by trypan blue exclusion (A), and total RNA was isolated (B). Northern analysis was performed using the following cDNAs as probes: bcl-xL, et2, PU.1, lysozyme M (lysM), and S26 as a control for loading. The sizes of the corresponding transcripts in kilobases (kb) are indicated.
RESULTS

Expression of ets2, PU.1, and bcl-xL upon CSF-1 Induction of Macrophage Differentiation—CSF-1 induces primary bone marrow-derived precursor cells to proliferate and then to differentiate into macrophages (BMM). We (23) and others (24) have shown that cotreatment of hematopoietic progenitors or myeloblastic cells with leukemia inhibitory factor enhances the hematopoiesis process. We obtained murine BMM using different concentrations of CSF-1 (12 and 120 ng/ml) in the presence of 0.1 ng/ml leukemia inhibitory factor. Based on morphology (data not shown) and the expression of a macrophage-specific marker with bacteriolytic functions (lysozyme M (lysM)), BMM were obtained with both concentrations of CSF-1 used (Fig. 1A). However, fewer macrophages were obtained with low doses of CSF-1 (Fig. 1A). Northern analysis revealed that PU.1 was abundantly expressed under both culture conditions, paralleling the expression of lysozyme M. However, ets2 expression was detected when a high concentration of CSF-1 was used. It is worth noting that even small increases in Ets2 expression are sufficient to induce profound biological changes since a >2-fold induction of ets2 mRNA expression has been shown to greatly affect bone and cartilage development in ets2 transgenic mice (25). Interestingly, bcl-xL expression was clearly activated when ets2 was expressed (Fig. 1B). These results show that there is a correlation of ets2 and bcl-xL expression upon treatment of primary precursor cells with concentrations of CSF-1 necessary to induce maximal proliferation and subsequent differentiation.

Depriving Primary Macrophages of CSF-1 Results in the Down-regulation of ets2 and bcl-xL Expression and Induces Apoptosis of These Cells—In addition to inducing proliferation and differentiation, CSF-1 is required for the survival of BMM. To determine the expression patterns of PU.1, ets2, and bcl-xL upon death or survival signals, we first treated bone marrow-derived cells with conditioned medium containing CSF-1 for 5 days to obtain BMM (control BMM, 0 h). BMM were then either starved of CSF-1 (0 ng) or maintained with decreasing amounts of CSF-1 (120, 12, or 6 ng/ml) for 36 h. Macrophages were photographed at 24 and 36 h. Visualized in Fig. 2A is the morphology of primary macrophages upon the different treatments. At 24 and 36 h, a higher number of floating, round, refractile cells corresponding to dying cells was observed at 0, 6, or 12 ng of CSF-1. The number of adherent macrophages also decreased in the absence or presence of low doses of CSF-1 compared with the number of cells maintained at 120 ng/ml CSF-1. Total numbers of viable cells were confirmed by trypan blue exclusion for each test condition at 36 h (Fig. 2B). To demonstrate that the cell death observed in the absence of CSF-1 was indeed due to programmed cell death by apoptosis, macrophages maintained with or starved of CSF-1 were incubated with annexin V, an early marker of apoptosis. As shown in Fig. 3, in the absence of CSF-1, 15% of the remaining cells were labeled with annexin V. The lower number of 4,6-diamidino-2-phenylindole-stained CSF-1-depleted cells (52% fewer compared with CSF-1-treated cells) and the typical compacted aspect of these nuclei reflect that the apoptotic process was underway.

To confirm that the cell death observed in CSF-1-starved BMM is accompanied by decreases in Bcl-xL protein expression, Western blot analysis was performed using lysates obtained from BMM starved of CSF-1 for 24 h and then restimulated with decreasing amounts of CSF-1 (120, 60, 12, or 0 ng/ml). After migration and transfer, the blot was incubated
with an anti-Bcl-xL antibody recognizing both anti-apoptotic Bcl-xL and pro-apoptotic Bcl-xS proteins. As shown in Fig. 4A, Bcl-xL levels decreased in the absence of CSF-1, correlating with increases in cell death. The pro-apoptotic Bcl-xS product migrating at ~25 kDa was not detected in these experiments. The amount of Ets2 and PU.1 proteins also decreased with decreasing concentrations of CSF-1. The conclusion from these results is that there is a tight correlation between the levels of Ets2, PU.1, and Bcl-xL protein expression and CSF-1. p42 MAPK was used as a control for the amount of protein loaded on the gel.

