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Apoptosis Triggered by Myc-Induced Suppression of Bcl-X_L or Bcl-2 Is Bypassed during Lymphomagenesis

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Enforced Bcl-2 expression inhibits Myc-induced apoptosis and cooperates with Myc in transformation. Here we report that the synergy between Bcl-2 and Myc in transforming hematopoietic cells in fact reflects a Myc-induced pathway that selectively suppresses the expression of the Bcl-X_L or Bcl-2 antiapoptotic protein. Myc activation suppresses Bcl-X_L RNA and protein levels in cultures of primary myeloid and lymphoid progenitors, and Bcl-X_L and Bcl-2 expression is inhibited by Myc in precancerous B cells from Eμ-*myc* transgenic mice. The suppression of *bcl-X* RNA levels by Myc requires de novo protein synthesis, indicating that repression is indirect. Importantly, the suppression of Bcl-2 or Bcl-X_L by Myc is corrupted during Myc-induced tumorigenesis, as Bcl-2 and/or Bcl-X_L levels are markedly elevated in over one-half of all lymphomas arising in Eμ-*myc* transgenic mice. Bcl-2 and/or Bcl-X_L overexpression did not correlate with loss of ARF or p53 function in tumor cells, indicating that these two apoptotic pathways are inactivated independently. Therefore, the suppression of Bcl-X_L or Bcl-2 expression represents a physiological Myc-induced apoptotic pathway that is frequently bypassed during lymphomagenesis.

Many cancers harbor alterations that directly or indirectly lead to constitutive overexpression of the c-Myc oncoprotein (reviewed in reference 3). In most cell types, c-Myc enforces S phase entry (10, 37, 54), although activation of c-Myc also triggers the apoptotic program (reviewed in reference 47). In vivo, activation of apoptosis by c-Myc is evident in the B cells of Eμ-*myc* transgenic mice, which have intrinsically high proliferative and apoptotic rates (26). Ultimately, secondary genetic changes make these B cells refractory to the Myc apoptotic response, resulting in the outgrowth of clonal pre-B- and B-cell lymphomas (1).

c-Myc activates the ARF-Mdm2-p53 tumor suppressor pathway, which is frequently disabled in human cancers (reviewed in reference 56). c-Myc activation leads to the rapid accumulation of $p19^{ARF}$ (64), a nucleolar protein encoded by an alternative reading frame of the *Ink4a/ARF* locus (49). In turn, ARF activates p53 both through nucleolar sequestration of p53's inhibitor Mdm2 (59, 62) and by interference with Mdm2 E3 ubiquitin ligase activity (22). Mdm2 is a transcriptional target of p53 that inhibits p53-dependent transactivation (43) and induces p53 ubiquitination (21) and its shuttling to the cytoplasm for destruction by the 26S proteasome (52). Thus, in the presence of oncoproteins such as c-Myc, high ARF levels inhibit Mdm2, allowing a robust p53 transcriptional response that triggers apoptosis (64).

In the majority of lymphomas that arise in Eμ-*myc* transgenic mice, c-Myc overexpression selects for loss of ARF and/or p53 function (11, 55). Moreover, loss of ARF or p53 markedly accelerates Myc-induced tumor development (11, 23, 25, 55). Although these cooperative effects are associated with a decreased apoptotic rate, even rapidly arising tumors from ARF-null Eμ-*myc* transgenic mice are clonal (C. M. Eischen and J. L. Cleveland, unpublished data), indicating that additional alterations are required during Myc-induced lymphomagenesis. Furthermore, in primary fibroblasts and pre-B cells the loss of ARF or p53 impairs, but does not fully abolish, c-Myc-induced apoptosis (11, 64). Thus other targets must contribute to the c-Myc apoptotic response.

Bcl-2 or Bcl-X_L overexpression blocks many cell death pathways, including those induced by c-Myc (6, 13). Apoptosis induced by c-Myc in hematopoietic cells is effectively suppressed by cytokines, yet high levels of c-Myc override the protective effects of these survival factors (12, 47). Therefore, Myc-induced cell death also likely hinges on proteins regulated by hemopoietins. Potential targets include the Bcl-2 family of proteins, which can either suppress (e.g., Bcl-2, Bcl-X_L, and Mcl-1) or augment (e.g., Bax, Bad, and Bak) the apoptotic program (reviewed in reference 17). Although these proteins are regulated by posttranslational modifications and changes in their subcellular localization (reviewed in reference 33), alterations in their steady-state levels also play a pivotal role in hematopoietic cell survival. First, in myeloid progenitors cytokines selectively regulate Bcl-X_L expression and apoptosis by a Jak2 kinase-dependent pathway (48). Second, loss of *bcl-X* in mice results in high levels of apoptosis in embryonic hematopoietic cells (44), whereas *bcl-2*-deficient mice display profound apoptosis of mature lymphocytes, which disappear by 4 to 6 weeks of age (45, 61). Finally, Bcl-2 transgenes effectively block the severe defects in T-cell lymphopoiesis seen in mice lacking either the interleukin-7 (IL-7) receptor or the common γ chain (2, 32) and enable macrophage production in mice lacking macrophage colony-stimulating factor (CSF-1) (34).

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Therefore, the appropriate expression of antiapoptotic Bcl-2 family proteins is critical for hematopoietic cell survival.

In vivo, the programmed expression of Bcl-2 in B cells blocks the intrinsically high rates of apoptosis of Eμ-*myc* transgenic B cells and cooperates with Myc to induce rapid primitive lymphoid tumors (57). We now report that this cooperation reflects an apoptotic pathway induced by c-Myc that selectively suppresses the expression of Bcl-X_L or Bcl-2 in hematopoietic cells. Furthermore, Myc-induced suppression of Bcl-2 or Bcl-X_L is disabled in over half of the lymphomas arising in Eμ-*myc* transgenic mice and occurs independently of ARF/p53 status.

