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LYMPHOID NEOPLASIA

MCL-1 but not BCL-XL is critical for the development and sustained expansion of thymic lymphoma in p53-deficient mice

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Key Points

- MCL-1 is critical for thymic lymphoma development mediated by loss of p53.
- MCL-1 is essential for sustained growth of p53-deficient thymic lymphoma cells.

Apoptosis plays a role in normal lymphopoiesis and lymphoid malignancies. Pro-survival MCL-1 is essential for survival of T-cell progenitors, BCL-XL for immature thymocytes, and BCL-2 for mature T cells. Conversely, little is known about the regulators that are required for the survival of T-cell lymphomas. We used constitutive and conditionally gene-targeted mice to investigate which pro-survival BCL-2 family member is required for the sustained survival of thymic lymphomas initiated by loss of p53. Constitutive loss of a single *Mcl-1* allele delayed tumor onset. In contrast, lymphomas emerging in *p53*$^{-/-}$ mice in which *Mcl-1* could be conditionally deleted had been selected for retention of MCL-1 expression. In contrast, complete loss of BCL-XL had no impact on lymphoma development in *p53*$^{-/-}$ mice. These results demonstrate that thymic lymphomas elicited by loss of p53 must arise from cancer-initiating cells that require MCL-1 for their survival. Acute deletion of both *Mcl-1* alleles abrogated the expansion of *p53*$^{-/-}$ lymphomas in mice, whereas inducible loss of BCL-XL had little impact. This reveals that MCL-1 is essential for the sustained survival of these malignant cells and suggests that targeting MCL-1 may be an attractive strategy for the treatment of T-cell lymphoma. (*Blood*. 2014;124(26):3939-3946)

Introduction

Mutations in p53 have been found in ~50% of sporadic human cancers.[1] Moreover, inherited mutations in 1 *p53* allele cause Li–Fraumeni syndrome, a rare autosomal disorder characterized by development of diverse cancer types at a young age after loss of the wild-type *p53* allele.[2,3] p53 is a transcription factor that controls diverse cellular responses to stress, including proliferation arrest, senescence, and apoptosis, by direct regulation of ~200 target genes.[4] Mice with homozygous germline loss or mutation of *p53* develop tumors (mostly thymic lymphomas) with 100% incidence within 300 days.[5-8]

Evasion of cell death is a prerequisite for the development of cancer. Apoptosis is a genetically programmed process for the killing of cells that are no longer needed or potentially dangerous.[9,10] Mutations or epigenetic changes in regulators of apoptosis cause or contribute to the development of several diseases, particularly cancer, and render malignant cells resistant to a broad range of chemotherapeutic drugs. The BCL-2-regulated apoptotic pathway can be activated by developmental cues or a diverse range of stress stimuli (eg, cytokine withdrawal, oncogene activation, DNA damage). This pathway is controlled by the 3 subgroups of the BCL-2 protein family.[10] The pro-survival members BCL-2, BCL-XL, MCL-1, BCL-W and A1 (human BFL-1) are required for cell survival. The pro-apoptotic BH3-only proteins (BIM, PUMA, NOXA, BID, BAD, BIK, BMF, HRK) are essential for initiation of apoptosis with different death stimuli activating distinct members.[10,11] The proapoptotic multi-BH (BCL-2 homology) domain proteins BAX and BAK are required for activation of the downstream phases of apoptosis: mitochondrial outer membrane permeabilization and consequent caspase activation.[12,13] The BH3-only proteins bind to the pro-survival BCL-2 family proteins to unleash primed BAX/BAK allowing them to cause mitochondrial outer membrane permeabilization. Certain BH3-only proteins (eg, BIM and PUMA) can also activate BAX/BAK through direct binding.[14,15]

Although it has long been known that overexpression of BCL-2 (or its pro-survival relatives) can promote tumorigenesis, researchers have only recently started to explore which pro-survival BCL-2 family member expressed under endogenous control is required for the development and sustained expansion of which tumor. BCL-XL[16,17] but not BCL-2[18] is essential for the development of MYC-driven pre-B/B lymphoma, whereas MCL-1 is critical for the development of acute myeloid leukemia (AML).[19,20] Such information provided the impetus for the development of inhibitors of pro-survival BCL-2 family members (BH3-mimetics navitoclax/ABT-263: inhibits BCL-2, BCL-XL, and BCL-W; ABT-199: inhibits only BCL-2).[10]

In this study, we examined the impact of loss of BCL-XL or MCL-1 on the development and sustained growth of thymic lymphoma elicited by loss of p53 and show that only MCL-1 is critical.

