MCL-1–dependent leukemia cells are more sensitive to chemotherapy than BCL-2–dependent counterparts

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Myeloid cell leukemia sequence 1 (MCL-1) and B cell leukemia/lymphoma 2 (BCL-2) are anti-apoptotic proteins in the BCL-2 protein family often expressed in cancer. To compare the function of MCL-1 and BCL-2 in maintaining cancer survival, we constructed complementary mouse leukemia models based on *Eμ-Myc* expression in which either BCL-2 or MCL-1 are required for leukemia maintenance. We show that the principal anti-apoptotic mechanism of both BCL-2 and MCL-1 in these leukemias is to sequester pro-death BH3-only proteins rather than BAX and BAK. We find that the MCL-1–dependent leukemias are more sensitive to a wide range of chemotherapeutic agents acting by disparate mechanisms. In common across these varied treatments is that MCL-1 protein levels rapidly decrease in a proteosome-dependent fashion, whereas those of BCL-2 are stable. We demonstrate for the first time that two anti-apoptotic proteins can enable tumorigenesis equally well, but nonetheless differ in their influence on chemosensitivity.

Introduction

Cancer cells exhibit many properties that have been shown to cause apoptosis in normal cells. Among these is the activation of proliferative oncoproteins like MYC. Activation of MYC induces a transcriptional program that can result in increased proliferation, but often at the expense of cell death via the apoptotic pathway. Whether the net effect is increased proliferation or apoptosis appears to vary depending on cell context. Conditions which often occur in cancer, such as growth factor and/or oxygen deprivation, can sensitize MYC-expressing cells to apoptosis (Evan et al., 1992; Graeber et al., 1996; Brunelle et al., 2004). To survive in these conditions, the cells must find a way to prevent cell death. Cancer cells can select for ways to block the apoptotic signaling from MYC in order to tip the balance in favor of proliferation. A seminal observation of this principle was made when it was found that overexpression of the anti-apoptotic protein BCL-2 (B cell leukemia/lymphoma 2) could rescue cells from MYC-induced apoptosis (Bissonnette et al., 1992; Fanidi et al., 1992). However, the molecular mechanisms responsible for the apoptotic induction by MYC and its rescue by BCL-2 remain incompletely understood.

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Abbreviations used in this paper: BCL-2, B cell leukemia/lymphoma 2; BH, BCL-2 homology; MCL-1, myeloid cell leukemia sequence 1.

The BCL-2 family proteins control apoptosis at the mitochondrion. They are related by sequence homology and by participation in control of apoptosis (Danial and Korsmeyer, 2004). They contain one or more BCL-2 homology (BH) domains that are involved in protein–protein interactions. BCL-2 family proteins containing only a BH3 domain can be activated in response to critical aberrations in cellular physiology, including DNA damage, growth factor withdrawal, and oncogene activation (Puthalakath and Strasser, 2002). These BH3-only proteins are activated by mechanisms involving transcriptional up-regulation, subcellular localization, and/or posttranslational modification. In one model of apoptotic control, the so-called "direct" model, certain BH3-only proteins (including BID, BIM, and perhaps PUMA) are known as "activators." These proteins activate the pro-apoptotic BCL-2 family proteins BAX and BAK (Wei et al., 2000; Certo et al., 2006; Letai, 2008). Activated BAX and BAK then homo-oligomerize and cooperate in the permeabilization of the outer mitochondrial membrane. Pro-apoptotic contents of the mitochondrial intermembrane space, including cytochrome *c*,

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 187 No. 3 429–442
www.jcb.org/cgi/doi/10.1083/jcb.200904049

are released to the cytosol. These contents drive the activation of caspases, which are proteases that cleave and disable critical proteins throughout the cell. Anti-apoptotic proteins (BCL-2, MCL-1, BCL-XL, BFL-1, BCL-w) inhibit cell death by intercepting activator BH3-only proteins and preventing their interaction with BAX and BAK (Cheng et al., 1996; Certo et al., 2006). Anti-apoptotic proteins may also bind activated, monomeric BAX and BAK before they can oligomerize (Willis et al., 2005).

Other BH3-only proteins called sensitizers (including BAD, NOXA, and BIK) cannot activate BAX and BAK directly (Letai et al., 2002; Certo et al., 2006). Sensitizers promote death by displacing activators from the hydrophobic binding pocket of anti-apoptotic proteins. PUMA is a pro-apoptotic BH3-only protein first identified as a transcriptional target of p53 (Nakano and Vousden, 2001). Whether PUMA acts primarily as a sensitizer or an activator remains the subject of debate. An alternative model of apoptosis control, the so-called "indirect" model, holds that BAX and BAK are not activated by activator BH3-only proteins. A key principle of this model is that the anti-apoptotic function of proteins like BCL-2 resides solely in their binding to BAX and BAK (Willis et al., 2007).

Certain cancers depend upon BCL-2 for survival. Understanding the mechanism of this dependence not only provides information to guide therapeutic targeting of such dependence, but also provides important insights into the function of BCL-2 (Deng et al., 2007; Letai, 2008). We have previously studied BCL-2–dependent cancers including human chronic lymphocytic leukemia (CLL), lymphoma cell lines, small cell lung cancer cell lines, and a murine lymphocytic leukemia (Certo et al., 2006; Del Gaizo Moore et al., 2007; Deng et al., 2007). We have found that BCL-2 dependence can be identified in cancer cells using a strategy we call BH3 profiling (Certo et al., 2006; Letai, 2008). The main principle of BH3 profiling is to quantify mitochondrial dysfunction after treatment with a panel of BH3 peptides. This affords the opportunity to isolate the mitochondrial precondition as a contributor to subsequent death decisions. By treating with measured death signals, in the form of BH3 peptides, we can identify mitochondria that more readily undergo apoptosis than others. Because anti-apoptotic proteins each have a distinct pattern of interaction with the BH3 peptides, the pattern of response to individual BH3 domains can identify the anti-apoptotic protein required to maintain survival in a cell.

A common property of the BCL-2 protein in BCL-2–dependent cells is that it is largely bound to pro-apoptotic proteins, specifically activator BH3-only proteins like BIM (Del Gaizo Moore et al., 2007; Deng et al., 2007). We refer to cells in this state as "primed for death," or more simply "primed." These proteins can be displaced by competition with peptides derived from the BH3 domains of BH3-only proteins. Mitochondrial dysfunction ensues and its measurement is the readout for BH3 profiling. We have previously found that primed cells are more sensitive to chemotherapy than unprimed cells (Deng et al., 2007).

The anti-apoptotic MCL-1 (myeloid cell leukemia sequence 1) protein is gaining attention as a potential therapeutic target in cancer (Reynolds et al., 1994). Although MCL-1 has been implicated in both tumorigenesis and chemoresistance, there is little known about MCL-1 dependence in cancer. There is evidence that MCL-1 is important for survival of myeloma cells, but the mechanism behind this requirement is obscure (Derenne et al., 2002). An MCL-1 transgenic murine model exists that demonstrates an increased risk of lymphoma, but the long latency (>18 mo on average) renders such a model inconvenient for mechanistic studies (Zhou et al., 2001). In murine bone marrow viral infection models, MCL-1 was found to accelerate both myeloid and lymphoid malignancies (Wendel et al., 2007; Beverly and Varmus, 2009).

To better understand MCL-1 dependence in cancer, we compared MCL-1– and BCL-2–dependent leukemia models. We find that BH3 profiling confirms the dependence in these tumors, and that the molecular basis to the dependence involves binding of BH3-only proteins rather than BAX or BAK. Furthermore, we find that MCL-1–dependent leukemias are more sensitive to a wide variety of treatments than are their BCL-2–dependent counterparts.

Results

MCL-1 facilitates myc-mediated leukemogenesis

It has been established previously that targeting Bcl-2 expression in mice to lymphoid cells that also overexpress the c-myc oncogene results in rapid onset of a lymphoid leukemia (Strasser et al., 1990). This leukemia is less mature and more rapidly fatal than the lymphoma generated by the c-myc oncogene alone (Adams et al., 1985). By using a Bcl-2 promoter that could be turned off when doxycycline was added to the drinking water, we showed that the resulting leukemia was explicitly dependent on BCL-2 for survival (Letai et al., 2004). An important question remains whether other BCL-2 family anti-apoptotic transgenes could function similarly to facilitate lymphoid leukemogenesis driven by c-Myc in this transgenic model. To test whether MCL-1 overexpression results in a leukemia, the H2K promoter was used to target MCL-1 expression to many tissues, including blood, spleen, bone marrow, thymus, and lymph nodes. As expected, MCL-1 protein levels were significantly increased in spleen and bone marrow samples of H2K-Mcl-1 mice when compared with those of wild-type mice (Fig. 1 A). H2K-*Mcl-1* mice were crossed with *Eμ-Myc* mice and a cohort was analyzed, henceforth called *Mcl-1/Eμ-Myc* mice. *Mcl-1/Eμ-Myc* mice survived only an average of 72 ± 21 d. Mice bearing the *Eμ-Myc* transgene survived an average of 134 ± 35 d, whereas both mice bearing only the H2K-*Mcl-1* transgene and wild-type mice survived well beyond 200 d (Fig. 1 B).