MATERIALS AND METHODS

Primary cells. Myeloid progenitors from the fetal livers of embryonic day 15 (E15) to E17 embryos or from the bone marrow of 6- to 8-week-old *bcl-2*$^{-/-}$, *p53*$^{-/-}$, *ARF*$^{-/-}$, *p53 ARF* double-null, and wild-type mice were cultured in RPMI 1640 medium supplemented with IL-3 (20 U/ml), IL-6 (10 ng/ml; R&D Systems), and stem cell factor (SCF) (10 ng/ml; R&D Systems) (48). The phenotypes of the cells by fluorescence-activated cell sorting (FACS) were uniformly CD34$^+$, c-Kit$^+$, and Sca-I$^+$. All antibodies used for phenotyping were from Southern Biotechnology (Birmingham, Ala.) or PharMingen (San Diego, Calif.).

Primary pre-B-cell cultures were generated from the bone marrow of wild-type, *bcl-2*$^{-/-}$, *ARF*$^{-/-}$, and/or *p53*-null mice as described previously (11). Immunophenotyping established that all cultures were greater than 98% pre-B cells (i.e., CD19$^+$ CD43$^-$ CD24$^+$ immunoglobulin M$^-$ [IgM$^-$]). B cells (IgM$^+$ CD19$^+$) and B-cell precursors (IgM$^-$ CD19$^+$) from bone marrow and spleens of age- and gender-matched wild-type and Eμ-*myc* transgenic mice (prior to signs of disease) were sorted by FACS after being stained with anti-IgM–fluorescein isothiocyanate and anti-CD19–phycoerythrin.

Virus infection. Primary myeloid and pre-B cells were infected with the murine stem cell virus (MSCV) Myc-estrogen receptor^{TM} (ER^{TM})-internal ribosome entry site (IRES)-green fluorescent protein (GFP) virus or with the MSCV-IRES-GFP control virus, as previously described (11). Comparable levels of Myc-ER^{TM} fusion protein in all cultures were established by immunoblotting. The phenotype of the Myc-ER^{TM} virus-infected myeloid progenitors was indistinguishable from that of uninfected or MSCV-GFP virus-infected myeloid cell cultures. The Myc-ER^{TM} chimeric protein was activated by the addition of 1 μM 4-hydroxytamoxifen (4-HT) (Sigma, St. Louis, Mo.). Addition of 1 μM 4-HT to uninfected or MSCV-GFP virus-infected cells had no effect on myeloid or pre-B-cell growth or viability.

Viability and apoptosis assays. Cell viability was determined at specific intervals by trypan blue dye exclusion following cytokine deprivation or the addition of 1 μM 4-HT to activate Myc-ER^{TM}. Apoptosis was quantitated by measuring fragmented DNA (sub-G_1) by flow cytometry following propidium iodide staining.

Transgenic and knockout mice. The inbred C57BL/6 Eμ-*myc* transgenic mouse strain was provided by Alan Harris (Walter & Eliza Hall Institute, Melbourne, Australia) and Charles Sidman (University of Cincinnati). We generated *ARF*$^{+/-}$ Eμ-*myc* and *ARF* $^{-/-}$ Eμ-*myc* transgenic mice as previously described (11). Fifth-generation Teconic 129S6/SvEv backcrossed *bcl-2*$^{-/-}$ mice were generated, and the control 129S6/SvEv strain was purchased from Taconic Laboratories (Germantown, N.Y.). *p53*- and *ARF*-null mice were kindly provided by Gerard Grosveld and Charles Sherr (St. Jude Children's Research Hospital), respectively.

Western blotting. Whole-cell protein extracts were isolated as previously described (11). Equal amounts of protein (20 to 125 μg/lane) were separated in sodium dodecyl sulfate–7.5 or 10% polyacrylamide gel electrophoresis gels. Proteins were transferred to nitrocellulose (Protran; Schleicher & Schuell, Dassel, Germany) and blotted with antibodies specific for murine c-Myc (06-340) and Bak (both from Upstate Biotechnology, Lake Placid, N.Y.); p19^{ARF} (49); p53 (AB-7; Calbiochem, La Jolla, Calif.); Bcl-2 (15021) and Bax (13686E) (both from PharMingen); Bcl-X_L (B2260), Mcl-1 (B54020), and Bad (B36420) (all three from Transduction Labs, San Diego, Calif.); and β-actin (Amersham, Arlington Heights, Ill.). Bound immunocomplexes were detected by enhanced chemiluminescence.

Northern blotting. Following addition of 1 μM 4-HT, primary pre-B and myeloid cells were harvested at the intervals indicated on the figures and total RNA was isolated using Trizol reagent (Life Technologies, Grand Island, N.Y.). Forty or 20 μg of total RNA from pre-B cells or myeloid cells, respectively, was run into formaldehyde agarose gels and transferred to nitrocellulose (Protran; Schleicher & Schuell). The membranes were probed sequentially with the coding portions of murine *bcl-X*, *bcl-2*, and β-*actin* cDNAs and stripped after each hybridization and autoradiography. For the cycloheximide experiment, primary pre-B cells were pretreated for 30 min at 37°C with 10 μg of cycloheximide/ml or vehicle control prior to the addition of 4-HT.

RESULTS

Loss of ARF and/or p53 protracts, but does not abolish, Myc-induced apoptosis of hematopoietic cells. In primary mouse embryo fibroblasts (MEFs) and pre-B cells, c-Myc-induced apoptosis involves the activation of the ARF-Mdm2-p53 apoptotic pathway (11, 64). However, MEFs lacking ARF or p53 are not totally resistant to Myc-induced apoptosis (64), indicating that Myc also activates apoptosis independent of ARF or p53. To study such effects in primary hematopoietic cells, we isolated fetal liver- and bone marrow-derived myeloid progenitors and bone marrow-derived pre-B cells from wild-type, *ARF*-null, *p53*-null, and/or *ARF p53* double-null mice. Primary myeloid progenitors (CD34$^+$ c-Kit$^+$ *Sca*I$^+$ Lin$^-$) derived from E15 to E17 fetal livers or bone marrow were cultured in IL-3, IL-6, and SCF (48), whereas primary pre-B cells (CD19$^+$ IgM$^-$ CD24$^+$ CD43$^-$) derived from bone marrow were grown in medium containing IL-7 (11). FACS analysis demonstrated that loss of *ARF* and/or *p53* had no overt effect on the myeloid or pre-B-cell phenotype (11) (data not shown).