Submitted September 16, 2014; accepted October 22, 2014. Prepublished online as *Blood* First Edition paper, November 3, 2014; DOI 10.1182/blood-2014-09-601567.

The online version of this article contains a data supplement.

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Materials and methods

Mice

Experiments with mice were conducted according to the guidelines of the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. The *Mcl-1*^{fl},[21] *Bcl-x*^{fl},[22] *Lck-Cre*,[23] *Rosa26CreERT2*,[24] and *p53*$^{-/-}$[6] mice (all maintained on a C57BL/6-Ly5.2 background) have been described previously.

Transplantation studies

For transplantation studies, 6 C57BL/6-Ly5.1 recipient mice were injected intraperitoneally with 3×10^6 thymic lymphoma cells (TCRβ$^+$CD4$^+$CD8$^+$ Ly5.2$^+$). A total of 3 to 6 independent thymic lymphoma samples were tested. Three of the recipient mice were treated by oral gavage with 4 mg tamoxifen/day (in peanut oil[25]) on days 7 and 8 (the remaining 3 recipients were left untreated) and then monitored for lymphoma development. Mice that had developed lymphoma were killed, and the lymphomas were analyzed by fluorescence-activated cell sorter analysis and western blotting. The experiment was concluded at 90 days after lymphoma injection. Mice bearing lymphoma were examined for organ weights.

Genotyping

Genotyping of mice was performed on DNA samples obtained from tail biopsies that had been digested with tail digestion buffer (Viagen Biotech, Los Angeles, CA) supplemented with proteinase K (Sigma Aldrich, Castle Hill, NSW, Australia). Oligonucleotides for genotyping were obtained from GeneWorks (Hindmarsh, SA, Australia) at polymerase chain reaction grade quality. GoTaq Green Master Mix (Promega, Alexandria, NSW, Australia) was supplemented with 10 pmol of the appropriate oligonucleotide primer pair to perform the polymerase chain reaction. Oligonucleotide sequences for genotyping will be provided on request.

Flow cytometric analysis

Lymphoid organs were harvested, and single-cell suspensions were prepared using 100-μM sieves (BD BioSciences, San Jose, CA). Red blood cells were depleted using red blood cell lysis buffer. Cells (5×10^4) were stained for surface markers using fluorochrome (fluorescein isothiocyanate, Ag-presenting cells, R-phycoerythrin; Life technologies, Mulgrave, VIC, Australia)-conjugated monoclonal antibodies (WEHI) to mouse CD4 (YTA3.2.1), CD8 (YTS169), CD3 (145-2C11), B220 (RA3-6B2), and human CD4 (RPA-T4) for 30 minutes in balanced salt solution supplemented with 2% fetal calf serum (Life Technologies, Mulgrave, VIC, Australia) and analyzed in a FACS-Calibur (BD BioSciences).

Western blot analysis

Extracts from thymic lymphomas were prepared. Blots were probed using the following monoclonal antibodies: hamster anti-mouse BCL-2 (3F11; WEHI), mouse anti-mouse BCL-XL (2F12; BD BioSciences), rat anti-mouse MCL-1 (19C4-15[26]), mouse anti-actin (AC-40; Sigma Aldrich, Castle Hill, NSW, Australia; used as a loading control), mouse anti-HSP-70 antibody (gift from Dr Robin Anderson, Peter MacCallum Cancer Research Institute, Melbourne, Australia; used as a loading control), polyclonal rabbit anti-mouse BIM (9292; Sapphire Bioscience, Waterloo, NSW, Australia) or polyclonal rabbit anti-mouse PUMA (Ab-27669; Abcam, Melbourne, VIC, Australia), and polyclonal anti-mouse ERα (HC-20; Santa Cruz Biotechnologies, Dallas, TX). Polyclonal goat antibodies against mouse, rat, hamster, or rabbit immunoglobulin (Ig)G antibodies coupled to horseradish peroxidase (In Vitro Technologies, Noble Park North, VIC, Australia) were used as secondary reagents, and enhanced chemiluminescence (GE Healthcare Life Sciences, Pittsburgh, PA) was used for detection of protein bands.

