Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia

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Acute myeloid leukemia (AML) frequently relapses after initial treatment. Drug resistance in AML has been attributed to high levels of the anti-apoptotic Bcl-2 family members Bcl-x_L and Mcl-1. Here we report that removal of Mcl-1, but not loss or pharmacological blockade of Bcl-x_L, Bcl-2, or Bcl-w, caused the death of transformed AML and could cure disease in AML-afflicted mice. Enforced expression of selective inhibitors of prosurvival Bcl-2 family members revealed that Mcl-1 is critical for survival of human AML cells. Thus, targeting of Mcl-1 or regulators of its expression may be a useful strategy for the treatment of AML.

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Acute myeloid leukemia (AML) is a heterogeneous disease, with treatment outcome after intensive chemotherapy strongly influenced by cytogenetic and molecular characteristics of the cancer. Several recurrent AML oncogenic fusion lesions have been linked to a high risk of relapse after chemotherapy, including chromosomal translocations involving the *Mll* (mixed-lineage leukemia) gene (MLL-ENL and MLL-AF9, particularly found in infants or individuals that had previously been treated with topoisomerase inhibitors) (Ayton et al. 2004) or *Aml1* gene (AML1-ETO9, found in ~15% of human AML cases) (Yan et al. 2006; Jiao et al. 2009). Mice transplanted with bone marrow-derived hematopoietic stem/progenitor cells that had been retrovirally transduced with the AML1-ETO9a (Yan et al. 2006) oncogene succumb to myeloblastic AML highly reminiscent of the human disease induced by the same oncogenic lesion. Similar transduction/transplantation models for expression of MLL-ENL (Lavau et al. 2000), MLL-AF9 (Somervaille and Cleary 2006), Mixl1 (Glaser et al. 2006), or Hoxa9 (Thorsteinsdottir et al. 2002) oncogenes cause monocytic and myelomonocytic AML in mice, recapitulating the human subtypes of AML often associated with these oncogenes. Like human AML (Estey and Dohner 2006), these murine leukemias often respond only transiently to anti-cancer therapeutics, with relapse of drug refractory disease in a significant proportion (Zuber et al. 2009). Anti-cancer therapy kills many tumor cells (at least in part) through induction of apoptosis, and defects in the programmed cell death machinery are frequently associated with poor response (Kaufmann et al. 1998; Johnstone et al. 2002). Recent studies have identified somatic amplification of the genomic regions containing the anti-apoptotic genes *mcl-1* or *bcl-x* (Beroukhim et al. 2010) or post-translational stabilization of Mcl-1 protein (Schwickart et al. 2010) in diverse human cancers. We used mouse as well as human AMLs to determine which anti-apoptotic Bcl-2 family members are essential for development and sustained growth of this hematological malignancy to gain insight into which of these proteins should be targeted for therapeutic benefit.

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Results and Discussion

Deletion of mcl-1 but not bcl-x kills transformed myeloid cells in culture

We employed gene targeted mice expressing a tamoxifen-regulated Cre recombinase estrogen receptor fusion protein (Rosa26-Cre-ERT2 mice, hereafter called Cre) (Seibler et al. 2003) to allow controlled inactivation of *loxP* targeted (*floxed*, hereafter denoted *fl*) *bcl-x* (Wagner et al. 2000) or *mcl-1* (Vikstrom et al. 2010) alleles (Supplemental Fig. 1A). We used cells of six different genotypes for analysis: wild-type (wt) to reveal toxic effects of tamoxifen, *cre*/+ heterozygotes to detect toxicity of the activated Cre recombinase, and *cre*/+ cells that are either heterozygous (*fl*/+) or homozygous (*fl*/*fl*) for conditional *bcl-x* or *mcl-1* alleles to examine the consequences of loss of the corresponding anti-apoptotic proteins (Bcl-x_L and Mcl-1). Bone marrow-derived hematopoietic stem/progenitor cells were infected with retroviruses encoding MLL-ENL, MLL-AF9, AML1-ETO9a, Mixl1, or Hoxa9 plus GFP (from an IRES) as a marker (Supplemental Fig. 1B). Each transduced cell population readily yielded immortalized cell lines exhibiting an immature myeloid profile, with the exception of AML1-ETO9a transduced cells, which exhibited a more differentiated morphology (Supplemental Fig. 1C). Treatment with tamoxifen resulted within 24 h in near complete recombination of both conditional *bcl-x*^{fl} or *mcl-1*^{fl} alleles (Supplemental Fig. 1D). Moreover, this treatment caused nearly complete loss of Bcl-x_L protein