Primary myeloid and pre-B cells were infected with the MSCV-Myc-ER^{TM}-GFP recombinant retrovirus or with the MSCV-GFP control virus. The MSCV-Myc-ER^{TM}-GFP retrovirus encodes a conditionally active form of c-Myc, in which c-Myc is fused to the hormone binding domain of the ER (Myc-ER^{TM}) modified to respond to 4-HT (36). Furthermore, GFP is expressed in *cis* via an IRES, allowing for direct selection of infected cells. Immunoblotting analysis revealed that comparable levels of Myc-ER^{TM} protein were expressed in cells with the different genotypes (Fig. 1A, inset) (11). When grown in complete medium containing both serum and cytokines, wild-type myeloid and pre-B cells infected with the Myc-ER^{TM} retrovirus exhibited a higher apoptotic index (20 to 30%) than uninfected cells, those infected with control vector, and *ARF*- and *p53*-null cells infected with the Myc-ER^{TM} retrovirus (Fig. 1 and data not shown). This basal level of Myc-induced apoptosis in wild-type hematopoietic cells is likely due to the somewhat leaky nature of the Myc-ER^{TM} construct (64). Activation of Myc-ER^{TM} by 4-HT induced rapid cell death of wild-type myeloid (Fig. 1A) and pre-B (Fig. 1B) cells, despite the presence of potent survival factors in the medium. Importantly, although myeloid and pre-B cells lacking ARF and/or p53 were more resistant to 4-HT-induced Myc-ER^{TM} activation, they ultimately underwent apoptosis in the presence of cytokines (Fig. 1). By 48 or 72 h following Myc activation, <40% of the *ARF*- or *p53*-null myeloid and pre-B cells, respectively, were alive, and all cells eventually died. Thus, effectors other than ARF and p53 must contribute to c-Myc-induced hematopoietic cell apoptosis.

Hematopoietic cell apoptosis induced by cytokine deprivation is independent of ARF and p53 but is augmented by the loss of *bcl-2*. Myc activation renders cells hypersensitive to many apoptotic insults including the withdrawal of survival factors, which suppress c-Myc-induced apoptosis (4, 12, 19). To

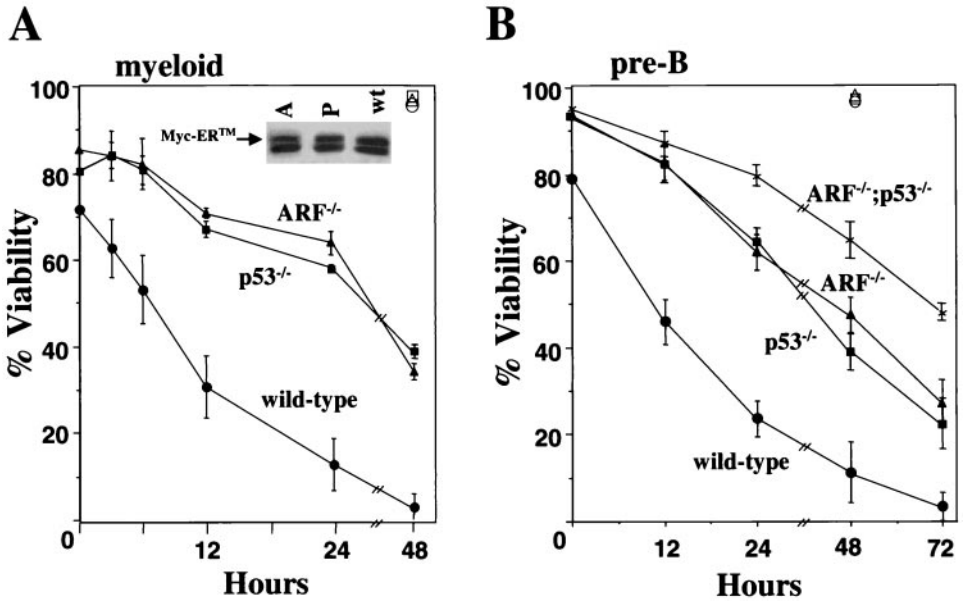

FIG. 1. Myc-induces apoptosis in hematopoietic cells lacking *ARF* and/or *p53*. 4-HT was added to the culture medium of wild-type, *ARF*-null, and/or *p53*-null primary myeloid precursors (A) and pre-B cells (B) infected with a retrovirus encoding Myc-ER™, and at the indicated intervals the percentages of viable cells were assessed by trypan blue dye exclusion. Data shown for the myeloid cells are the means of five (wild type), four ($ARF^{-/-}$), and two ($p53^{-/-}$) independent experiments, and three independent experiments were performed with pre-B cells. Error bars represent one standard deviation. Open symbols, viability of wild-type (triangle), *ARF*-null (circle), and *p53*-null (square) cells containing GFP vector controls following 4-HT addition for the indicated intervals. (Inset) Immunoblotting of $ARF^{-/-}$ (A), $p53^{-/-}$ (P), and wild-type (wt) Myc-ER™ retrovirus-infected primary myeloid cells with a Myc-specific antibody. Arrow, location of Myc-ER™. Myc-ER™ was expressed at similar levels in pre-B cells derived from wild-type, *ARF*-null, *p53*-null, and *ARF p53* double-null mice (11).

address the relevance of ARF and p53 to apoptosis that follows the withdrawal of survival factors, we compared the rates of death of wild-type, *ARF*-null, and *p53*-null primary hematopoietic cells following removal of their required hemopoietins. Both *ARF*- and *p53*-null primary myeloid and pre-B cells grew at accelerated rates (data not shown) (11). However, when deprived of cytokines these myeloid (Fig. 2A) and pre-B (Fig. 2B) cells died at rates essentially identical to those derived from wild-type mice. Thus, ARF and p53 do not regulate hematopoietic cell apoptosis triggered by cytokine deprivation.