Given the results from prior models noted above, we suspected that the early mortality in the *Mcl-1/Eμ-Myc* mice was due to the development of a lymphoid leukemia. White blood cell counts for 4–6-wk-old *Mcl-1/ Eμ-Myc* mice averaged 367,000 ± 156,000 per microliter of blood. Counts from *Eμ-Myc* only, H2K-*Mcl-1* only, and wild-type mice of the same age were significantly lower and nearly normal: 12,000 ± 2,000, 12,000 ± 3,000, and 7,900 ± 1,600 per microliter of blood, respectively (Fig. 1 C). White blood cells isolated from *Mcl-1/ Eμ-Myc* mice

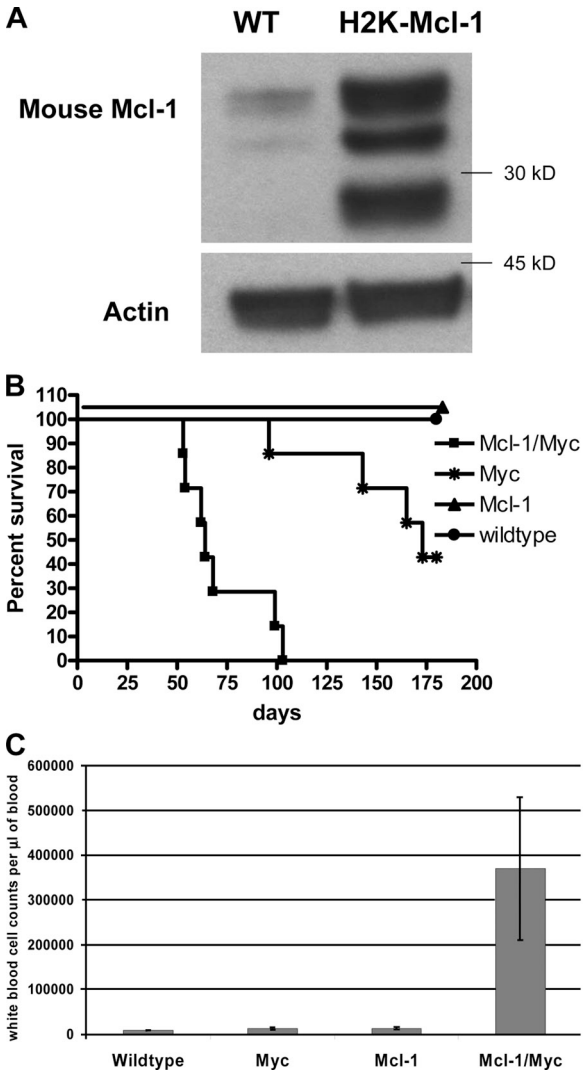

Figure 1. **MCL-1 overexpression targeted to hematopoietic cells by the H2K promoter in conjunction with c-myc results in the development of leukemia in mice.** (A) Levels of MCL-1 protein expression in wild-type and H2K-*Mcl-1* mouse spleenocytes. (B) Kaplan-Meier plot showing the survival in number of days for *Mcl-1/Eμ-Myc*, H2K-*Mcl-1* only, *Eμ-Myc* only, and wild-type mice. This is from a cohort of mice containing seven of each genotype. (C) Number of white blood cells per μl of blood from *Mcl-1/Eμ-Myc*, H2K-*Mcl-1* only, *Eμ-Myc* only, and wild-type mice. This is from a cohort of mice containing seven of each genotype.

were positive for B220 and CD19, negative for CD4, IgG, IgM, and IgD, and variable for AA4.1 and CD43 (unpublished data). This pattern confirms their B lymphocyte origin. Their cell surface marking corresponds to a pre- or pro-B cell, class C or D in the Hardy scheme (Hardy and Hayakawa, 2001), consistent with the majority of leukemias derived from previously reported combination of BCL-2 and c-Myc (Letai et al., 2004). The earliest white blood cell counts were taken after weaning and genotyping. Therefore, *Mcl-1/Eμ-Myc* mice show evidence of leukemia by 4 wk of age and may well have leukemia even earlier.

Spleen and bone marrow samples from 1–3-mo-old *Mcl-1/Eμ-Myc* mice contained a monotonous population of lymphoblasts crowding out the normal trilineage hematopoiesis, similar to the *BCL-2/Eμ-Myc* marrow samples (Fig. 2). Note that the bone marrow and spleen populations of comparably aged H2K-*Mcl-1*, *Eμ-Myc*, or wild-type mice are essentially normal. These results show that MCL-1 facilitates c-MYC–driven leukemogenesis in a manner clinically and morphologically indistinguishable from BCL-2. The bone marrow from leukemic mice is, however, quite distinct from that of *Eμ-Myc* mice.

BH3 profiling distinguishes MCL-1– and BCL-2–dependent leukemias

We have previously used BH3 profiling to detect dependence on individual anti-apoptotic family proteins in several systems (Certo et al., 2006; Del Gaizo Moore et al., 2007; Deng et al., 2007). An important test of this technique is whether it can distinguish between dependence on two different anti-apoptotic proteins in primary cancer cells. Our murine leukemia models provided an ideally controlled test case for performance of BH3 profiling. Briefly, mitochondria were isolated from the white blood cells of *Mcl-1/Eμ-Myc* mice. The mitochondria were treated with peptides corresponding to the BH3 domain of several BCL-2 family proteins: BIM, BID, BAD, BIK, NOXA A, NOXA B, HRK, BNIP3, PUMA, and BMF. After peptide treatment, release of cytochrome *c* was measured. The resulting profile showed high levels of cytochrome *c* release when treated with BIM, BID, NOXA, PUMA, and BMF peptides (Fig. 3 A). This matches fluorescence polarization data indicating that MCL-1 has high affinity for BH3 domains derived from BIM, BID, NOXA, PUMA, and BMF (Certo et al., 2006). This pattern thus corresponds to one of MCL-1 dependence. We also performed BH3 profiling on leukemia cells derived from the leukemia in *BCL-2/Eμ-Myc* mice. We had previously explicitly shown that these leukemias were BCL-2 dependent, as they vanished when BCL-2 was removed using the tet-off switch (Letai et al., 2004). The BH3 profile of *BCL-2/Eμ-Myc* white blood cells shows high levels of cytochrome *c* release when treated with BIM, BID, BAD, PUMA, BMF (Fig. 3 D). This pattern is consistent with mitochondrial BCL-2 dependence and consistent with the cellular BCL-2 dependence already established. Thus, using BH3 profiling we have verified that we have engineered two distinct models of murine leukemia, differing in that one model is dependent on BCL-2 and the other model is dependent on MCL-1. We detected no other difference between the two leukemia models. These results increase our confidence in the ability of BH3 profiling to make the subtle distinction between dependence on different anti-apoptotic proteins in primary cancer samples.

Although MCL-1– and BCL-2–dependent primary leukemia models were identified, it is convenient to perform replicate experiments with the same cell over time and primary leukemia cells are not useful reagents for such work. Thus, we established cell lines, three from each genotype that spontaneously immortalized in culture from these primary leukemias. The BH3 profiles of these cell lines correspond to the BH3 profile from the primary leukemias (examples in Fig. 3, B and E). Therefore, the cell lines were validated as cancer cell line models of MCL-1 and BCL-2 dependence.

The BH3 profiling in Fig. 3, A, B, D and E relies on the performance of a heavy membrane preparation that is enriched for mitochondria at the expense of lighter membranes like

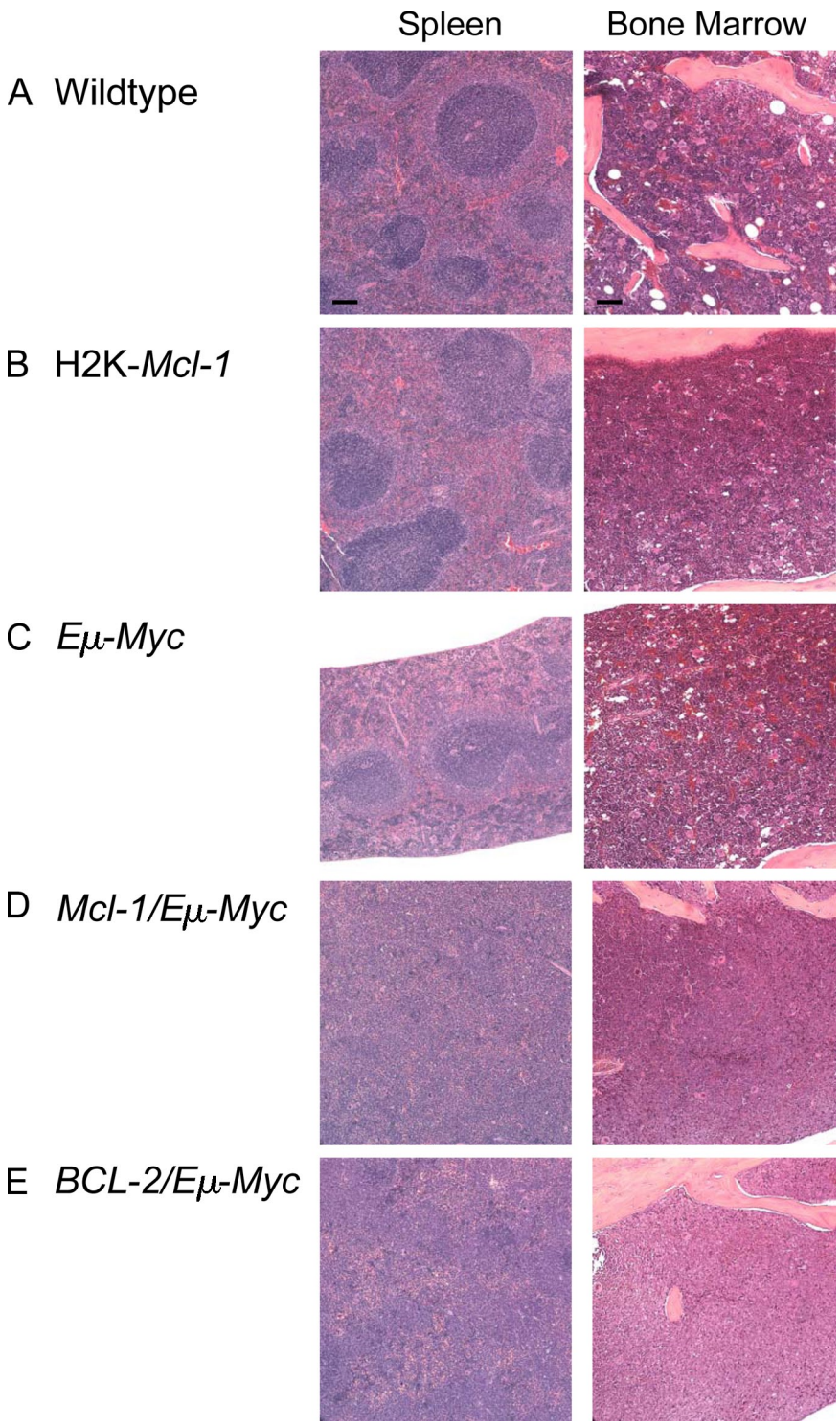

Figure 2. **Pathology reveals that the normal architecture of spleen and bone marrow is replaced by a monotonous population of lymphoblasts in *Mcl-1/Eμ-Myc* mice, similar to *BCL-2/Eμ-Myc* mice.** (A) Wildtype. (B) H2K-*Mcl-1* only. (C) *Eμ-Myc* only. (D) Mcl-1/Eμ-Myc. (E) BCL-2/Eμ-Myc. Bar for the spleen samples is 100 μm, and bar for the bone marrow samples is 50 μm.