in *bcl-x*$^{fl/fl}$;*cre*/+ cells and almost complete loss of Mcl-1 (and increase of Bim but not tBid or Puma) protein in *mcl-1*$^{fl/fl}$;cre/+ cells (Supplemental Fig. 1E). Tamoxifen exerted no toxicity on wild-type cells, but Cre activation reduced the viability of *cre*/+ cells to ~70% compared with untreated cultures (Fig. 1A; Supplemental Fig. 1F). *Bcl-x*$^{fl/+}$;*cre*/+ and *bcl-x*$^{fl/fl}$;*cre*/+ cells showed slightly increased death compared with the *cre*/+ cells, displaying an initial drop in viability to 57%–74% or 45%–57%, respectively (Fig. 1A; Supplemental Fig. 1F). Addition of tamoxifen reduced the viability of *mcl-1*$^{fl/+}$;cre/+ cells to 37%–55% and, remarkably, completely killed all *mcl-1*$^{fl/fl}$;cre/+ lines (Fig. 1A). These results show that Mcl-1 but not Bcl-x_L is essential for sustained survival of transformed myeloid cells in vitro.

We next examined the mechanism by which these transformed cells are killed upon *mcl-1* gene deletion. Overexpression of Bcl-2, Bcl-x_L, or Mcl-1 (Supplemental Fig. 1G) prevented this cell death (Fig. 1B). Among the apoptosis initiators tested, Bim was the most highly expressed in these cells, and, remarkably, knockdown of this BH3-only protein by shRNA (Supplemental Fig. 1H) prevented apoptosis of MLL-ENL transformed cells undergoing *mcl-1* deletion (Fig. 1B). These results demonstrate that Mcl-1 deletion kills transformed myeloid cells in vitro by activating the "Bcl-2-regulated" (also called "mitochondrial," "intrinsic," or "stress") apoptotic pathway in a Bim-dependent manner (Youle and Strasser 2008).

Murine AML are more sensitive to Mcl-1 deletion than nontransformed stem cells and myeloid progenitors

We next generated AML in vivo by reconstituting lethally irradiated mice with MLL-ENL or MLL-AF9 retrovirus-infected bone marrow-derived hematopoietic stem/progenitor cells. We used the Cre/loxP system to induce loss of Bcl-x_L or Mcl-1 (Supplemental Fig. 2A), or tetracycline-regulated RNAi (Zuber et al. 2011a) for knockdown of Mcl-1 (Supplemental Fig. 2B). All recipient mice developed AML within 5 wk after reconstitution (Supplemental Fig. 2C,D). Knockdown of Mcl-1 by RNAi caused apoptosis and rapid depletion of (dsRed$^+$/Venus$^+$) *mcl-1* shRNA-expressing AML cells that had been transformed by combined expression of the MLL-AF9 and N-ras oncogenes (Fig. 1C). In agreement with the data described above (Fig. 1B), this death could be inhibited by overexpression of Bcl-2 or Mcl-1 (Fig. 1C).

For Mcl-1 inhibition to become a feasible strategy for AML therapy, there needs to be a "therapeutic window" in which leukemic cells are more sensitive than normal hematopoietic stem cells (which require Mcl-1 for survival) (Opferman et al. 2005). Treatment with tamoxifen caused efficient recombination of *mcl-1*^{fl} alleles in FACS-sorted nontransformed LSK (lineage marker$^-$Sca-1$^+$c-Kit$^+$) stem and myeloid progenitors from *mcl-1*$^{fl/+}$;cre/+ or *mcl-1*$^{fl/fl}$;cre/+ mice, as demonstrated by flow-cytometric detection of the hCD4 reporter (Supplemental Fig. 3A). Mcl-1 deletion lowered the viability of *mcl-1*$^{fl/+}$;cre/+ LSK cells to 80% and that of *mcl-1*$^{fl/fl}$;cre/+ LSK cells to 34%, compared with untreated cells (Fig. 2A). Myeloid progenitors were slightly more susceptible to Mcl-1 deletion, showing viability of 91% for cre/+, 66% for *mcl-1*$^{fl/+}$;cre/+, and 15% for *mcl-1*$^{fl/fl}$;cre/+ cells (Fig. 2A). When MLL-ENL and MLL-AF9 transformed AML cells were treated in vitro with tamoxifen for 48 h, no effect was observed in wild-type cells, whereas the viability of *cre*/+ cells dropped to ~65% (Fig. 2B). The *bcl-x*$^{fl/+}$;*cre*/+ and *bcl-x*$^{fl/fl}$;*cre*/+ leukemic cells displayed a reduction in viability that was slightly higher compared with that of *cre*/+ cells (Fig. 2B). In contrast, the viability of *mcl-1*$^{fl/+}$;*cre*/+ cells dropped to ~35%, and most *mcl-1*$^{fl/fl}$;*cre*/+ cells were killed within 72 h (Fig. 2B). These results demonstrate that while Mcl-1 is important in both normal stem/myeloid progenitor cells and AML cells, the AML cells were significantly more susceptible to loss of Mcl-1, even when they were cocultured (Supplemental Fig. 3B). Thus, it might be possible to establish a "therapeutic window" for Mcl-1 inhibitors in AML therapy.