Potential mediators of apoptosis induced by cytokine deprivation include members of the Bcl-2 family. Gene targeting studies have demonstrated that Bcl-2 and Bcl-X are rate limiting for hematopoietic cell survival (44, 45, 61). However, only *bcl-2*-deficient mice are amenable to analyses, as *bcl-X*-deficient mice die at E13.5 (44). We therefore derived primary pre-B cells and myeloid progenitors from the bone marrow of $bcl\text{-}2^{-/-}$ mice and assessed their rates of apoptosis following cytokine deprivation. Strikingly, $bcl\text{-}2^{-/-}$ pre-B cells had a very high apoptotic index and therefore could not be expanded in tissue culture (Fig. 2C). After 12 to 17 days in culture, pre-B cells lacking *bcl-2* were only 25 to 35% viable, whereas cells from wild-type mice were healthy and readily expanded (Fig. 2C). Although $bcl\text{-}2^{-/-}$ primary myeloid progenitors only had a slightly higher apoptotic index in complete medium (Fig. 2D), they died at an accelerated rate when deprived of cytokines (Fig. 2D). The majority of the *bcl-2*-deficient myeloid cells were dead within 24 h after cytokines were removed, whereas only a small fraction of the wild-type progenitors died during this interval (Fig. 2D). Thus, *bcl-2* loss potentiates the apoptotic program initiated when hematopoietic cells are deprived of survival factors.

c-Myc suppresses Bcl-X_L expression in primary myeloid and pre-B cells. Given the profound effects of *bcl-2* loss on hematopoietic cell survival, we assessed the effects of c-Myc on the expression of Bcl-2 family proteins. Activation of Myc-ER™ by 4-HT in wild-type primary myeloid cells revealed, as expected, the induction of ARF and p53 in wild-type myeloid cells, whereas the induction of p53 and p53 transcriptional target $p21^{Cip1}$ was severely impaired in *ARF*-null cells (Fig. 3A). As previously reported for MEFs and pre-B cells (11, 64), Myc activation induced ARF in wild-type and *p53*-null primary myeloid cells. Notably, Myc activation in primary myeloid and pre-B cells led to an obvious and selective reduction in Bcl-X_L levels without affecting the expression of Bcl-2 or of proapoptotic proteins Bax, Bad, and Bak (Fig. 3 and data not shown). The levels of the antiapoptotic Mcl-1 protein in the primary myeloid cells were also not altered (Fig. 3A), and Mcl-1 was not detected in any of the pre-B cell cultures (data not shown). One prediction was that Myc-mediated suppression of Bcl-X_L was ARF and/or p53 dependent. However, Myc activation in $ARF^{-/-}$, $p53^{-/-}$, and *ARF p53* double-null myeloid and pre-B cells also resulted in rapid and similar reductions in Bcl-X_L levels (Fig. 3). Therefore, Myc activation selectively represses Bcl-X_L protein expression in primary hematopoietic cells, and this occurs independent of ARF and/or p53 status.

To address whether the decrease in Bcl-X_L protein levels following Myc activation was due to changes in *bcl-X* transcripts, we performed Northern blot analysis of RNA isolated from primary myeloid and pre-B cells infected with the Myc-

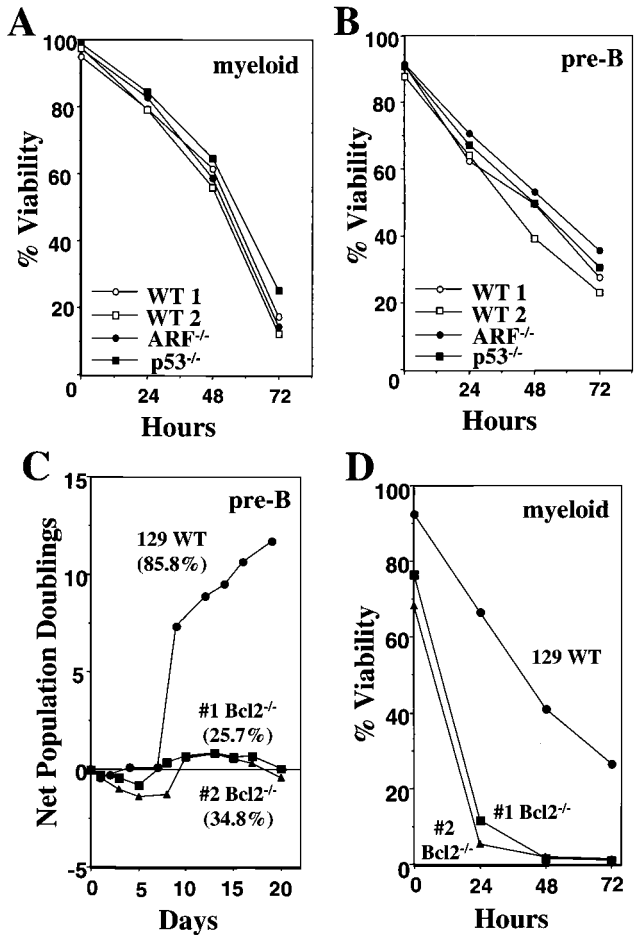

FIG. 2. Apoptosis induced by cytokine withdrawal is independent of ARF or p53 yet is accelerated by *bcl-2* loss. Wild-type (WT), *ARF*-null, or *p53*-null myeloid progenitors were deprived of IL-3, IL-6, and SCF (A), and wild-type, *ARF*-null, or *p53*-null pre-B cells were deprived of IL-7 (B). At the indicated intervals the percentages of viable cells were assessed by trypan blue dye exclusion. Results shown are representative of three independent experiments. (C) Growth curves of bone marrow cells from two *bcl-2*-deficient mice and one 129/SvEv wild-type mouse in media containing IL-7. The mean percentages of viable cells between days 12 to 17 of culture are in parentheses; standard deviations were less than 5% for all three cultures. (D) Wild-type or *bcl-2*$^{-/-}$ myeloid progenitors were deprived of IL-3, IL-6, and SCF, and at indicated intervals the percentages of viable cells were assessed by trypan blue dye exclusion. Data shown are representative of two independent experiments. Apoptosis was confirmed by analysis of subdiploid DNA content after propidium iodide staining.