**IFN-γ and LPS Induce the Up-regulation of bcl-x, ets2, and PU.1 Transcripts**—Macrophage cytotoxic activity for killing neoplastic cells or microorganisms requires both priming and triggering signals. Priming and triggering by IFN-γ and LPS, respectively, rapidly down-regulate CSF-1R expression even in the presence of CSF-1 (26, 27). In addition, IFN-γ and LPS up-regulate bcl-xL, whose expression depends on de novo protein synthesis (14). To determine whether the up-regulation of bcl-xL correlates with PU.1 and ets2 expression independent of CSF-1, we treated primary macrophages with IFN-γ and LPS. As shown in Fig. 4B, a 4-h treatment with IFN-γ or LPS up-regulated the expression of bcl-xL, ets2, and PU.1. IP10 was used as a positive control of an mRNA abundantly induced after IFN-γ or LPS treatment (28). These results demonstrate that bcl-xL, ets2, and PU.1 are coexpressed in macrophages not only after a CSF-1 growth, differentiation, and survival stimulus, but also upon activation of macrophage functions independent of CSF-1.

**Synergistic Effects of Ets2 and PU.1 on the bcl-x Promoter**—Our Northern and Western results show that PU.1, Ets2, and Bcl-xL are expressed upon CSF-1 survival, proliferation, or differentiation signals in primary macrophages. In addition, treatment of macrophages with IFN-γ and LPS, which down-regulates CSF-1 signaling (26, 27), also up-regulates ets2, PU.1, and bcl-xL expression. Because PU.1 (17) and Ets2 (17, 29) individually transactivate the bcl-x promoter, and PU.1 and Ets2 were present when we detected the bcl-xL transcript, we asked whether these transcription factors could compete or work together in activating bcl-x transcription. Human 293 cells were used in these studies for two reasons. First, it is not possible to transiently transfect primary macrophages due to their rapid cell death following addition of DNA to these cells (30). Second, no endogenous Ets2 or PU.1 was detected by Western analysis (data not shown) in 293 cells, thereby eliminating potential contributions from endogenously expressed proteins.

By Western analysis, we verified that exogenously added HA-tagged Ets1, ΔEts2, Ets2, ΔPU.1, and PU.1 were expressed at comparable levels in transiently transfected 293 cells (Fig. 5A). Visualized in Fig. 5B (left panels) are the levels of the tagged Ets2 and PU.1 proteins following transfection of human 293 cells. In these experiments, the levels of transfected HA-PU.1 decreased as the levels of transfected HA-Ets2 were increased to keep the exogenously added amounts of total Ets proteins constant (transfected Ets DNAs at 400 ng). Corresponding transactivation studies from the same transfected samples are also shown using the 5′-regulatory sequences of the bcl-x gene upstream of the luciferase gene as the reporter (Fig. 5B). The fold inductions were higher when both Ets2 and PU.1 were equally expressed (200 ng of each DNA) than when either protein was expressed alone, but at twice the amount (400 ng of DNA) (see Fig. 5B). To verify that this observation is valid in other cell systems, similar experiments were performed using murine NIH3T3 cells (Fig. 5B, right panels). Similar results were obtained using NIH3T3 cells, in which
Transcriptional activities reported as relative bcl-xL are arbitrarily set to 1. pCMV-βgal was used in all experiments as an internal control for transfection efficiency. This is one representative experiment out of five different experiments, all giving similar results.

Ets2 and PU.1 transactivated the bcl-x promoter better (8-fold) than Ets2 (3-fold) or PU.1 (2-fold) alone. These experiments indicate that, under constant levels of EBS activity, transcriptional activation is more efficient when both PU.1 and Ets2 are present. In other words, keeping the exogenous levels of Ets proteins constant, PU.1 and Ets2 transactivate the bcl-x promoter better than PU.1 or Ets2 alone in two different cell types from two different species.