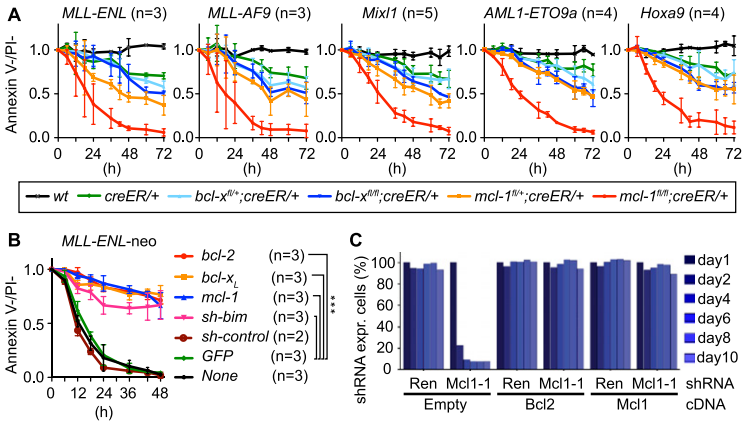

Figure 1. Impact of conditional deletion of *bcl-x* or *mcl-1* in transformed myeloid and AML cells in vitro. (*A*) Cells of the indicated genotypes, transformed with the oncogenes indicated, were grown for 72 h in medium with or without tamoxifen. Viable (AnnexinV$^-$/PI$^-$) cells were enumerated by flow cytometry. Graphs represent the means ± SEM of the ratio of viable tamoxifen-treated cells versus viable untreated cells (n = 3–5 independently transduced and sorted cell lines for each transforming oncogene and genotype). (*B*) *MLL-ENL/neo* transformed *mcl-1*$^{fl/fl}$;*creER*/+ cells were secondarily transduced with retroviruses encoding GFP alone or GFP plus Bcl-2, Bcl-x_L, Mcl-1, or shRNA for knockdown of Bim and purified on the basis of GFP expression. Cells were then cultured in the presence or absence of tamoxifen and their survival was measured; data are presented as in *A*. (***) $P < 0.001$. (*C*) RNAi-mediated suppression of Mcl-1 in MLL-AF9 plus N-Ras AML cells. Graphs represent the percentages of cells coexpressing shRNA and the cDNAs indicated over time following doxycycline treatment.

Anti-apoptotic Bcl-x_L, Bcl-2, and Bcl-w are collectively dispensable for sustained in vitro survival of AML

Although loss of Bcl-x_L had only a minor impact, it may have a critical function in AML cell survival that overlaps with Bcl-2 and/or Mcl-1. We therefore treated MLL-ENL transformed AML cells with ABT-737, a BH3 mimetic that potently binds and inactivates Bcl-x_L, Bcl-2, and Bcl-w (but not Mcl-1 or A1) (Oltersdorf et al. 2005), but on its own this had only a minor impact on viability (Fig. 2C). Moreover, ABT-737 further decreased the viability of *mcl-1*$^{fl/+}$;*cre*/+ AML cells that had also been treated with tamoxifen to lose one *mcl-1* allele to only a limited extent (Fig. 2C). This indicates that, collectively, Bcl-2, Bcl-x_L, and Bcl-w have only a minor role in sustaining the survival of AML cells.