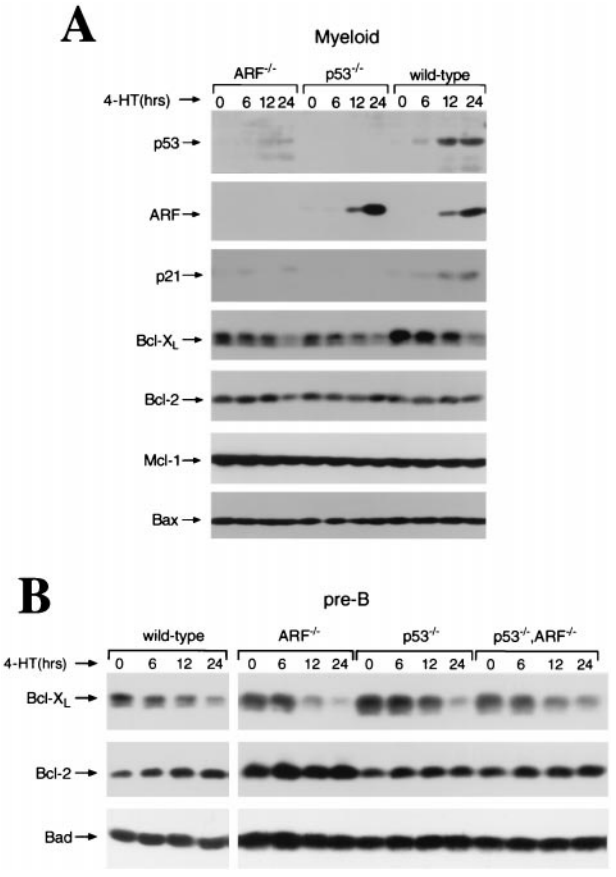

FIG. 3. Myc selectively suppresses the expression of Bcl-X_L protein in primary hematopoietic cells independent of ARF and p53 status. (A) The expression of p53, ARF, p21^{Cip1}, Bcl-X_L, Bcl-2, Mcl-1, and Bax protein was assessed at the indicated intervals by immunoblotting extracts prepared from Myc-ER^{TM} retrovirus-infected primary myeloid precursors following activation of Myc-ER^{TM} by 4-HT. (B) Expression of Bcl-X_L, Bcl-2, and Bad protein was assessed at the indicated intervals by immunoblotting extracts prepared from primary pre-B cells harboring Myc-ER^{TM} following activation by 4-HT.

ER^{TM} retrovirus. The levels of *bcl-X* RNA decreased rapidly (within 3 h) following Myc activation by 4-HT in both cell types, whereas the levels of *bcl-2* transcripts remained unchanged (Fig. 4). The selective suppression of *bcl-X* transcripts was not simply a secondary effect of cell death, as at 3 h Myc-ER^{TM}-activated cells were as viable as untreated cells (time zero) (Fig. 4A). Notably, new protein synthesis was required for Myc to suppress *bcl-X* RNA levels (Fig. 4B). Cycloheximide blocked the Myc-induced decrease in *bcl-X* expression in pre-B cells, indicating that Myc suppresses *bcl-X* levels by an indirect mechanism. Interestingly, *bcl-X* transcripts appear to be somewhat induced by cycloheximide, as levels of the transcripts were higher than those in untreated cells (Fig. 4B). Therefore, in primary hematopoietic cells Myc selectively suppresses *bcl-X* RNA expression and this occurs at either a transcriptional or posttranscriptional level and requires new protein synthesis.

Bcl-2 and Bcl-X_L expression is suppressed in precancerous Eμ-*myc* transgenic B cells. To establish whether Myc also influenced Bcl-X_L expression in vivo, we harvested bone marrow from wild-type and Eμ-*myc* transgenic littermates prior to any detectable disease and sorted B-cell subsets by FACS. B-cell populations were sorted for the pan-B-cell marker CD19 and for the expression of cell surface IgM. The promoter/enhancer used to express the Myc transgene is utilized at the pro- to pre-B-cell stage of B-cell differentiation and stays on throughout B-cell development (1). Therefore, as expected, the levels of Myc in IgM$^-$ precursor B cells and the more mature IgM$^+$ B cells were not different (Fig. 5A). Consistent with previous reports (16, 40), we observed high Bcl-X_L expression and low Bcl-2 expression in wild-type IgM$^-$ B-cell progenitors but low Bcl-X_L levels and high Bcl-2 levels in

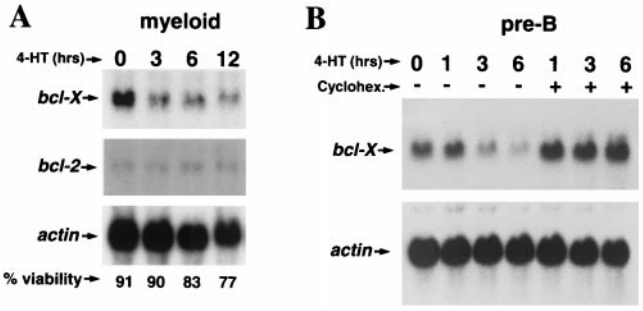

FIG. 4. New protein synthesis is required for the suppression of *bcl-X* RNA levels by Myc. The expression of *bcl-X* and *bcl-2* transcripts from primary myeloid cells (A) and *bcl-X* transcripts from pre-B cells (B) expressing Myc-ER^{TM} was assessed following no pretreatment (A) or a 30-min pretreatment with 10 μg of cycloheximide/ml or vehicle control followed by activation of Myc-ER^{TM} with 4-HT for the indicated intervals. Hybridization with a β-*actin* probe was used to equalize loading of RNA.