endoplasmic reticulum. However, these lighter membranes can also harbor MCL-1 and BCL-2, albeit usually at lower quantities. To better test the contribution of MCL-1 and BCL-2 at all membrane locations, we turned to a modification of BH3 profiling using whole cells. In this modification, cells were permeabilized with low concentrations of digitonin to permit peptide access to the cell interior, and mitochondrial permeabilization was measured with the JC1 dye. We have found across dozens of cell lines that there is a tight correlation between MOMP measured by cytochrome *c* release and by JC1 by this method (unpublished data). Dependence on MCL-1 and BCL-2 are again demonstrated. As expected, the Bad peptide caused a rapid decrease in mitochondrial membrane potential in the putatively Bcl-2–dependent cells (Fig. 3 F), whereas the Noxa-specific peptides (Noxa A and Bim 2A) caused a decrease in mitochondrial membrane potential in the putatively Mcl-1–dependent cells (Fig. 3 C). The results using whole cells confirm the initial BH3 profiling results obtained

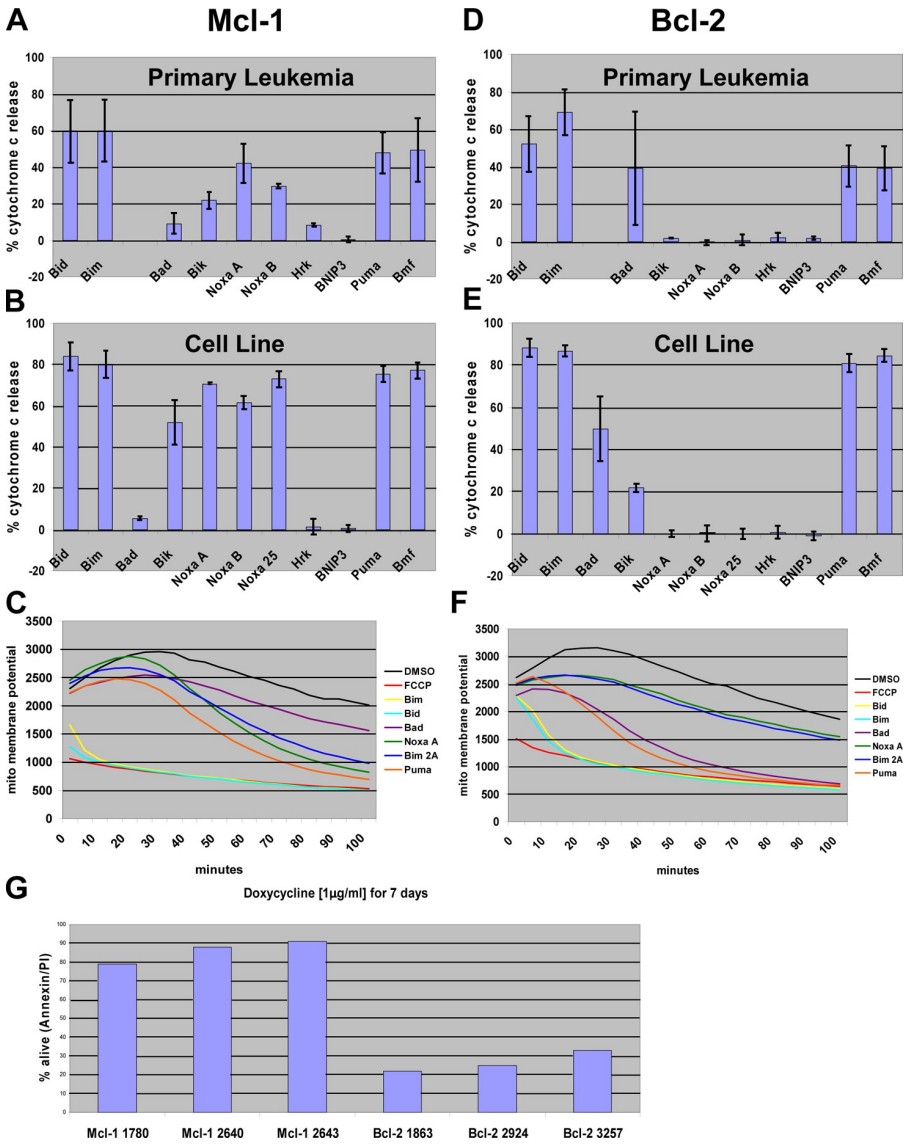

Figure 3. **BH3 profiling distinguishes BCL-2– and MCL-1–dependent leukemias.** Each profile is an average and standard deviation of three independent experiments, except for the JC1 whole-cell assay. The JC1 whole-cell assay is one experiment done in triplicate, with the average of the three repeats presented here. (A) BH3 profile of *Mcl-1/Eμ-Myc* primary leukemia cells. (B) BH3 profile of *Mcl-1/Eμ-Myc* cell line. (C) BH3 profile of *Mcl-1/Eμ-Myc* cell line using the JC1 whole-cell assay. (D) BH3 profile of *BCL-2/ Eμ-Myc* primary leukemia cells. (E) BH3 profile of *BCL-2/Eμ-Myc* cell line. (F) BH3 profile of *BCL-2/Eμ-Myc* cell line using the JC1 whole-cell assay. (G) Cell lines were treated with doxycycline for 7 d and survival assessed as the Annexin V negative population, measured by FACS. Data presented are from a single experiment; the experiment was repeated twice.

from isolated mitochondria. Results from all six cell lines can be seen in Fig. S1.

To further ensure that key apoptotic phenotypes were preserved, we treated the cell lines with doxycycline, which turns off BCL-2 expression and induces killing of leukemia cells in vivo. We found that doxycycline efficiently induced killing of *BCL-2/Eμ-Myc* leukemia cell lines, but not *Mcl-1/Eμ-Myc* leukemia cell lines (Fig. 3 G). These cell lines provided us with a pair of models which differed only in whether they were dependent on BCL-2 or MCL-1. We are unaware of any other comparable pairing of primary or cancer cell line models contrasting defined BCL-2 and MCL-1 dependence. BH3 profiling demonstrated a remarkable ability to distinguish these two models.

MCL-1 and BCL-2 are primed with pro-death BIM and PUMA

Anti-apoptotic proteins inhibit apoptosis by binding and sequestering pro-apoptotic BH3-only proteins and possibly monomeric BAX or BAK, especially when BAX or BAK have been activated. If cells are dependent on MCL-1 or BCL-2, it is likely because the MCL-1 and BCL-2 are sequestering pro-death molecules that would induce death if MCL-1 or BCL-2 function were lost or abrogated. In this situation, we describe the pro-death proteins as "priming" MCL-1 or BCL-2, and cells or mitochondria or anti-apoptotic proteins in that situation to be "primed" (Certo et al., 2006; Deng et al., 2007). To determine what pro-death molecules are priming MCL-1 in *Mcl-1/Eμ-Myc* leukemia, coimmunoprecipitation was performed from CHAPS lysates of white blood cells from two primary samples and one cell line. MCL-1 did indeed coimmunoprecipitate with BIM and PUMA, but not with BID, BAX, or BAK (Fig. 4 A). Similar to the *Mcl-1/Eμ-Myc* leukemia and to prior results (Letai et al., 2004), *BCL-2/Eμ-Myc* white blood cell lysates show that BCL-2 coimmunoprecipitated with BIM and PUMA, but not BID, BAX, or BAK in two primary samples and one cell line (Fig. 4 B). In summary, it appears that anti-apoptotic proteins BCL-2 and MCL-1 are primed with BH3-only proteins BIM and PUMA. In contrast, priming by BAX and BAK is not detectable. Others have found that BAK and MCL-1 interact, and we and others have found that BAX and BCL-2 interact in

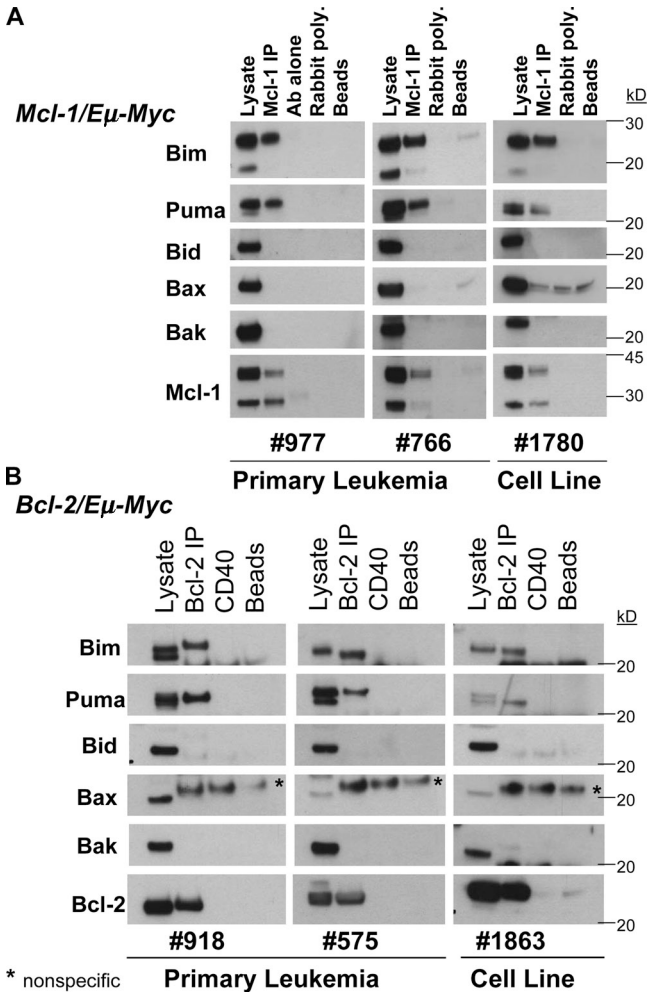

Figure 4. **MCL-1 and BCL-2 are primed with BIM and PUMA in leukemia cells.** (A) Coimmunoprecipiation of MCL-1 from CHAPS lysate of white blood cell primary sample or cell line from *Mcl-1/Eμ-Myc* mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and MCL-1. (B) Coimmunoprecipiation of BCL-2 from CHAPS lysate of white blood cell primary sample or cell line from *BCL-2/Eμ-Myc* mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and BCL-2.

other cell lines (Willis et al., 2005; Deng et al., 2007). Our results here do not rule out such interactions, but instead point out that many of the interactions between pro- and anti-apoptotic family proteins will be very dependent on the context, and specifically dependent on the abundance of other BCL-2 family proteins that might be competing as heterodimerization partners.