To investigate in greater detail this apparent cooperative effect, bcl-x promoter activity was monitored in 96-well plates to allow for the analysis of a wide range of Ets2 versus PU.1 concentrations. As the concentrations of either PU.1 or Ets2 alone increased, an increase in transcriptional activation was observed (Fig. 6). As the concentrations of both PU.1 and Ets2 increased, so did their capacities to transactivate the bcl-x promoter. The inductions observed with Ets2 and PU.1 together result from a synergistic (and not an additive) effect between these factors.

**Synergy Is Specific for Ets2 and PU.1 Transcription Factors**—To address whether other Ets proteins could synergize with PU.1 or Ets2, the following experiments were performed. 293 cells were transiently transfected with Ets2 and Ets1 (Fig. 7A) or Ets1 and PU.1 (Fig. 7B). In the 96-well assay at the concentrations of DNA used, Ets1 alone transactivated the bcl-x promoter approximately two times better than Ets2 (Fig. 7A). As the concentrations of Ets1 or Ets2 increased, so did the luciferase activities. The relative bcl-x promoter activity was not at all affected by cotransfections with increasing concentrations of both Ets1 and Ets2. Similar results were obtained using Ets1 and PU.1 (Fig. 7B). Cotransfection with Ets1 and PU.1 did not result in a synergistic response on the bcl-x promoter. These results indicate that the capacity to transactivate the bcl-x promoter in a synergistic manner is specific to the Ets proteins PU.1 and Ets2.

The full-length bcl-x promoter contains binding sites for other transcription factors, including an AP-1 site for Fos and Jun, an E-box for Myc and USF-1, and GAS sites for STAT proteins. Activation of macrophage competence is accompanied by increases in Fos and Jun expression (for review, see Ref. 31). Since CSF-1 stimulation of NIH3T3 fibroblasts exogenously expressing the CSF-1R also results in the up-regulation of Fos, Jun, and Myc (32), and CSF-1 activates STAT phosphorylation in BMM and in the BAC1.2F5 cell line (33), we asked whether these transcription factors could activate transcription on the bcl-x promoter in cooperation with Ets2. Cotransfection of Fos and c-Jun led to a 2–3-fold increase in luciferase activity compared with a 4–5-fold increase for Ets2. An additive effect was observed following cotransfections of the bcl-x promoter reporter construct with Fos, c-Jun, and Ets2 (Fig. 7C). Similar inductions (3-fold) have been obtained upon interleukin-3 or erythropoietin stimulation of pro-B or erythroid cells, respectively (reviewed in Ref. 34). But when STAT5a and Ets2 were coexpressed, the transactivation capacities of Ets2 appeared to be inhibited by STAT5a (Fig. 7D). Together, these results suggest that CSF-1 activation of these transcription factors is not necessary for the regulation of transcription of the bcl-x gene, but more likely for the regulation of other downstream target genes.

**Transactivation Domains of Both Ets2 and PU.1 Are Necessary for Their Synergistic Effects on the bcl-x Promoter**—To determine whether the transactivation domain or the DNA-binding domain of the Ets2 protein is necessary for this synergistic response with PU.1, we cotransfected PU.1 with ΔEts2. ΔEts2 bound to DNA, but remained inactive since its transactivation domain is absent. In agreement with the results obtained in Fig. 9A, the transactivation capacities of PU.1 alone increased with increasing concentrations of PU.1. However, these capacities were not significantly affected by increasing concentrations of ΔEts2 (Fig. 9A). These results show that ΔEts2 does not interfere negatively with the potential of PU.1 to transactivate the bcl-x promoter, but that the transactivation domain of Ets2 is required for its synergistic response with PU.1.