mature IgM$^+$ B cells (Fig. 5B). Comparison of Bcl-X_L and Bcl-2 expression revealed that IgM$^+$ B cells from Eμ-*myc* transgenic mice had markedly reduced levels of Bcl-2 protein relative to those expressed in IgM$^+$ cells from wild-type mice (Fig. 5B). The low levels of Bcl-2 in IgM$^-$ (CD19$^+$) B-cell precursors from transgenic and wild-type mice did not differ from each other (Fig. 5B). In contrast, Bcl-X_L protein levels were drastically reduced in the IgM$^-$ B-cell progenitors from Eμ-*myc* transgenic mice compared with those in B-cell progenitors from wild-type mice; these differences were less obvious in IgM$^+$ B cells (Fig. 5B). Therefore, c-Myc overexpression selectively suppresses Bcl-X_L or Bcl-2 expression in vivo in a cell context-specific fashion.

Bcl-2 or Bcl-X_L or both are overexpressed in lymphomas arising in Eμ-*myc* transgenic mice. If suppression of Bcl-X_L or Bcl-2 expression by c-Myc is relevant to apoptosis in vivo, then this pathway should be disabled in pre-B- and B-cell lymphomas arising in Eμ-*myc* transgenic mice. Previously we demonstrated that most of the fatal lymphomas (80%) of Eμ-*myc* transgenic mice have alterations in the ARF-Mdm2-p53 pathway (11). However, the remaining 20% of tumors arising in *ARF*$^{+/+}$ Eμ-*myc* transgenic mice and 8% of the tumors of *ARF*$^{+/-}$ Eμ-*myc* transgenic mice lacked alterations in ARF, p53, or Mdm2. We therefore assessed the expression of Bcl-2 and Bcl-X_L in this group of lymphomas. We compared their levels to those of Bcl-2 and Bcl-X_L present in FACS-sorted B cells derived from precancerous Eμ-*myc* bone marrow, which expressed reduced levels of Bcl-2 or Bcl-X_L relative to levels expressed by wild-type B cells (Fig. 5B). In tumors lacking alterations in ARF, p53, or Mdm2, only two tumors, one from an *ARF*$^{+/+}$ Eμ-*myc* transgenic mouse (DF296) and one from an *ARF*$^{+/-}$ Eμ-*myc* transgenic mouse (DF114), overexpressed Bcl-2 and none had elevated levels of Bcl-X_L (Fig. 6A and data not shown). However, 56% (14 of 25) of lymphomas from *ARF*$^{+/+}$ Eμ-*myc* transgenic mice expressed much higher levels of Bcl-2 and/or Bcl-X_L than those expressed in precancerous B cells (Fig. 6A and data not shown). Bcl-2 was overexpressed in 13 of 25 tumors (CR20, CR102, CR246, CR320, CR73, CR205, CR303, CR230, DF195, DF795, CR156, CR325, and DF296), whereas 2 of 25 tumors (CR73 and CR17) overexpressed Bcl-X_L. Only one tumor (CR73) overexpressed both Bcl-2 and

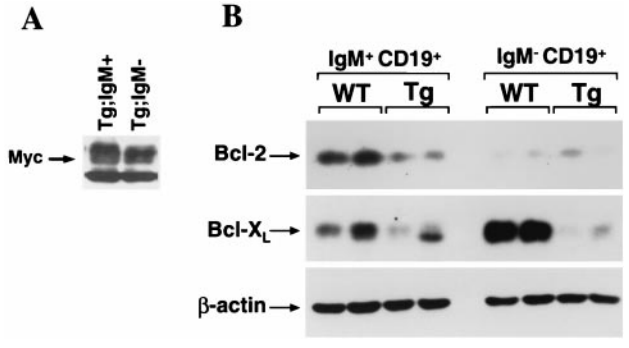

FIG. 5. Bcl-2 or Bcl-X_L levels are suppressed in B-cell subsets from precancerous Eμ-*myc* transgenic mice. The levels of Myc (A) and Bcl-2, Bcl-X_L, and β-actin (B) proteins in mature (IgM$^+$ CD19$^+$) and precursor (IgM$^-$ CD19$^+$) B cells from FACS-sorted bone marrow from two wild-type (WT) and two Eμ-*myc* transgenic (Tg) mice (age and gender matched) were assessed by immunoblotting with antibodies specific for each protein.

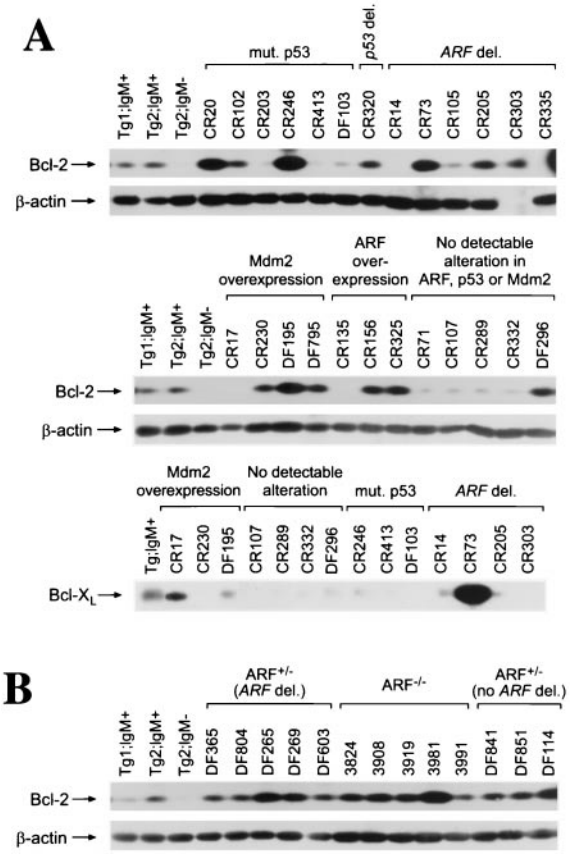

FIG. 6. Bcl-2 is overexpressed in over half of the lymphomas arising in Eμ-*myc* transgenic mice. Levels of Bcl-2, Bcl-X_L, and β-actin proteins in tumors from wild-type (A) and from *ARF*$^{+/-}$ and *ARF*$^{-/-}$ (B) Eμ-*myc* transgenic mice were assessed by immunoblotting with antibodies specific for each protein. IgM$^-$ B-cell precursors and mature IgM$^+$ B cells were sorted by FACS from bone marrow and spleens of precancerous Eμ-*myc* transgenic mice and run as controls for Bcl-2 and Bcl-X_L expression.