Statistical analysis

Kaplan-Meier survival curves, dot, and bar graphs for organ weights were generated and analyzed using the GraphPad Prism software using a 2-tailed Student *t* test comparing 2 groups with each other. Error bars are presented as standard error of mean (GraphPad Software, La Jolla, CA). Mouse survival curves were compared by log-rank Mantel Cox test. Disease incidence was calculated using χ^2 test. $P < .05$ was considered significant.

Results

Loss of BCL-XL does not delay thymic lymphoma development

We used the *loxP*-targeted *Bcl-x* conditional knockout mouse model to determine the role of BCL-XL in tumorigenesis caused by loss of p53. These animals were crossed to the *Lck-Cre* strain in which a constitutively active Cre recombinase is expressed from the early TN3 (TCRβ$^-$CD3$^-$CD4$^-$CD8$^-$CD25$^+$CD44$^-$) T-cell progenitor stage in the thymus.

Tumor incidence and rate of tumor development were comparable between *p53*$^{-/-}$ (median survival, 156 days) and *p53*$^{-/-}$*;Lck-Cre* mice (median survival, 161 days; $P = .2996$; supplemental Figure 1 available on the *Blood* Web site), demonstrating that expression of a constitutively active Cre recombinase had no adverse effect on tumor development in *p53*$^{-/-}$ mice. The survival and rate of tumor development were also comparable between *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$ (median survival, 146 days; $P = .6703$), *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/fl}$ (median survival, 134 days; $P = .3236$), and *p53*$^{-/-}$*;Lck-Cre* mice (median survival, 161 days) (Figure 1A). Although all *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$ mice developed cancer, the proportion of mice exhibiting thymic lymphoma was reduced compared with *p53*$^{-/-}$*;Lck-Cre* mice (Figure 1B). This reduction in thymic lymphoma incidence was compensated by an increase in other tumors (eg, splenomegaly and sarcoma). The *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/fl}$ mice displayed a minor (~10%) increase in thymic lymphoma incidence but a ~15% decrease in splenomegaly compared with *p53*$^{-/-}$*;Lck-Cre* mice (Figure 1B). There were no significant differences in thymus or spleen weights between sick *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$ and *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/-}$ animals vs sick *p53*$^{-/-}$*;Lck-Cre* controls (supplemental Figure 2A-B). Western blot analysis confirmed that BCL-XL expression was substantially reduced or even abrogated in thymic lymphomas from *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$ (5/6 lymphomas) and *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/fl}$ mice (10/10 lymphomas; representative blots shown; Figure 1C). These lymphomas did not show consistent alterations in the expression of other pro-survival (BCL-2, MCL-1) or proapoptotic (BIM and PUMA) BCL-2 family members examined (Figure 1C). These results demonstrate that BCL-XL is not required for the development of thymic lymphoma or other cancers elicited by loss of p53.

Constitutive loss of a single allele of *Mcl-1* significantly delays thymic lymphoma development in *p53*$^{-/-}$ mice

Because most thymic lymphomas in *p53*$^{-/-}$ mice express readily detectable levels of MCL-1 (Figure 1C), we generated *p53*-deficient mice that lack 1 allele of *Mcl-1* (loss of both *Mcl-1* alleles causes early embryonic lethality[27]) to examine the role of this BCL-2 pro-survival family member in T lymphomagenesis (Figure 2). In comparison with *p53*$^{-/-}$ mice (median survival, 156 days), *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice (median survival, 194 days) showed significantly prolonged tumor-free survival (Figure 2A; $P = .0006$). Compared with *p53*$^{-/-}$ controls, thymic lymphoma incidence was reduced by ~50% in *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice. Instead the *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice were more likely to develop mature T-cell lymphoma in the spleen with liver metastasis and occasionally sarcoma (Figure 2B).