Heterogeneous dependence on BCL-2 and MCL-1 in lymphomas from Eμ-Myc mice

Forced transgenic expression of BCL-2 and MCL-1 apparently buffers death signaling in a way that fosters the development of a MYC-driven leukemia that is more aggressive than the lymphoma that appears in *Eμ-Myc* mice later in life. Yet even the less aggressive lymphomas in the *Eμ-Myc mice* likely have death signals to buffer. It is unknown how *Eμ-Myc* lymphomas block apoptosis. We asked whether the lymphomas in *Eμ-Myc* mice also showed dependence on anti-apoptotic proteins, or whether they selected an alternative type of block in apoptosis (Deng et al., 2007). To answer this question, we turned again to BH3 profiling. This technique shows that dependence on anti-apoptotic proteins in these lymphomas varies from case to case. The lymphoma from mouse #859 demonstrates MCL-1 dependence (Fig. 5 A). Combinations of BCL-2, BCL-XL, and/or MCL-1 dependence were discovered in the lymphomas of three other mice tested (Fig. 5, B–D). To test whether patterns of protein expression bore a discernable relationship to the dependence observed in BH3 profiling, we examined immunoblots of whole-cell lysates of the lymphomas (Fig. 5 E). Consistent with the MCL-1 dependence seen by BH3 profiling, MCL-1 protein levels are high and BCL-2 protein levels are relatively lower in tumor #859 (Fig. 5 A). In the remaining lymphoma cells with a more mixed picture of dependence by BH3 profiling, expression of BCL-2, MCL-1, and BCL-XL appeared more mixed. In the absence of a transgene driving overexpression of a particular anti-apoptotic protein, c-MYC–driven lymphomas demonstrate a heterogeneous pattern of dependence on anti-apoptotic proteins. It is a formal possibility that some of this heterogeneity might derive from oligo- or poly-clonality in the tumors. However, although we did not directly demonstrate clonality for the tumors studied here, others have shown that tumors from this model are almost always monoclonal (Adams et al., 1985).

Western blotting was also used to compare Bcl-2 family protein levels among *BCL-2/Eμ-Myc*–, *Mcl-1/Eμ-Myc*–, and *Eμ-Myc*–only tumors (Fig. 6 A). Notably, BIM and PUMA levels were much higher in the leukemias in which BCL-2 or MCL-1 was overexpressed. This suggests the possibility that the anti-apoptotic buffering capacity permitted a more aggressive phenotype by fostering tolerance of greater level of pro-death signaling. The low levels of BIM and PUMA in the *Eμ-Myc*–only tumors also supports the conjecture that there may be proteins that activate BAX and BAK and are sequestered by MCL-1 and BCL-2 other than BIM and PUMA. Levels of BAX and BAK remained similar among all the samples, whereas levels of the sensitizer BAD greatly varied among the samples. Relative levels of Phospho-Bad 112 mirrored total Bad levels (Fig. 6 B). There was very low signal detected using Phospho-Bad 136 (not depicted).

Leukemias that rely on MCL-1 for survival are more chemosensitive than those that rely on BCL-2

Although biochemical details are important in understanding leukemogenesis, it is of paramount practical clinical interest to understand how these changes affect response to chemotherapy. Although both MCL-1 and BCL-2 can cooperate with c-MYC to form leukemias, it has not been tested whether cellular exploitation of one over the other might confer greater chemosensitivity. To put this question another way, with all other things being equal, do leukemia cells that depend on MCL-1 for survival have a different sensitivity to conventional chemotherapy agents than those that depend on BCL-2 for survival? To answer this question, we compared the sensitivity of our *Mcl-1/Eμ-Myc* leukemia cell lines with those of our *BCL-2/Eμ-Myc* cell lines to a range of chemotherapeutic agents that work via diverse mechanisms (Fig. 7). The agents used included etoposide (topoisomerase II inhibitor), vincristine (microtubule disruptor), staurosporine (broad spectrum

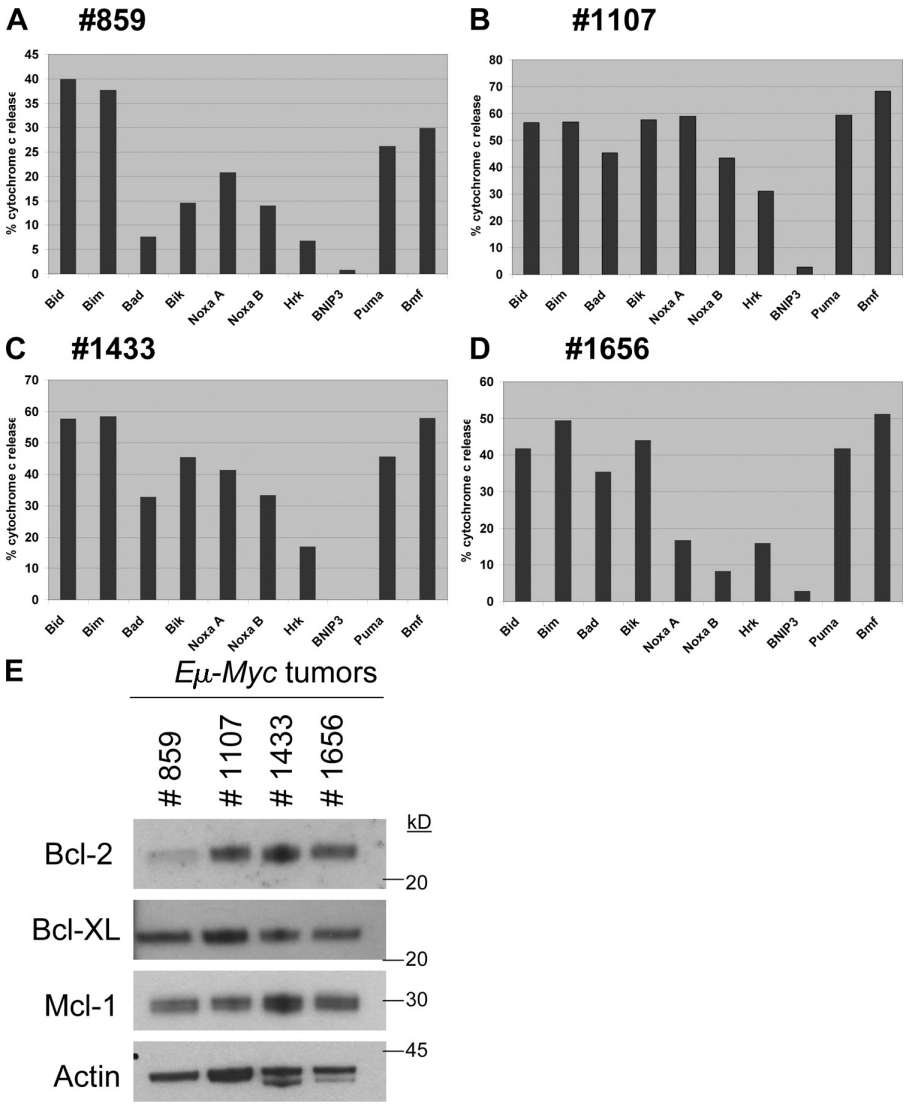

Figure 5. **BH3 profiling of *Eμ-Myc* tumors reveals variable dependence on anti-apoptotic proteins.** Data presented are from one single experiment. (A) *Eμ-Myc* tumor #859 shows a MCL-1 profile. (B) *Eμ-Myc* tumor #1107 shows a profile where MCL-1 > BCL-2/BCL-XL. (C) *Eμ-Myc* tumor #1433 shows a profile where MCL-1 > BCL-2/BCL-XL. (D) *Eμ-Myc* tumor #1656 shows a profile where BCL-2/BCL-XL > MCL-1. (E) Western blot containing CHAPS lysates of the *Eμ-Myc* tumors in order to compare protein levels of anti-apoptotic proteins BCL-2, BCL-XL, and MCL-1. Note low BCL-2 protein level in *Eμ-Myc* tumor #859.

kinase inhibitor), flavopiridol (CDK9 inhibitor), fludarabine (purine analogue), MNNG (DNA alkylating agent), and bortezomib and MG132 (proteosome inhibitors). We found that generally, *Mcl-1/Eμ-Myc* leukemias were more sensitive to drug. Comparison of treatment with MNNG, vincristine, staurosporine, and etoposide all demonstrated differences between the MCL-1– and BCL-2–dependent leukemias with P value <0.05. The other treatments showed differences that were close, but slightly above this level of statistical significance. These results suggested that when c-MYC leukemogenesis is facilitated by MCL-1, a more chemosensitive leukemia results than when it is facilitated by BCL-2.

MCL-1 levels decrease before commitment to death

Our BH3 profiling results showed that the leukemias are dependent on continuous function of either MCL-1 or BCL-2 for survival. MCL-1 protein is known to have a much shorter half-life than BCL-2 (<1 h compared with 16–24 h) (Maurer et al., 2006). We therefore hypothesized that the *Mcl-1/Eμ-Myc* leukemia lines might be more sensitive due to loss of MCL-1 protein.