Bcl-X_L. Therefore, over half of the lymphomas arising in Eμ-*myc* transgenic mice overexpress Bcl-2 and/or Bcl-X_L, indicating that the suppression of Bcl-2 or Bcl-X_L expression by Myc in precancerous B cells is bypassed during lymphoma progression.

Bcl-2 levels are higher in IgM^+ B cells than in IgM^- B-cell precursors (Fig. 5B). We determined by FACS that approximately 73% (27 of 37) of Eμ-*myc* lymphomas arising in Eμ-*myc* C57BL/6 transgenic mice are IgM^+ or a mixture of IgM^+ and IgM^- ($CD19^+$), whereas only 27% (10 of 39) are IgM^- ($CD19^+$) (C. M. Eischen and J. L. Cleveland, unpublished data). The fact that the majority of our Eμ-*myc* transgenic mice develop B-cell instead of pre-B-cell lymphomas differs from an early report indicating that only 19% of Eμ-*myc* transgenic mice develop B-cell lymphomas (20). The genetic backgrounds of our Eμ-*myc* transgenic mice (congenic C57BL/6) and their Eμ-*myc* transgenic mice (C57BL/6J Wehi × SJL/J Wehi F_1 hybrids) are significantly different, and that is the most likely explanation for these discrepancies. Nevertheless, the expression of Bcl-2 is markedly suppressed by Myc in precancerous IgM^+ mature B cells (Fig. 5B). The preponderance of mature B-cell lymphomas and Bcl-2 overexpression in these tumors thus reflects a bypass of this pathway in the more mature B cell and may explain why Bcl-2 is more frequently overexpressed in Eμ-*myc* lymphomas than Bcl-X_L (Fig. 6).

In the lymphomas we found no correlation between Bcl-2 and Bcl-X_L expression levels and Mdm2 overexpression, *p53* mutation, or *ARF* deletion (Fig. 6A). Eμ-*myc* lymphomas with mutated or deleted *p53* or deleted *ARF* were analyzed, and 8 of 13 (62%) expressed high levels of Bcl-2 and/or Bcl-X_L. Furthermore, in many lymphomas from $ARF^{+/-}$ Eμ-*myc* transgenic mice, where 80% harbor deletions of the wild-type *ARF* allele (11), and in the rapidly arising tumors of *ARF* nullizygous Eμ-*myc* transgenic mice, Bcl-2 or Bcl-X_L or both were also expressed at abnormally high levels (9 of 13, 69%) (Fig. 6B and data not shown). Thus, disabling the ARF-Mdm2-p53 pathway and loss of Myc-mediated suppression of Bcl-2 or Bcl-X_L occur independently during Myc-induced lymphomagenesis.

DISCUSSION

Myc suppresses Bcl-X_L and Bcl-2 expression in hematopoietic cells. Acquiring resistance to Myc-induced apoptosis must occur as cells proceed toward malignancy. The ability of Myc to induce ARF and activate p53 leads to apoptosis, and this inhibits tumor development (11, 55, 64). The ARF-Mdm2-p53 pathway is therefore disabled in most of the lymphomas from Eμ-*myc* transgenic mice (11, 25, 55). Here we demonstrate that Myc activation in wild-type primary pre-B and myeloid progenitor cells results in a reduction of Bcl-X_L levels and that this also occurs in cells lacking ARF and/or p53. In precancerous B-cell subsets of Eμ-*myc* transgenic mice, Myc suppresses either Bcl-X_L or Bcl-2 expression, depending on cell context, whereas over half of the lymphomas arising in these transgenic mice overexpress Bcl-2 or Bcl-X_L. Furthermore, the corruption of this second Myc-induced apoptotic pathway occurs independent of ARF, Mdm2, or p53 status in these lymphomas.

Prior to overt lymphoma, B-cell precursors from Eμ-*myc* transgenic mice have high apoptotic indices in the bone marrow, which are offset by the elevated proliferative rates of premalignant B cells (26). FACS-sorted B-cell subsets from precancerous Eμ-*myc* transgenic mice had decreased levels of Bcl-2 or Bcl-X_L protein (Fig. 5B), whereas the levels of proapoptotic Bcl-2 family members did not change (C. M. Eischen and J. L. Cleveland, unpublished data). This logically should result in apoptosis, as *bcl-2*- and *bcl-X*-deficient hematopoietic progenitors are highly prone to apoptosis (Fig. 2) (44). Most Bcl-2 family members appear to control apoptosis by regulating the release of cytochrome *c* from mitochondria, which activates the caspase-9 regulator Apaf-1 (17). The ratio of pro- and antiapoptotic Bcl-2 family members regulates the susceptibility of cells to apoptosis (33); thus, decreased levels of Bcl-2 or Bcl-X_L without changes in proapoptotic proteins in the B cells of Eμ-*myc* transgenic mice should account for their increased susceptibility to apoptosis. Furthermore, the suppression of Bcl-2 or Bcl-X_L expression by Myc independent of ARF or p53 status supports observations by Juin and colleagues that in fibroblasts Myc induces a p53-independent release of cytochrome *c* from mitochondria, thus facilitating apoptosis (27).