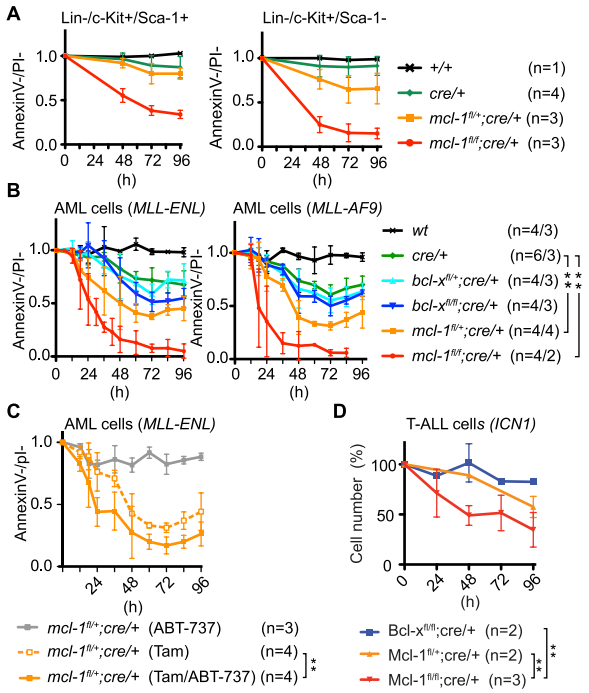

Figure 2. Impact of conditional in vitro deletion of *bcl-x* or *mcl-1* in normal hematopoietic stem and progenitor cells and in vivo derived AML cells. (*A*) Normal LSK (*left* panel) and Lin$^-$c-Kit$^+$Sca-1$^-$ myeloid progenitors (*right* panel) were cultured in the presence or absence of tamoxifen and cell survival was determined; data are presented as in Figure 1A. (*B*) Leukemic cells from mice bearing MLL-ENL-induced (*left* panel) or MLL-AF9-induced (*right* panel) AML were cultured in the presence or absence of tamoxifen and cell survival was determined; data are presented as described in Figure 1A. (**) $P < 0.01$. (*C*) MLL-ENL transformed AML cells of the indicated genotypes were grown in medium containing tamoxifen, ABT-737 (1 μM), or tamoxifen plus ABT-737 (1 μM). Cell survival was determined, and data are presented as described in Figure 1A. (**) $P < 0.01$. (*D*) Leukemic cells from mice bearing ICN1-induced T-ALL of the indicated genotypes were cultured in the presence or absence of tamoxifen. Cell survival was determined, and data are presented as described in Figure 1A. (**) $P < 0.01$.

Sustained survival of T-ALL is possible in the absence of Mcl-1

To examine whether Mcl-1 is generally essential for the sustained survival and growth of all types of leukemic cells, we generated T-ALL by reconstituting lethally irradiated mice with *bcl-x$^{fl/fl}$;cre/+*, *mcl-1$^{fl/+}$;cre/+*, or *mcl-1$^{fl/fl}$;cre/+* bone marrow cells that had been retrovirally transduced with an expression vector encoding intracellular NOTCH1 (ICN1), an oncogene implicated in human T-ALL (Supplemental Fig. 4A; Allman et al. 2001). Treatment of GFP$^+$ leukemic T cells maintained on OP9-DL1 feeder cells with tamoxifen led to efficient recombination of the *mcl-1^{fl}* allele, as evidenced by hCD4 reporter expression (Supplemental Fig. 4B). Although loss of Mcl-1 (but not loss of Bcl-x$_L$) elicited some reduction in T-ALL viability in vitro, a large fraction of T-ALL cells could survive longer term in the absence of Mcl-1 (Fig. 2D). This is in striking contrast to the obliteration of AML cells caused by Mcl-1 loss (Figs. 1A,C, 2B), and thereby demonstrates that the requirement for Mcl-1 for sustained growth and survival of transformed cells is specific to certain types of leukemia.