We examined MCL-1 levels after treatment with vincristine, etoposide, and flavopiridol and found that in each case MCL-1 levels were dramatically reduced over the first 24 h (Fig. 8, A–C), the time over which almost all cells were killed. In addition, it can be seen that the quicker MCL-1 levels were reduced, the more rapidly cells died. For instance, in cells treated with flavopiridol, MCL-1 levels were nearly undetectable at 8 h (Fig. 8 A) and nearly all cells were killed by 8 h (Fig. 9 B). Etoposide was next-most efficient at reducing MCL-1 levels, followed by vincristine, again corresponding to how rapidly the cells were killed (Fig. 8, B and C; Fig. 9 B). Note that although the MCL-1 decrease for etoposide-treated 2640 cells appears modest, it was consistent, as demonstrated by densitometric analysis of four independent experiments. MCL-1 levels (average [standard deviation]) at 8, 16, and 24 h were 95% [18%], 76% [25%], and 72% [33%] of starting levels. In contrast, treatment with these same three agents had little effect on BCL-2 levels in the BCL-2–dependent cell lines, with concomitantly slower killing kinetics and reduced toxicity to the drugs (Fig. 8, D–G; Fig. 9 C). It is important to note that although Mcl-1 levels are driven by the H2K promoter in the *Mcl-1/Eμ-Myc* mouse

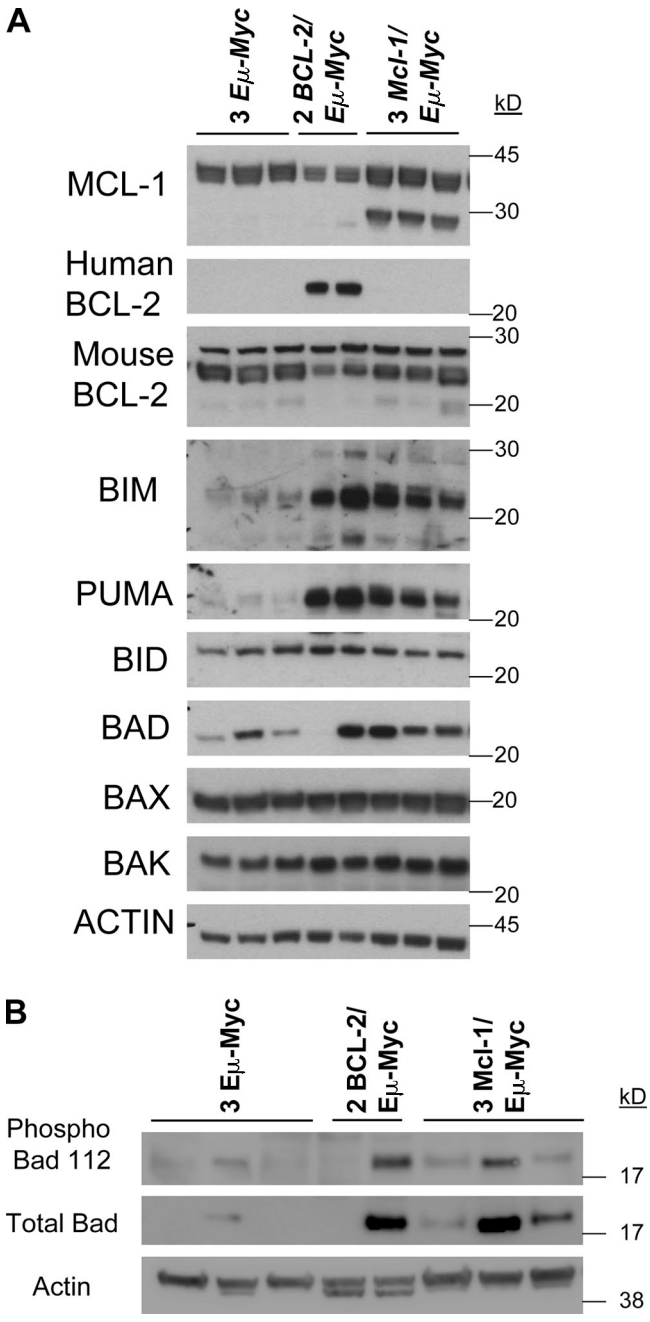

Figure 6. **Higher BIM and PUMA expression observed in MCL-1– and BCL-2–overexpressing leukemias.** Western blot comparing protein levels among three *Eμ-Myc* tumors, two *BCL-2/Eμ-Myc* leukemias, and three *Mcl-1/Eμ-Myc* leukemias. (A) The proteins blotted were MCL-1, BCL-2, BIM, PUMA, BID, BAD, BAX, and BAK. Please note that the *BCL-2* transgene in the *BCL-2/EμMyc* lymphomas is human. (B) The proteins blotted were BAD, Phospho-Bad (Ser112), and Actin.

and MCL-1–dependent cell lines, endogenous levels of MCL-1 are found in the *BCL-2/Eμ-Myc* mouse and BCL-2–dependent cell lines. MCL-1 levels do decrease in the Bcl-2–dependent cells when treated with etoposide, vincristine, or flavopiridol (Fig. 8, E–G), suggesting that the changes in MCL-1 levels are not dependent on the promoter from which protein expression derives.

After death, one might expect the levels of many short half-life proteins to decrease, potentially including MCL-1. Thus, it is important to distinguish whether MCL-1 loss occurs upstream or downstream of cellular death. To that end, it is important to note that the experiments in Fig. 8 were performed in the presence of the broad spectrum caspase inhibitor Q-VD-OPH, which was used at concentrations sufficient to inhibit manifestations of death including membrane permeabilization as measured by propidium iodide staining and phosphatidylserine exposure as measured by Annexin V staining (Fig. 9 A). Thus, MCL-1 loss is seen in the absence of overt manifestations of cell death, suggesting that MCL-1 loss is a cause, rather than a consequence, of cell death in these experiments.

MCL-1 protein half-life remains constant after treatment

Like all proteins, MCL-1 levels depend on an equilibrium between production and degradation. A decrease in MCL-1 can thus be due either to an increase in degradation or a decrease in production, or both. It has been shown that MCL-1 is subject to proteosomal degradation, both tonically and in response to numerous cellular perturbations (Nijhawan et al., 2003; Maurer et al., 2006). This can be seen here in the increase in MCL-1 levels caused by treatment with the proteosome inhibitor bortezomib (Fig. 8 A). Loss of MCL-1 after chemotherapy treatment could therefore be due to an induced increase in proteosomal degradation. To test whether degradation was increasing, we compared MCL-1 half-life in cells treated with cycloheximide in the presence and absence of flavopiridol, the drug that most rapidly caused MCL-1 loss. Fig. 10, A and B shows that the half-life is unaffected by flavopiridol, indicating that an increase in degradation is unlikely to play an important role in MCL-1 loss. This suggests that the loss of MCL-1 after chemotherapy treatment in these leukemias is not due to an increase in its rate of degradation, but rather due to a decrease in its production caused by the toxic agents.

It has been found that NOXA can bind to MCL-1 and increase the rate of proteosomal degradation of MCL-1 (Czabotar et al., 2007). Although our results here suggest that the rate of MCL-1 degradation is not increasing, we nonetheless tested whether changes in NOXA levels correlate to changes in MCL-1 levels. We were unable to identify any antibody to recognize murine NOXA, so we used RT-PCR to quantitate NOXA message levels. We found that although NOXA transcript levels increased in response to bortezomib, etoposide, and vincristine, they decreased in response to flavopiridol (Fig. S2), where the most rapid MCL-1 decrease was seen. Thus, although we cannot rule out a role for NOXA, an increase in NOXA transcript levels are not a requirement for MCL-1 loss.

MCL-1 and BCL-2 have similar a priori anti-apoptotic potency in the leukemias

The *Mcl-1/ Eμ-Myc* and *BCL-2/ Eμ-Myc* leukemias were constructed by the same basic method, and leukemias of identical morphology, cell surface phenotype, and clinical behavior were obtained. However, as the proteins were untagged, we could not directly compare absolute levels of BCL-2 and MCL-1. We could, however, test their relative "potency." That is, we could test whether *Mcl-1/Eμ-Myc* cells contained mitochondria that

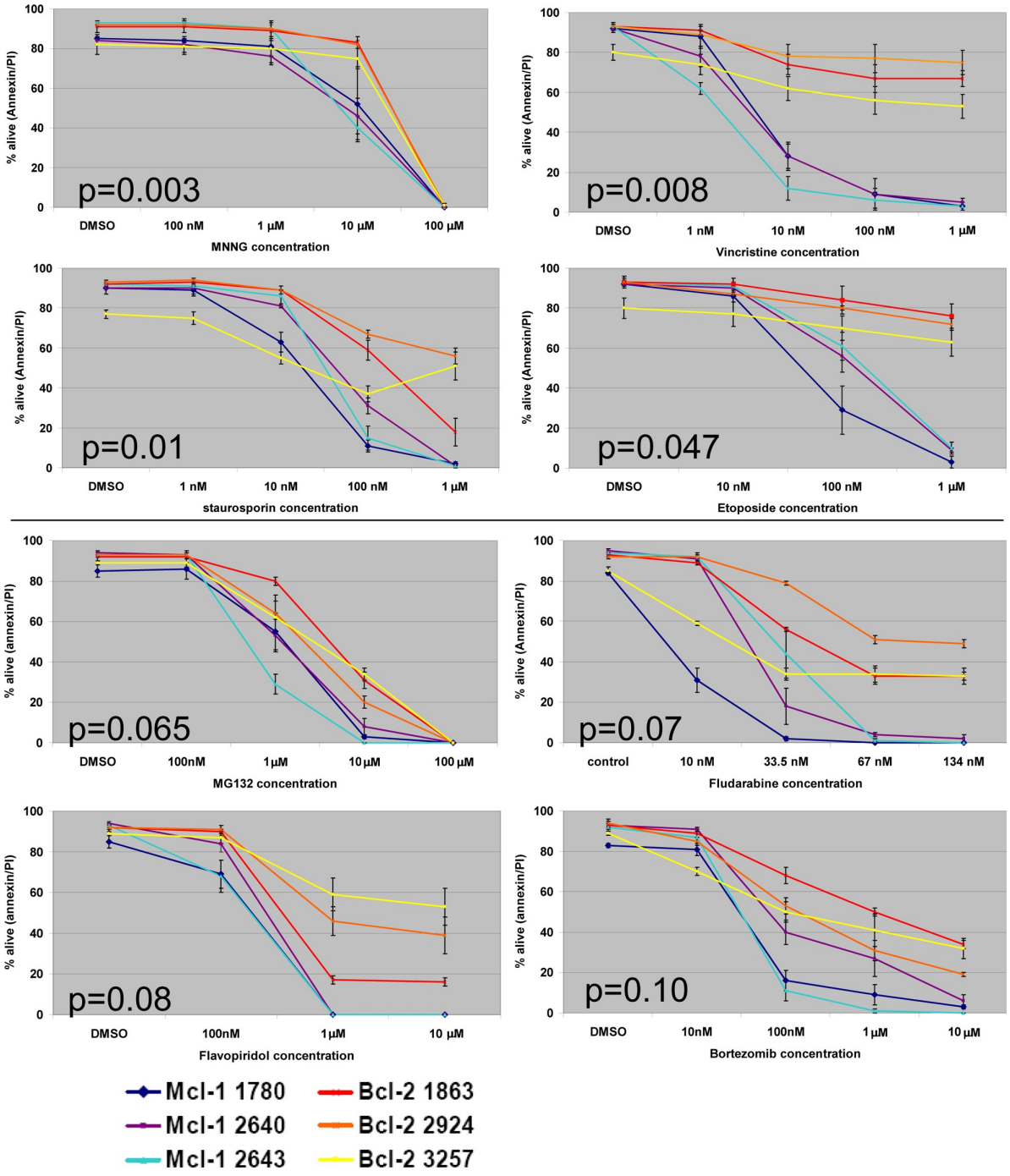

Figure 7. **MCL-1–dependent leukemia lines are more sensitive to chemotherapy drugs than BCL-2–dependent leukemia lines.** Dose response curves use the following drug treatments: MNNG, Vincristine, Staurosporin, Etoposide, MG132, Fludarabine, Flavopiridol, and Bortezomib. After 24 h of drug treatment, the cells were stained with Annexin/PI and the percentage of viable cells was graphed. Each time point is an average and standard deviation of three independent experiments. For the statistics, the three Mcl-1 cell lines and the three Bcl-2 cell lines were averaged and area under the curve was determined. P value was analyzed by using a paired *t* test of area under the curve.