Bcl-2 family members play important roles in programmed cell deaths that occur when cells are deprived of survival factors (48). This is underscored by the high apoptotic index of Bcl-2- and Bcl-X_L-deficient hematopoietic progenitors and their accelerated rates of death following deprivation of cytokines (Fig. 2) (44). By contrast, the rates of apoptosis of primary hematopoietic cells lacking p53 or ARF are identical to those of wild-type progenitors when deprived of cytokines. Therefore the suppression of Bcl-2 or Bcl-X_L by Myc is more likely to mediate Myc's ability to override the protective effects of survival factors in hematopoietic cells.

Mechanism of Myc-induced Bcl-2 and/or Bcl-X_L suppression. The mechanism by which Myc induces Bcl-2 or Bcl-X_L suppression in primary cells is not resolved, yet is most likely transcriptional and indirect. In support of this notion, Myc activation in primary pre-B and myeloid cells results in rapid reductions in *bcl-X* RNA levels. This Myc-induced decrease in *bcl-X* RNA requires new protein synthesis, which suggests that Myc controls the expression of a regulator of *bcl-X*. Whether Myc's transactivation or transrepression functions are required for this response is not resolved. However, it is interesting that cycloheximide induces increases in *bcl-X* transcripts (Fig. 4B), suggesting that it may remove a labile repressor and that Myc's transrepression functions appear necessary for Myc-induced apoptosis (8). Thus, a model emerges whereby Myc transrepresses a gene or a set of genes that are necessary for maintaining *bcl-X* expression.

The underlying mechanism(s) by which Bcl-2 or Bcl-X_L or both are no longer suppressed by Myc but are rather overexpressed in the lymphomas arising in Eμ-*myc* transgenic mice is also not resolved but is not a result of gene amplifications or gross rearrangements of the genes by translocations or retrovirus insertions (data not shown). Inactivation of the c-Myb or Pim-1 oncogenes or the induction of the p53 or $p16^{Ink4a}$ tumor suppressors has been shown to down-regulate levels of *bcl-2* transcripts (15, 29, 35, 42, 60). We have thus far been unable to implicate any of these proteins in the suppression of Bcl-2 or Bcl-X_L by Myc. c-Myc has no effect on c-Myb or Pim-1 expression in myeloid cells (J. L. Cleveland, unpublished data), and the inactivation of p53 or the deletion of the *INK4a*/*ARF* locus

in lymphomas from Eμ-*myc* transgenic mice does not necessarily restore Bcl-2 or Bcl-X_L levels (Fig. 6). However, our data do support the concept that oncoproteins and tumor suppressors that regulate the apoptotic program do indeed target Bcl-2 or Bcl-X_L expression. Notably, overexpression of Bcl-2 cooperates with Myc in accelerating lymphomagenesis in Eμ-*bcl-2* Eμ-*myc* double-transgenic mice (57), and our studies now provide an explanation for this observation. Moreover, these findings may explain why oncogenes that induce Bcl-2, such as v- or c-Myb (15, 60), Pim-1 (35), Ras (30), and BCR-ABL (53), cooperate with Myc in transformation (reviewed in references 24 and 63).

Cooperation of Myc with the ARF-p53 pathway and Bcl-2 and/or Bcl-X_L in lymphomagenesis. Analyses of lymphomas arising in Eμ-*myc* transgenic mice indicate that Bcl-2 and/or Bcl-X_L overexpression and *p53* mutation or *ARF* loss are selected for independently during Myc-induced tumorigenesis. p53 or ARF inactivation occurs in over 70% of human cancers (18), while Bcl-2 or Bcl-X_L is overexpressed in many tumor types (51). Genetic studies of mice have demonstrated essential roles for p53 and ARF in inhibiting tumor development (5, 9, 28), whereas the evidence linking the Bcl-2 family of proteins to cancer is less obvious. Although Bcl-2 was cloned as the translocation product in human follicular lymphomas, Bcl-2 overexpression alone is poor at inducing tumors in transgenic mice (38, 58). Nonetheless, inactivating frameshift mutations in proapoptotic Bcl-2 family member Bax are found in adenocarcinomas of the colon (50) and in some human hematopoietic malignancies (39). In addition, Bcl-X_L expression is activated by retrovirus insertions in murine myeloid and T-cell leukemias (48).

Myc's induction of the ARF-Mdm2-p53 pathway or the suppression of Bcl-2 or Bcl-X_L expression alone results in apoptosis, but when combined both events ensure a complete and rapid cell death response. The ARF-Mdm2-p53 pathway mediates cell cycle arrest and apoptosis, whereas Bcl-2 and Bcl-X_L inhibit cell death. Thus, cells that have lost p53 or ARF function and that overexpress Bcl-2 and/or Bcl-X_L should resist growth arrest and apoptosis and continue to cycle under growth-limiting conditions, such as those that occur in the tumor microenvironment. Therefore, inactivating both pathways should provide cells with an even greater survival advantage. Forty-four percent of the lymphomas arising in Eμ-*myc* transgenic mice that are inactivated in the ARF-Mdm2-p53 pathway, 60% of tumors from $ARF^{+/-}$ Eμ-*myc* transgenic mice bearing deletions of the wild-type allele of *ARF*, and 80% of tumors from $ARF^{-/-}$ Eμ-*myc* transgenic mice overexpressed Bcl-2 and/or Bcl-X_L. Therefore disabling both Myc-induced pathways appears to provide a selective advantage to Myc-overexpressing B cells. Consistent with this notion, *p53* mutation and Bcl-X_L overexpression cooperate to favor the accumulation of cells with genetic damage (41).

Myc appears to independently target Bcl-2 and/or Bcl-X_L expression and the ARF-Mdm2-p53 pathway, yet the precise mechanisms by which Myc regulates these pathways are unresolved and remain important issues. It will also be interesting to further evaluate Myc-induced tumors that have disabled both pathways, as they should prove more resistant to treatment. Finally, these findings appear to be directly relevant to human cancers such as Burkitt's lymphoma, where *p53* mutations, *Ink4A/ARF* inactivation, and Mdm2 and Bcl-2 overexpression have all been observed (7, 14, 31, 46).

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