Similarly, the transactivation domain of PU.1 was deleted to...
The BAC1.2F5 macrophage cell line is dependent on CSF-1 for its growth and survival, although CSF-1 depletion of these cells results in slightly slower kinetics of cell death than observed with primary macrophages. We previously described a BAC1.2F5 clone that constitutively expresses Ets2 even in the absence of CSF-1 (BACets2.1D) (17). To determine whether PU.1 and Ets2 work together in macrophages to inhibit apoptosis induced by CSF-1 depletion, we electroporated BAC1.2F5 cells with PU.1 and Ets2 expression plasmids. In parallel experiments, BAC1.2F5 cells were electroporated with transcriptionally inactive ΔPU.1 and ΔEts2 mutants. In a second set of experiments, electroporations of BACets2.1D cells with a control empty expression plasmid or with APU.1 were performed. Electroporated cells were split into two dishes and were first cultured with CSF-1 to allow cells to adhere to culture dishes. Cells were washed in PBS and then cultured in either the presence or absence of CSF-1. 48 h post-transfection, the numbers of viable cells were determined by trypan blue exclusion.

The first striking observation is that cell survival of BAC1.2F5 cells, as well as of the Ets2-expressing cell line BACets2.1D, depends on the transfected plasmids. Indeed, electroporation of constructs encoding dominant negative forms of PU.1 and Ets2 results in a dramatic increase in cell death compared to electroporation of a control vector or expression vectors encoding full-length Ets2 and PU.1 proteins (54–69% fewer cells) (Fig. 10A). This massive increase in cell death is independent of CSF-1, reinforcing the notion that Ets2 and PU.1 may be involved in other cell survival processes in macrophages independent of CSF-1, as shown above with LPS and IFN treatment. It is worth noting that in parallel experiments, 293 cells were transfected with a control expression plasmid or ΔPU.1, and cell viability was determined 24 and 48 h after transfection. No difference in cell numbers was observed, demonstrating that the effect of ΔPU.1 is specific to macrophages and is not due to the toxicity of ΔPU.1 in other cell types (data not shown).

The second observation is that, among the remaining cells, sensitivity to CSF-1 depletion is doubled in the Ets2-expressing BACets2.1D line when the dominant negative form of PU.1 is expressed (Fig. 10B). Similarly, CSF-1 depletion of parental BAC1.2F5 cells resulted in 4 times more cell death when both Ets2 and PU.1 are expressed.
PU.1 and Ets2 dominant negative forms are expressed (Fig. 10B). Taken together, these results show that Ets2 and PU.1 participate in macrophage survival, in both a CSF-1-dependent and -independent manner.

Previous studies showed that the expression of the pro-apoptotic Bax protein in 293 cells induces apoptosis, whereas apoptosis is inhibited when Bax heterodimerizes with Bcl-x<sub>L</sub> in these cells (35). Since Ets2 and PU.1 activate the transcription of the bcl-x gene and, as a consequence, the expression of the Bcl-x<sub>L</sub> protein, we asked whether Ets2 and PU.1 could functionally replace the exogenous expression of Bcl-x<sub>L</sub> to inhibit Bax-induced apoptosis. 293 cells were transfected in the presence of an enhanced green fluorescent protein expression plasmid and with an empty expression plasmid as a control; with Bax; with Bcl-x<sub>L</sub> and Bax together; or with Ets2, PU.1, and Bax together. 20 h after transfection, the cells were labeled with annexin V, fixed, and analyzed by FACS. Visualized in Fig. 11 are the enhanced green fluorescent protein-positive cells labeled with annexin V. In agreement with previous studies (35), Bax induced apoptosis as visualized by the detection of annexin V-positive cells within the green fluorescent protein-positive ones. In contrast, the proportion of annexin V-positive cells in cotransfections with Bax and Bcl-x<sub>L</sub> was comparable to control cells. The profile obtained with Ets2, PU.1, and Bax was identical to that obtained with Bax and Bcl-x<sub>L</sub>. In other words, Ets2 and PU.1 functionally replaced Bcl-x<sub>L</sub> in these studies. Taken together, these results clearly demonstrate that both PU.1 and Ets2 function together to inhibit apoptosis and do so by transcriptionally activating the expression of the bcl-x gene, which results in the induction of Bcl-x<sub>L</sub> protective functions.