were intrinsically more sensitive to apoptotic stimuli, even before perturbation with toxic agents. To test whether this was the case, we isolated mitochondria from *Mcl-1/Eμ-Myc* and *BCL-2/Eμ-Myc* leukemia cell lines, and compared their response to the PUMA BH3 peptide. The PUMA BH3 peptide behaves as a sensitizer and interacts with all of the anti-apoptotic proteins (Certo et al., 2006). Thus, it can be used alone as an index of how primed for death a mitochondrion is. This test provides a functional comparison that interrogates simultaneously the possible differential contribution of many BCL-2 family proteins in the two leukemias. In Fig. 10 C, we show that there is no significant difference in the priming of *Mcl-1/Eμ-Myc* and *BCL-2/Eμ-Myc* at the heavy membranes. In Fig. 10 D, we show a similar result using the whole-cell BH3 profiling technique. This implies that before treatment, the intrinsic sensitivity of the *Mcl-1/Eμ-Myc* and *BCL-2/Eμ-Myc* mitochondria to apoptotic

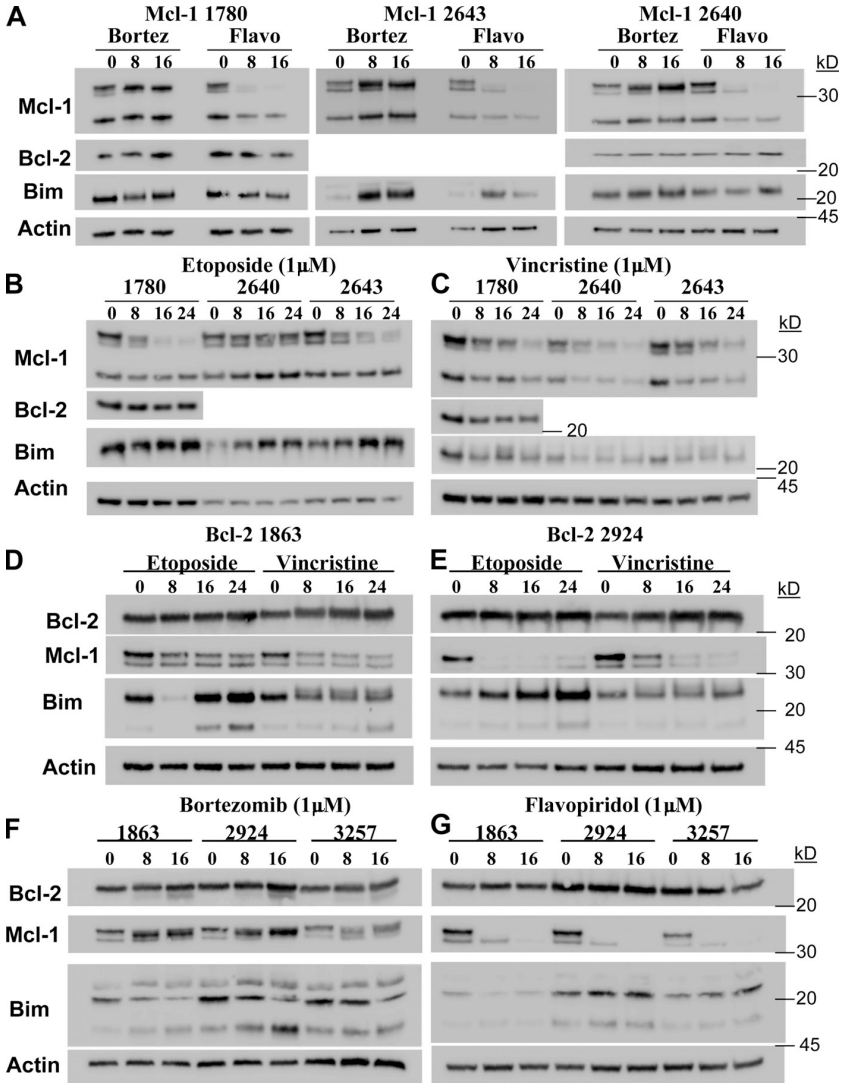

Figure 8. **Varied chemotherapeutics induce MCL-1 before cell death.** All cell lines pretreated with 20 μM of caspase inhibitor Q-VD-OPH for 1 h. CHAPS lysates were prepared and MCL-1, BCL-2, BIM, and Actin protein levels were determined using Western blotting. (A) *Mcl-1/Eμ-Myc* cell lines treated with 1 μM Bortezomib or 1 μM Flavopiridol for 0, 8, and 16 h. (B) *Mcl-1/Eμ-Myc* cell lines treated with 1 μM Etoposide for 0, 8, 16, and 24 h. (C) *Mcl-1/Eμ-Myc* cell lines treated with 1 μM Vincristine for 0, 8, 16, and 24 h. (D and E) *BCL-2/Eμ-Myc* cell lines treated with 1 μM Etoposide or 1 μM Vincristine for 0, 8, 16, and 24 h. (F) *BCL-2/Eμ-Myc* cell lines treated with 1 μM Bortezomib for 0, 8, and 16 h. (G) *BCL-2/Eμ-Myc* cell lines treated with 1 μM Flavopiridol for 0, 8, and 16 h.

signaling is the same. This is consistent with the hypothesis that net loss of MCL-1 after drug treatment is responsible for the selective sensitivity of the MCL-1–dependent leukemias.

Our data suggest that Mcl-1–dependent cells are killed easier than those that are Bcl-2 dependent, all other things being equal. However, the ARF/Mdm2/p53 pathway can also play an important role in apoptosis, and it has previously been found that selection of alterations of this pathway can be altered by anti-apoptotic protein expression in a myc-driven lymphoma model (Schmitt et al., 2002). We measured expression of ARF and p53 to test whether MCL-1 expression systematically resulted in a different proportion of p53- or ARF-overexpressing tumors compared with BCL-2 expression. In Fig. S3, we show that MCL-1 and BCL-2 expression result in similar proportions of p53 and ARF up-regulation (6/12 vs. 7/12, 5/12 vs. 5/12, respectively). Note that p53 up-regulation, indicating a defect in the ARF/Mdm2/p53 tumor suppressor pathway, was found in 10 of 11 *Eμ-Myc* samples tested, a higher proportion than was reported previously (Eischen et al., 1999). These results are consistent with prior results which found that forced expression of an anti-apoptotic protein decreased the rate of selection for abnormalities in the ARF/Mdm2/p53 pathway in a myc-driven lymphoma model (Schmitt et al., 2002).

In summary, we observed that MCL-1–dependent cancer cells were more sensitive to treatment with a range of chemotherapeutic agents than were matched BCL-2–dependent counterparts. The mechanism behind the difference appears to be that MCL-1 is a short half-life protein subjected to ongoing proteosome-dependent degradation. Although the rate of this degradation does not change after chemotherapy treatment, chemotherapy apparently disrupts efficient MCL-1 protein production, resulting in a rapid net loss of MCL-1 protein. BCL-2 has a longer half-life and seems to be spared chronic proteosomal degradation, so its levels are relatively stable after treatments, resulting in less chemosensitivity.

Discussion

Here we report a comparison of murine models of MCL-1– and BCL-2–dependent lymphoid leukemias. MCL-1 dependence has not been previously demonstrated in a murine cancer model. We now have a system of cell lines and murine leukemia models that are apparently identical except for their different dependence

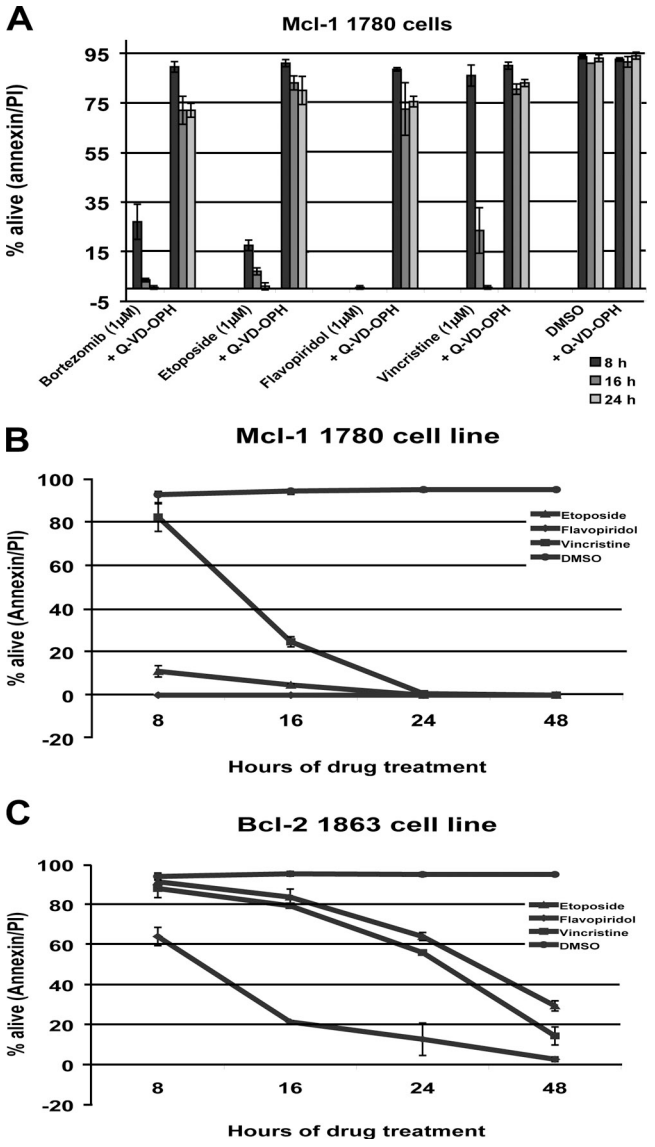

Figure 9. **The *Mcl-1/Eμ-Myc* cell line dies faster than the *BCL-2/Eμ-Myc* cell line.** (A) Annexin/PI measurement used to determine cell viability of *Mcl-1/Eμ-Myc* cell line using Etoposide, Flavopiridol, Vincristine, and DMSO drug treatments with and without 20 μM Q-VD-OPH. Data presented are an average and standard deviation of three independent experiments. (B) Dose-response curve of *Mcl-1/Eμ-Myc* cell line treated with 1 μM of Etoposide, Flavopiridol, or Vincristine over 8, 16, 24, and 48 h. Data presented are an average and standard deviation of three independent experiments. (C) Dose-response curve of BCL-2/Eμ-Myc cell line treated with 1 μM Etoposide, Flavopiridol, or Vincristine over 8, 16, 24, and 48 h. Data presented are an average and standard deviation of three independent experiments.