Deletion of mcl-1 but not bcl-x kills murine AML within the whole animal

The impact of loss of Mcl-1 or Bcl-x$_L$ on AML within the whole animal was assessed by administering AML-burdened mice with tamoxifen. TUNEL staining of the marrow 3 d after tamoxifen treatment revealed significant (albeit moderate) apoptosis of *bcl-x$^{fl/fl}$;cre/+* AML cells, more abundant death of *mcl-1$^{fl/+}$;cre/+* AML cells, and widespread apoptosis of *mcl-1$^{fl/fl}$;creER/+* leukemic blasts (Fig. 3A, top panels; Supplemental Fig. 5A). Accordingly, almost no leukemic blasts were evident in histological examination 5 d after tamoxifen treatment of mice that had previously developed MLL-ENL transformed *mcl-1$^{fl/fl}$;cre/+* AML (Fig. 3A, bottom panels). On the fifth day after starting treatment, we found 24×10^6 to 70×10^6 GFP$^+$ wild-type, *cre/+*, *bcl-x$^{fl/+}$;cre/+*, or *bcl-x$^{fl/fl}$;cre/+* cells and $26 \times 10^6 \pm 21 \times 10^6$ GFP$^+$ *mcl-1$^{fl/+}$;cre/+* AML cells but almost no ($0.2 \times 10^6 \pm 0.2 \times 10^6$) GFP$^+$ *mcl-1$^{fl/fl}$;cre/+* AML cells (Fig. 3B). All mice treated with tamoxifen 3 wk after transplantation with MLL-ENL transduced bone marrow cells relapsed with AML. In contrast, when treated with tamoxifen at day 10 or day 5 post-transplantation, only half or none, respectively, of the *mcl-1$^{fl/fl}$;cre/+* AML-burdened mice became sick during observation for >1 year. In contrast, their untreated counterparts survived for only 49 ± 5 d ($P < 0.001$), and untreated or tamoxifen-treated *mcl-1$^{fl/+}$;cre/+* AML-burdened mice lived for 49 ± 3 d or 66 ± 1 d, respectively (Fig. 3C). These findings were confirmed by demonstrating that tetracycline-induced RNAi-mediated knockdown of Mcl-1 in vivo caused clearance of AML cells from the liver and spleen and substantially prolonged survival of such tumor-burdened mice (Supplemental Fig. 5B–D).

All relapsed *bcl-x$^{fl/fl}$;cre/+* and *mcl-1$^{fl/+}$;cre/+* AML from tamoxifen-treated mice had efficiently deleted their *floxed* alleles, as reflected by the absence of Bcl-x$_L$ protein (Fig. 3D) or by Southern blot analysis and hCD4 reporter expression (Supplemental Fig. 6A,B). In contrast, all relapsed *mcl-1$^{fl/fl}$;cre/+* AML arose from variant leukemic cells bearing mutations that debilitate CreERT2-mediated recombination. Most (eight out of nine) of these relapsed *mcl-1$^{fl/fl}$;cre/+* AML had not recombined both *mcl-1^{fl}* alleles due to mutated CreERT2, as evidenced by the absence of hCD4 reporter (Supplemental Fig. 6B) and Cre-ERT2 protein expression (Fig. 3E). Retroviral reintroduction of CreERT2 and tamoxifen treatment allowed *mcl-1^{fl}* recombination and consequently rapidly killed such cells in vitro (Supplemental Fig. 6C). Another *mcl-1$^{fl/fl}$;cre/+* AML (#890) contained CreERT2 protein but had recombined only one *mcl-1* allele due to a deletion mutation in one *loxP* site, as reflected by Western blotting (Fig. 3E), hCD4 expression, and DNA sequencing (Supplemental Fig. 6B,D). These results show that Mcl-1 is essential for sustained survival and expansion of AML within the whole animal—only AML cells that had escaped *mcl-1* deletion could cause tumor relapse.

Mcl-1 is critical for sustained survival of human AML-derived cell lines and primary human AML

These gene deletion studies in murine AML indicated that pharmacological inhibition of Mcl-1 might be effective for treatment of this malignant disease in humans. However, small organic BH3 mimetic compounds specifically targeting Mcl-1 have yet to be developed. To test