**DISCUSSION**

CSF-1 is necessary for the survival, proliferation, and differentiation of myeloid cells. In this report, we show that there is a tight correlation of expression of ets2 and bcl-x<sub>L</sub> in macrophages differentiated from primary bone marrow-derived progenitor cells. When ets2 is up-regulated, so is bcl-x<sub>L</sub>, but only when doses of CSF-1 are sufficient to induce maximal differentiation. Depriving fully differentiated primary BMM of CSF-1 induces these cells to die by apoptosis. This cell death is accompanied by decreased levels of Ets2, PU.1 and Bcl-x<sub>L</sub>, and when primary BMM are CSF-1-starved and then restimulated with CSF-1, the expression of both Ets2, PU.1 and Bcl-x<sub>L</sub> is up-regulated, and survival/proliferation pathways are restored in a CSF-1 dose-dependent manner.

The induction of macrophage tumoricidal and microbicidal activities by IFN-γ and LPS is independent of CSF-1 signaling (26, 27). IFN-γ and LPS induce Bcl-x<sub>L</sub> expression in peritoneal macrophages (14) and increase PU.1 DNA binding in tissue macrophages (36). In this report, we show that IFN-γ and LPS induces bcl-x<sub>L</sub>, ets2, and PU.1 in BMM.

We previously showed that the bcl-x<sub>L</sub> transcript has a short half-life (17). In this report, we show that programmed cell death of primary BMM induced by CSF-1 withdrawal correlates with decreasing Bcl-x<sub>L</sub> protein expression and is not due to the generation of the pro-apoptotic Bcl-x<sub>S</sub> form. Instead, growth factor withdrawal-induced apoptosis of primary BMM results from silencing of the bcl-x<sub>L</sub> gene as evidenced by decreased bcl-x<sub>L</sub> transcript expression. Our results using terminally differentiated macrophages are in agreement with those of Packham et al. (15), who showed that apoptosis of primary myeloid stem cells induced upon interleukin-3 withdrawal results in decreased bcl-x<sub>L</sub> mRNA levels and not in caspase cleavage of the Bcl-x<sub>L</sub> protein. Together, these studies demonstrate that Bcl-x<sub>L</sub> is more ubiquitous than previously thought, but is tightly regulated in myeloid cells independent of their maturation state or their specific cell survival factors.

Although the expression of PU.1 is necessary to induce macrophage differentiation from immature precursor cells, the expression of PU.1 alone is not sufficient to keep the BAC1.2F5 macrophage cell line alive in the absence of CSF-1. In contrast,
constitutive Ets2 expression protects CSF-1-depleted BAC1.2F5 macrophages from cell death by apoptosis while PU.1 levels remain elevated (17). The question that we addressed in this study is what are the functions of these two members of the Ets family in terminally differentiated macrophages. To answer this question, we investigated the transcriptional activity of both factors individually and together on the bcl-x promoter. We showed that although both Ets2 and PU.1 were able to individually transactivate the bcl-x promoter, a strong synergistic activation was observed when both were present. It thus appears that although a constitutively elevated level of PU.1 would cause some basal bcl-x expression, the concomitant expression of the highly regulated Ets2 protein results in a dramatic increase in bcl-x activation. Abundant levels of Bcl-xL expression would then enable cell survival and ensure a long life span of macrophages when both Ets proteins are simultaneously expressed.

It is striking to note that the synergy between Ets family members PU.1 and Ets2 on the bcl-x promoter is specific since cotransfections of Ets2 and Ets1 or of PU.1 and Ets1 do not result in increased transcriptional activity. Interestingly, although Ets1 is not expressed in macrophages, Ets1 is highly expressed during certain stages of developing thymocytes (37, 38) when bcl-x is not expressed (39, 40). Together, these observations suggest that the expression patterns of Ets family members parallel functions on the bcl-x promoter. Finally, we showed that both PU.1 and Ets2 transcriptional domains are required for this synergy. The integrity of the bcl-x promoter is necessary for maximal synergy between Ets2 and PU.1 since deletion of two or more sites greatly reduces the capacity of these two transcription factors to synergize (data not shown).

We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown). We observed that other transcription factors like Fos and Jun or Myc (data not shown), which are up-regulated after CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter through their AP-1 or E-box sites, respectively. Cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a cotransfections with Ets2 result in only an additive effect in increasing transcriptional activation. STAT3 and STAT5a are only transactivated by CSF-1 stimulation, surprisingly only weakly transactivate the bcl-x promoter (33). We also demonstrated that two transcription factors to synergize (data not shown).