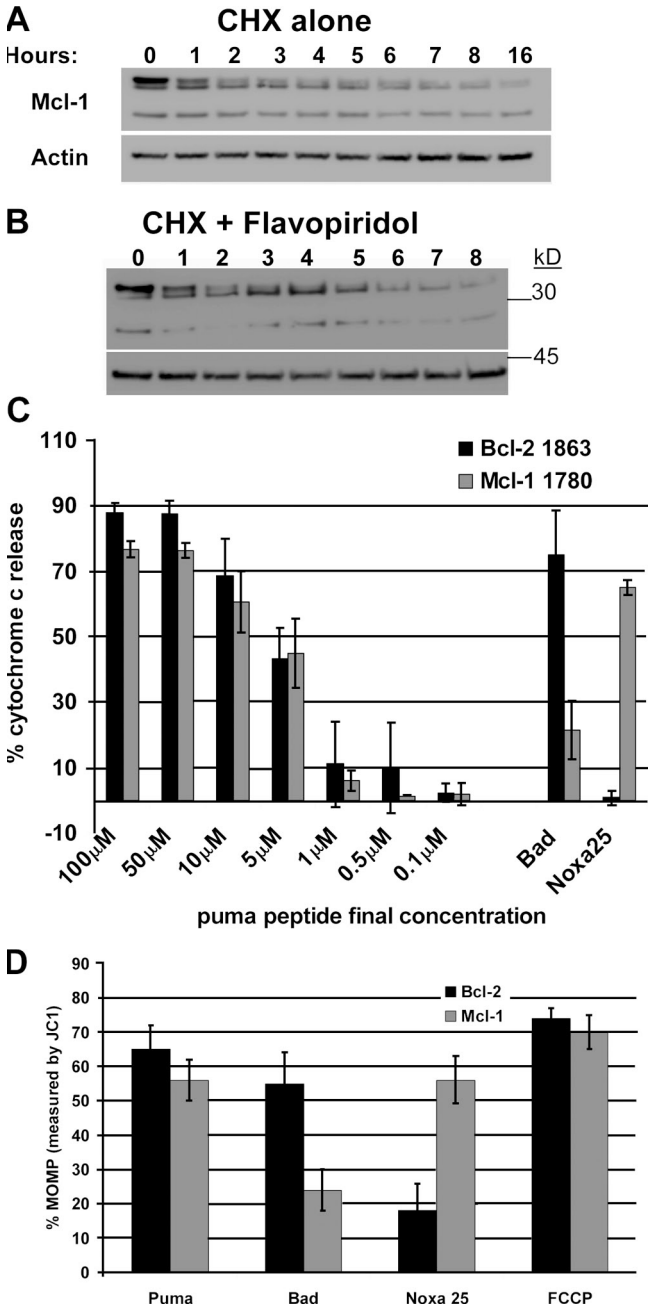

Figure 10. **MCL-1 half-life is unchanged by flavopiridol treatment; MCL-1– and BCL-2–dependent mitochondria demonstrate equivalent pretreatment "priming."** (A) A *Mcl-1/Eμ-Myc* cell line pretreated with 20 μM of caspase inhibitor Q-VD-OPH for 1 h, followed by treatment with 20 μM cycloheximide (CHX). CHAPS lysates were made after each hour of treatment. (B) A *Mcl-1/Eμ-Myc* cell line pretreated with 20 μM of caspase inhibitor Q-VD-OPH for 1 h, followed by a combined treatment with 20 μM cycloheximide (CHX) and 1 μM Flavopiridol. CHAPS lysates were made after each hour of treatment. (C) BH3 profiling of a *Mcl-1/Eμ-Myc* and *BCL-2/Eμ-Myc* cell line using decreasing concentrations of the PUMA peptide. BAD and NOXA peptides were included as controls. Three independent experiments were averaged. (D) BH3 profiling by the whole-cell JC1 method at the 60-min time point. The results of the three Bcl-2 cell lines and three Mcl-1 cell lines were averaged for the following peptides: Puma, Bad, Noxa25, and FCCP.

on either BCL-2 or MCL-1. Such a system has potential utility as in vivo and in vitro test systems for putative selective antagonists of either BCL-2 or MCL-1 that are currently in preclinical and clinical development for cancer therapy. In addition, as we have exploited it in this paper, such a system is useful in understanding the mechanisms by which cancer cells escape apoptosis, and in comparing the similarities and differences in biological function between BCL-2 and MCL-1.

We have previously reported testing BH3 profiling in model systems in which BH3 profiling accurately identified dependence on different individual anti-apoptotic proteins, but have not previously identified a purely MCL-1–dependent cancer. Here we find that BH3 profiling could usefully validate the

distinction between MCL-1 and BCL-2 dependence in leukemia models based on MCL-1 or BCL-2 overexpression in combination with the *Eμ-Myc* transgene. However, the *Eμ-Myc* transgene by itself drives formation of lymphomas, leading us to ask how these tumors escaped apoptosis. Although the *Eμ-Myc* lymphoma model has been studied for decades, the tools were not previously available to investigate its dependence on anti-apoptotic proteins (Adams et al., 1985). Using BH3 profiling, we find that in the absence of forced overexpression of any single anti-apoptotic protein, *Eμ-Myc* lymphomas are nonetheless dependent on anti-apoptotic proteins for survival. The specific anti-apoptotic protein dependence varies from lymphoma to lymphoma, and can be mixed in a single lymphoma. The source for the differences may lie in a simple stochastic selection for greater or lesser expression of one or the other of the anti-apoptotic proteins, as seems to be the case for lymphoma #859 (Fig. 5). Alternatively, the differences in expression may reside in inherent differences in differing initiating cells selected for tumorigenesis in each animal.

We also asked if the anti-apoptotic proteins in our leukemias are primed, what pro-death molecules are they primed with? We were unable to observe sequestration of BAX or BAK in our leukemias. Instead, we observed sequestration of the pro-death BH3-only proteins BIM and PUMA. This observation is more consistent with a model in which the most important anti-apoptotic function of anti-apoptotic proteins like BCL-2 or MCL-1 is to sequester BH3-only proteins (Cheng et al., 1996; Cheng et al., 2001; Letai et al., 2002; Kuwana et al., 2005; Certo et al., 2006). The pure indirect model would suggest that we should have observed sequestration of BAX and BAK. Although we observed sequestration of both BIM and PUMA, it is yet possible that in these leukemias BCL-2 and MCL-1 sequester other BH3-only proteins, perhaps some not yet even identified as BH3-only proteins. If indeed the sequestration of BIM or PUMA is critical to the function of MCL-1 or BCL-2, one would expect that loss of PUMA or BIM might also be able to cooperate with *Eμ-Myc* to drive development of the less mature leukemias. In fact, loss of PUMA or BIM combined with the *Eμ-Myc* transgene does cause development of the immature leukemia, supporting their sequestration as a key role performed by MCL-1 and BCL-2 (Egle et al., 2004; Michalak et al., 2009).

Despite the similarity in phenotype and in biochemical properties, the *BCL-2/Eμ-Myc* and *Mcl-1/Eμ-Myc* leukemias differed consistently in their sensitivity to cytotoxic agents. Our results suggest that this is because proteosomal degradation of MCL-1 persists while new synthesis of MCL-1 is reduced by treatment with a wide variety of agents. This changes the steady state so that there is a net loss of MCL-1, resulting in accelerated death of MCL-1–dependent cells. A similar observation has been made in the response of HeLa cells to ultraviolet radiation (Nijhawan et al., 2003). Here, we show that MCL-1 loss is a more general phenomenon following a wide variety of insults. Such a net loss does not occur for the longer half-life BCL-2 protein, which is more stable. Killing the BCL-2–dependent cells thus requires more time and more drug (Figs. 7 and 9). Note that our studies cannot address issues of which tumors are cured because this depends on issues like fractional kill and stem cell biology, still poorly understood for individual tumors. Yet it is important to understand chemosensitivity because this is a necessary, though not sufficient, condition for cure by chemotherapy.

Chemosensitivity of the *Eμ-myc* and *Eμ-myc/Eμ-BCL-2* leukemia/lymphoma models was compared in a recent manuscript (Mason et al., 2008). In contrast with our *BCL-2/Eμ-Myc* model, the expression of BIM in the *Eμ-myc/Eμ-BCL-2* cancers was similar to that in the *Eμ-myc* cancers. Consistent with our expectation that tumors from the Eμ-myc model would be more frequently MCL-1 dependent (see Fig. 5), MCL-1 levels were higher in these malignancies than in the *Eμ-myc/Eμ-BCL-2* malignancies. Both models were subjected to treatment with cyclophosphamide, an alkylating agent. Consistent with our findings that Eμ-myc tumors are frequently MCL-1 dependent, and the leukemias based on myc and *BCL-2* transgenes BCL-2 dependent, and that MCL-1–dependent tumors tend to respond better to chemotherapy, the *Eμ-myc* mice demonstrated vastly superior survival after therapy, with a 50% long-term survival compared with 0% in the *Eμ-myc/Eμ-BCL-2* mice. These results support an in vivo drug sensitivity consequence to MCL-1 dependence versus BCL-2 dependence in cancers.