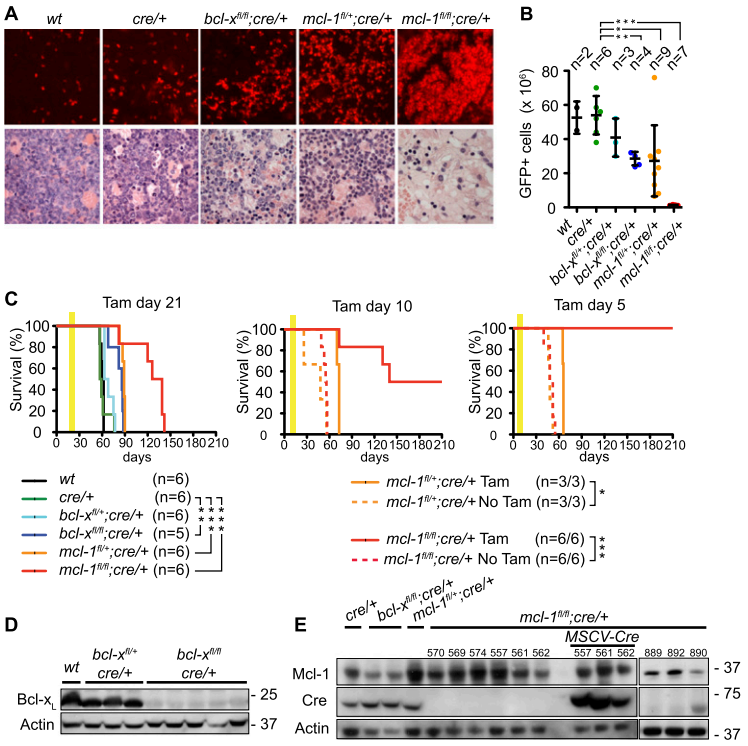

Figure 3. Conditional deletion of *mcl-1* causes regression of AML in tumor-burdened mice. (*A*) Histological examination of bone marrow from mice burdened with MLL-ENL-induced AML of the indicated genotypes. The *top* panels show apoptosis of cells (detected by TUNEL staining) after 3 d of treatment with tamoxifen. The *bottom* panels show the presence of leukemic blasts after 5 d of treatment with tamoxifen. (*B*) Symptomatic (elevated leukocyte counts, thrombocytopenia, and anemia) mice bearing MLL-ENL-induced AML of the indicated genotypes were treated for 5 d with tamoxifen. Shown are the total numbers of GFP$^+$ leukemic cells collected from two femora and two tibiae on the sixth day. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. (*C*) Survival of AML-burdened mice that were treated with tamoxifen (treatment window indicated by yellow bar) 21 d (*left* panel) 10 d (*middle* panel) or 5 d (*right* panel) after transplantation with MLL-ENL transformed AML cells of the indicated genotypes. Untreated mice (dotted lines) from the same cohort treated with tamoxifen at 10 d or 5 d all developed disease, thus confirming the presence of AML at that time pointl (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. (*D*,*E*) Western blot analysis to detect Bcl-x$_L$ (*D*) or Mcl-1 and CreER proteins (*E*) in leukemic cells of the indicated genotypes from mice that relapsed with AML after tamoxifen treatment.

the impact of functional inactivation of Mcl-1 in human AML, we employed lentiviral expression of Bim$_S$-derived BH3 variant ligands: Bim$_S$4E, an inert Bim variant (negative control) (Chen et al. 2005); Bim$_S$2A to target Mcl-1 but not Bcl-2, Bcl-x$_L$, Bcl-w, or A1 (Lee et al. 2008); Bim$_S$Bad to target Bcl-2, Bcl-x$_L$, and Bcl-w but not Mcl-1 or A1 (Merino et al. 2009); and Bim$_S$wt to target all prosurvival Bcl-2 proteins (Chen et al. 2005) (Supplemental Fig. 7A). Doxycycline-induced expression of RFP or Bim$_S$4E for 72 h in MLL-ENL transformed mouse AML or multiple human AML- and chronic myeloid leukemia (CML)-derived cell lines did not reduce the numbers of lentivirally transduced (GFP$^+$) cells compared with uninduced cultures (Fig. 4A; Supplemental Fig. 7B). In contrast, Bim$_S$wt expression greatly reduced the viability of all tested cell lines (Fig. 4A; Supplemental Fig. 7B). Importantly, blockade of Mcl-1 by Bim$_S$2A potently killed six of seven AML-derived cell lines but had only minor impact on the human erythroleukemia-derived (HEL92.1.7) and CML-derived (KU-812 and K562) cell lines (Fig. 4A). Interestingly, the few GFP$^+$ MV4,11 cells that remained viable after 3 d of doxycycline treatment exhibited low or no Bim$_S$2A expression (Supplemental Fig. 7C), indicating that selection against Mcl-1 blockade had occurred. In contrast, Bim$_S$Bad expression (Fig. 4A) or ABT-737 treatment (Fig. 4B), both of which block Bcl-2, Bcl-x$_L$, and Bcl-w (Oltersdorf et al. 2005; Merino et al. 2009), did not reduce the viability of most AML lines, except the ML-1, HL-60, and MV4,11 cells (small but significant effect), but they potently killed the human erythroleukemia (HEL92.1.7) and CML (KU-812) cell lines. Bim$_S$2A-mediated Mcl-1 blockade in combination with ABT-737 treatment for 40 h led to almost complete killing of all cell lines tested (Fig. 4B), consistent with the notion that cells cannot survive when a broad range of prosurvival Bcl-2 proteins are inactivated.

We next transduced primary AML cells from patients (Supplemental Fig. 7D) with the aforementioned expression constructs to block Mcl-1 (Bim$_s$2A) or all prosurvival Bcl-2 proteins (Bim$_s$wt), using Bim$_s$4E as a negative control. Interestingly, two primary human AMLs (#1 and #3) showed substantially reduced viability after doxycycline-mediated induction of Bim$_S$2A or Bim$_S$wt but not after induction of (inactive) Bim$_S$4E (Fig. 4C). Some primary human AML, however, did not show a significant drop in viability after doxycycline treatment. Although in some AML cells this may have been due to insufficient induction of Bim$_s$2A or Bim$_s$wt, we remarkably found mutated Bak (abnormal protein size) and abnormally low Bax levels in AML #2 and very low expression levels of both Bax and Bak in AML #5 (Fig. 4C). It appears likely that the therapeutic treatments that these patients received prior to our analysis selected for outgrowth of AML with such generalized defects in the "Bcl-2-regulated" apoptotic pathway.

Collectively, these results demonstrate that Mcl-1 is critical for the sustained survival and expansion of mouse as well as human AML resulting from a broad range of oncogenic lesions, whereas Bcl-x$_L$, Bcl-w, or Bcl-2, as reported (Zuber et al. 2011b), play minor prosurvival roles in these cancers. As such, these findings encourage the development of pharmacologic inhibitors that target Mcl-1 or regulators of its expression for treatment of this hematological malignancy.

Materials and methods

Animals

Experiments with mice were conducted according to the guidelines of The Walter and Eliza Hall Institute Animal Ethics Committee. *bcl-x$^{fl/fl}$* (Wagner et al. 2000), *mcl-1$^{fl/fl}$* (Supplemental Fig. 1A; Vikstrom et al. 2010) and the Rosa26-CreERT2 (Seibler et al. 2003) (Taconic Artemis) gene targeted mice (all on a C57BL/6 background) have been described. Mice received one aliquot containing 4 mg of tamoxifen (Sigma, T5648) per day for five consecutive days by oral gavage as described previously (Anastassiadis et al. 2010).

Generation and culture of transformed leukemic cells

MLL-ENL and MLL-AF9 retroviral constructs were obtained from Drs. R. Slany and M. Cleary, the AML1-ETO9a retroviral construct was obtained from Dr. D.-E. Zhang (Addgene plasmid 12433) (Yan et al. 2006), the Hoxa9