It may well be that using BH3 profiling or other strategies, other tumors, perhaps even primary tumors, will be revealed as MCL-1 dependent. Indeed, there is already reason to believe that many myeloma cells may be MCL-1 dependent (Derenne et al., 2002). Superficially, our results may suggest that such cells will be readily killed by a wide variety of agents. More exactly stated, however, our prior results and those here suggest that primed cells that are dependent on any anti-apoptotic protein will be killed easier that unprimed, and that primed cells that are MCL-1 dependent will be killed easier that those that are BCL-2 dependent, all other factors being equal. Achieving comparisons where all other factors are equal, outside of purposefully constructed models, will indeed be difficult, but perhaps with sufficient numbers, informative testing of the hypothesis may be possible. It will be interesting to test this hypothesis in the coming years as relevant cancers are identified. However, we already know that our use of cytotoxic drugs is limited by toxicity, so that more directly targeted therapy may be superior. Thus, selective antagonists of MCL-1 may eventually play an important role in treating MCL-1–dependent cancers.

A key generalizable finding of this study is that the type of block in apoptosis selected by a cancer during tumorigenesis can significantly influence chemosensitivity. Though two different anti-apoptotic proteins might both enable leukemogenesis, the leukemias derived differ critically in their chemosensitivity. As shown here and previously, BH3 profiling can identify types of blocks in apoptosis, and thus may be useful in prediction of chemosensitivity.

Materials and methods

Transgenic mice

The MMTV-rtTA/tet-BCL-2/Eμ-Myc mouse has been described previously (Letai et al., 2004).

Mcl-1 transgenic mice

A minigene containing the H2K promoter/enhancer and the Moloney MuLV enhancer/poly(A) site (a gift from Dr. Jos Domen, Medical College

of Wisconsin, Milwaukee, WI) driving the expression of mouse Mcl-1 cDNA was injected into zygotes obtained from crosses between F1 (C57BL/6 3 C3H) mice. Resulting progeny were tested by Southern blotting, and positive mice were backcrossed to C57BL/6 mice. Four independent founder lines were generated with different expression levels as tested by Western blot. The transgenic mice were generated, housed, and bred in the Dana-Farber Cancer Institute (DFCI) animal facility. All animal experiments were approved by the Dana-Farber Cancer Institute IACUC (protocol #05-001).

Mice containing the H2K-*Mcl-1* transgene (usually female) were bred to *Eμ-Myc* mice (usually male).

Monitoring leukemia

Blood from a razor nick was diluted in buffer (saponin 0.3%, Hoechst 33258 1 μg/ml, EDTA 25 mM in PBS) and manual counts of white blood cells were performed.

Pathology

Spleen and bone marrow samples were stored in formalin and sent to the Dana Farber Harvard Cancer Center Pathology Core for processing with hematoxylin and eosin stain.

Microscopy and image analysis

The microscope used was a Nikon Eclipse E600. To view the bone marrow samples the objective lens was a Nikon Plan Fluor 20x/0.75 NA. To view the spleen samples the objective lens was a Nikon Plan Fluor 10x/0.30 NA. The camera was a SPOT RT SE from Diagnostic Instruments, Inc. (model 9.0 monochrome-6) with SPOT software for image acquisition.

Mouse cell lines

White blood cells were isolated from blood using Ficoll gradient. Spleens were removed from the mouse and smashed between two glass slides. Smashed spleens and white blood cells were placed in 50 mL of RPMI, 10% FBS, 1% PenStrep, and β-mercaptoethanol. Media was changed 2–3 times per wk. After approximately 3 wk, cell mixture was split 1:2. As population of dividing cells emerged, cells were split 1:5 as necessary.

BH3 profiling using heavy membranes

Mitochondria were isolated from the white blood cells of *Mcl-1/Eμ-Myc* or *BCL-2/Eμ-Myc* mice, *Eμ-Myc* lymphomas, or cell lines. In brief, cells were placed in isolation buffer (250 mM sucrose, 10 mM Tris-HCL, pH 7.4, and 0.1 mM EGTA) and cell disruption was performed with several passes through a 27-gauge needle. After the sample was centrifuged at 600 *g* for 10 min, the resulting supernatant was centrifuged at 10,000 *g* for 10 min. Mitochondrial concentration of 0.3–0.5 mg/ml was mixed with peptides corresponding to the BH3 domain of BCL-2 family proteins in experimental buffer (125 mM KCl, 10 mM Tris-MOPS, pH 7.4, 5 mM glutamate, 2.5 mM malate, 1 mM KPO_4, and 10 μM EGTA-Tris, pH 7.4). Protocol details, including peptide sequence, have been previously published (Letai et al., 2002). The peptide stocks are diluted in DMSO. The peptide sequences are as follows: BID: EDIIRNIARHLAQVGDSMDR; BIM: MRPEIWIAQELRRIGDEFNA; BAD: NLWAAQRYGRELRRMSDEFVDSFKK; BIK: MEGSDALALRLACIGDEMDV; NOXA A: AELPPEFAAQLRKIGDKVYC; NOXA B: PADLKDECAQLRRIGDKVNL; NOXA 25: AELEVECATQLRRFGDKLNFRQKLL; Bim2A: DMRPEIWIAQEARRIGDEANAYYARR (Lee et al., 2008); HRK: SSAAQLTAARLKALGDELHQ; BNIP3: VVEGEKEVEALKKSADWVSD; PUMA: EQWAREIGAQLRRMADDLNA; BMF: HQAEVQIARKLQLIADQFHR.

After peptide treatment (~40 min), release of cytochrome *c* in the supernatant and mitochondrial pellet is measured via ELISA (R&D Systems).

BH3 profile using whole cells (JC1)

Cell densities range 5×10^4 cells per well were used. 15 μL of 200 μM peptides in T-EB (300 mM Trehalose, 10 mM Hepes-KOH pH 7.7, 80 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% BSA, and 5 mM succinate) (Yamaguchi et al., 2007) were deposited per well in a nontreated black 384-well plate, 1 treatment per well. Single cell suspensions were washed once in T-EB before being resuspended at 4x their final density. One volume of the 4x cell suspension was added to one volume of a 4x dye solution containing 4 μM JC-1, 40 μg/ml oligomycin, 0.02% digitonin, and 20 mM 2-mercaptoethanol in T-EB. The resulting 2x cell/dye solution was allowed to rest at ambient temperature for 5–10 min to allow permeabilization and dye equilibration. 15 μL of the 2x cell/dye mix was then added to each treatment well of the 384-well plate, shaken for 15 s inside the plate reader, and the fluorescence at 590 nM monitored every 5 min at ambient temperature.

Westerns and coIPs

MCL-1 IP. White blood cells isolated from *Mcl-1/Eμ-Myc* mice were put in 1% CHAPS lysis buffer. MCL-1 antibody (3 μg; Rockland) was added to 50 μg of lysate and incubated on a rotator for at least 1 h at 4 degrees. Control antibody used for IPs was MnSOD (SOD-111; Stressgen). Protein A beads (Sigma-Aldrich) were subsequently added and the resulting mixture was incubated for another hour. Beads were washed three times with CHAPS lysis buffer and then loading dye was added. Samples were heated and then run on a 10% gel.

BCL-2 IP. White blood cells isolated from *BCL-2/Eμ-Myc* mice were put in 1% CHAPS lysis buffer. BCL-2 6C8 (551051; BD) antibody (3 μg) was added to 10 μg of lysate and incubated on a rotator for at least 1 h at 4 degrees. Control antibody used for IP's was CD40 (553721; BD). Protein A/G beads (Santa Cruz Biotechnology, Inc.) were subsequently added and the resulting mixture was incubated for another hour. Beads were washed three times with CHAPS lysis buffer and then loading dye was added. Samples were heated and then run on a 10% gel.

Antibodies used in Western blots were: Actin (Millipore), Mcl-1 (Rockland), Human Bcl-2/100 (BD), Mouse Bcl-2 3F11 (BD), Bcl-XL (provided by Larry Boise, Emory University, Atlanta, GA), BIM 22–40 (EMD), BID (Santa Cruz Biotechnology, Inc.), BAD (Santa Cruz Biotechnology, Inc.), Phospho-Bad Ser112 (Cell Signaling Technology), BAX N20 (Santa Cruz Biotechnology, Inc.), BAK NT (Millipore), PUMA (Sigma-Aldrich), p53 (EMD), and p19ARF (Abcam). Secondary antibody used for coIPs was Protein A HRP linked (GE Healthcare). Secondary antibodies for all other blots were anti–mouse HRP linked (GE Healthcare), anti–rabbit HRP linked (GE Healthcare), anti–hamster HRP linked (Jackson ImmunoResearch Laboratories, Inc.), and anti–rat HRP linked (GE Healthcare).

Imaging was performed using Super Signal chemiluminescence developing agent (Thermo Fisher Scientific) either onto film or using an Image Reader (model LAS-4000; Fujifilm).

RT-PCR

RNA was isolated using Trizol (Invitrogen) and the Promega Access RT-PCR System was used to determine levels of Noxa and GAPDH. Noxa primers were forward: CGTCGGAACGCGCCAGTGAACCC and reverse: TCCTTCCTGGGAGGTCCCTTCTTGC. GAPDH primers were forward: TGATGACATCAAGAAGGTGGTGAAG and reverse: TCCTTGGAGGCCATGTAGGCCAT.

Drugs

Drugs are as follows: MNNG (Thermo Fisher Scientific), Vincristine (Sigma-Aldrich), Staurosporin (Sigma-Aldrich), Etoposide (Sigma-Aldrich), MG132 (Sigma-Aldrich), Fludarabine (Sigma-Aldrich), Flavopiridol (Alexis Biochemicals), and Bortezomib (LC Laboratories).

Online supplemental material

Fig. S1: the whole-cell method of BH3 profiling. Fig. S2: Noxa mRNA levels after drug treatment. Fig.S3: protein levels of p53 and ARF in *BCL-2/Eμ-Myc*, *Mcl-1/Eμ-Myc*, and *Eμ-Myc* samples. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904049/DC1.

We would like to thank Mark Anderson for helpful discussions and comments and Jill Fisher for assistance with murine husbandry.

The authors gratefully acknowledge financial support from National Institutes of Health grants P01 CA068484, U01 CA105423, and R01 CA129974. A. Letai is a Leukemia and Lymphoma Society Scholar. A. Letai is inventor on a patent application regarding BH3 profiling. A. Letai is a founder and head of the scientific advisory board for Eutropics Pharmaceuticals.

Submitted: 9 April 2009
Accepted: 7 October 2009

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