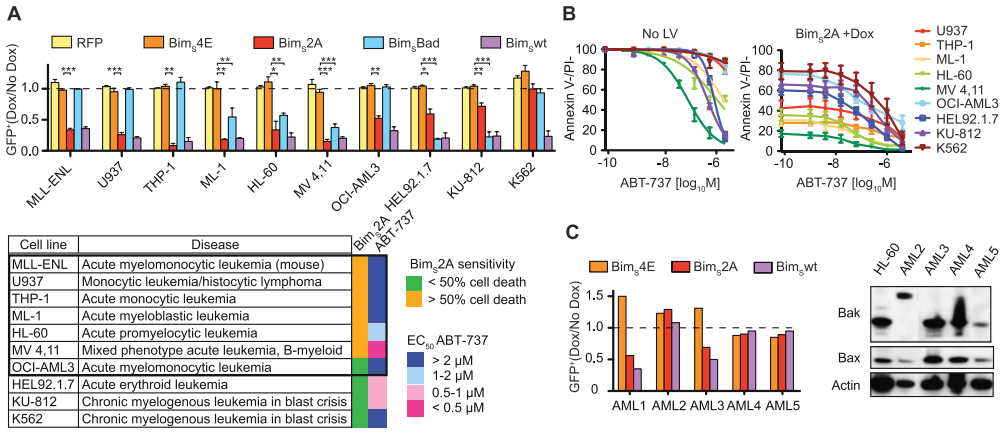

Figure 4. Impact of functional inactivation of Mcl-1 by inducible expression of Bim-derived BH3-like ligands that selectively neutralize different prosurvival Bcl-2 family members in human leukemia-derived cell lines and primary human AML cells. (*A*) Mouse AML cells and human leukemia-derived cell lines (U937, THP-1, ML-1, HL-60, MV4,11, OCI-AML3, HEL92.1.7, KU-812, and K562) were transduced with lentiviral constructs that allow inducible expression of Bim$_S$4E (negative control, no binding to Bcl-2-like proteins), Bim$_S$2A (binding only Mcl-1), Bim$_S$Bad (binding Bcl-2, Bcl-x$_L$, and Bcl-w but not Mcl-1 or A1), or Bim$_S$wt (binding all Bcl-2 prosurvival proteins) and were either left untreated or treated with doxycycline. Graphs represent the ratio of GFP$^+$ cells (doxycycline-treated compared with untreated) detected by flow cytometry of at least three experiments for each cell line. In the table, cell lines derived from AML are boxed; the heat map summarizes the sensitivity of all cell lines to Bim$_S$2A expression or ABT-737 treatment. (*B*) The cell lines indicated were treated with the indicated doses of ABT-737 alone (*left* panel) or in combination with induced Bim$_S$2A (inhibits Mcl-1) expression (*right* panel). (*C*) Primary human AML cells were transduced with Bim$_S$4E-encoding (negative control), Bim$_S$2A-encoding (inhibits Mcl-1), or Bim$_S$wt-encoding (inhibits all prosurvival Bcl-2 family members) lentiviruses and were either left untreated or treated with doxycycline. Shown is a graph representing the ratio of doxycycline-treated compared with untreated GFP$^+$ AML blast cells from one representative experiment for each patient sample. Western blot analysis to detect Bax and Bak protein levels in primary AML cells. Low levels of Bax and Bak were detected in sample #5; low levels of Bax and abnormal size of Bak were detected in sample #2.

retroviral construct was obtained from Dr. C. Largman (Addgene plasmid 8515) (Shen et al. 1997), and the Mixl1 retroviral construct was described previously (Glaser et al. 2006). The Bcl-2, Bcl-x$_L$, and Mcl-1 retroviral expression constructs were provided by Dr. D.C.S. Huang. The CreER-hCD2 retroviral construct was provided by Dr. M. Bussliner (IMP, Vienna, Austria), and the retroviral construct for shRNA-mediated knockdown of Bim has been described (Bouillet et al. 2005). Lentiviral expression constructs were generated by KpnI digestion, and by cloning a synthesized 1638-base-pair (bp) fragment containing GFP-WPRE and AgeI/ClaI digestion and cloning of Bim$_S$ variants into the TRIPZ vector (Open Biosystems). Viral supernatants were produced by $CaCl_2$ cotransfection of 293T cells with expression constructs and packaging plasmids. Fetal liver (E13.5 [embryonic day 13.5] embryos) or bone marrow cells from 5-FU-treated mice were enriched for immature cells by immunomagnetic depletion of mature lymphoid (CD3$^+$, CD4$^+$, CD8$^+$, and B220$^+$) myeloid (Mac1$^+$ and Gr1$^+$) and erythroid (Ter119$^+$) cells. Viral transduction was performed as described (Metcalf et al. 2007). Transduced cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% FBS (HyClone), 2 mM L-glutamine, 100 ng/mL murine stem cell factor, and 10 ng/mL IL-3 (Peprotech). Infected cells were either FACS-sorted on the basis of GFP expression for in vitro studies or injected unsorted into sublethally γ-irradiated (5.5 Gy) C57BL/6 mice. Cells retrovirally transduced with the MLL-ENL/neo expression vector were plated in methylcellulose medium M3234 (Stem Cell Technologies) supplemented with 100 ng/mL murine stem cell factor, 10 ng/mL IL-3, and 1 mg/mL G418 (InvivoGen). G418-resistant cells from the third plating were transduced with retroviral constructs encoding Bcl-2 family members or RNA hairpins for knockdown of Bim and were linked with IRES-GFP, and transduced cells were subsequently isolated by cell sorting. Cre-mediated recombination in vitro was induced by treatment with 10^{-7} M 4-hydroxy tamoxifen (Sigma, H7904) (Glaser et al. 2009). For analysis of protein levels by Western blotting, *mcl-1$^{fl/fl}$;creER/+* cells were treated with tamoxifen in the presence of the pan-caspase inhibitor qVD-OPh (25 μM; MP Biomedicals) to protect them from apoptotic death.

Detailed methodology is described in the Supplemental Material.

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