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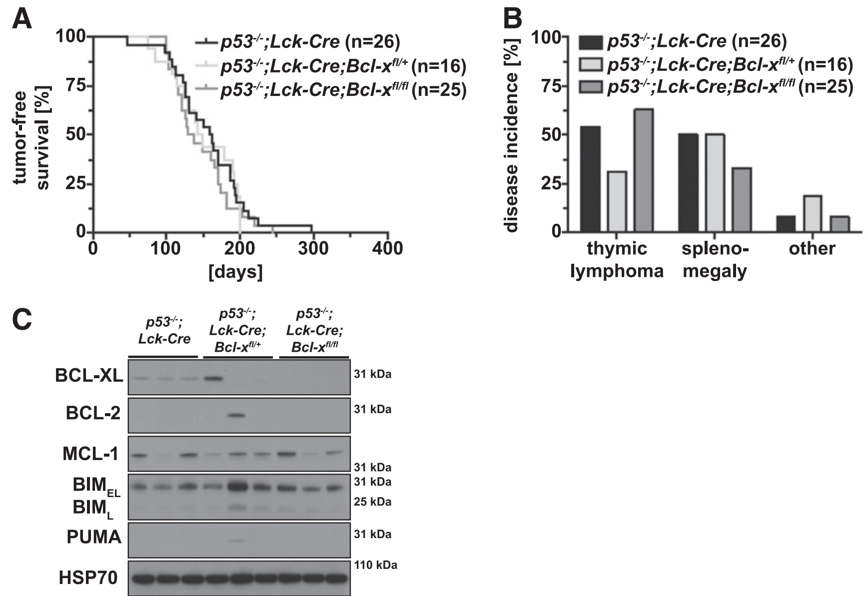

Figure 1. Loss of BCL-XL does not delay thymic lymphoma development in p53-deficient mice. (A) Tumor-free survival of *p53*$^{-/-}$*;Lck-Cre* (control; n = 26), *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$ (n = 16, compared with control; *P* = .6703), and *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/fl}$ mice (n = 26; compared with control; *P* = .3236); mouse cohorts were compared using the log-rank Mantel-Cox test. (B) Incidence (as % of total mice analyzed) of thymic lymphoma, splenomegaly, and other pathologies (*eg*, sarcoma) in cohorts of *p53*$^{-/-}$*;Lck-Cre* (n = 26), *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$ (n = 16), and *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/fl}$ mice (n = 24). (C) Analysis of the proteins indicated by western blotting in primary thymic lymphoma samples from sick *p53*$^{-/-}$*;Lck-Cre*, *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/+}$, and *p53*$^{-/-}$*;Lck-Cre;Bcl-x*$^{fl/fl}$ mice (representative of 6-10 samples for each genotype). Probing for HSP70 was used as a loading control.

Lymphoma burden (measured by the weight of the thymus and spleen) in sick *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice was comparable to control *p53*$^{-/-}$ animals (p_{thymus} = .0619; p_{spleen} = .6274; supplemental Figure 3A-B). Moreover, thymic lymphomas in both strains displayed a similar immunophenotype, mostly TCRβ$^+$CD4$^+$CD8$^+$ (Figure 2C). Western blot analysis revealed reduced levels of MCL-1 (5/7 lymphomas) and also BIM (5/7 lymphomas) in the lymphomas from the *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice compared with tumors from control *p53*$^{-/-}$ mice (representative blots shown; Figure 2D). These results show that loss of a single allele of *Mcl-1* can markedly delay the development of thymic lymphoma that is initiated by loss of p53.

Impact of conditional deletion of 1 or both alleles of *Mcl-1* on T-cell lymphoma development in *p53*$^{-/-}$ mice

The ability of loss of 1 allele of *Mcl-1* to significantly delay lymphoma development in *p53*$^{-/-}$ mice could be due to an impact on cells undergoing neoplastic transformation during T-lymphoid differentiation or might be a consequence of defects in the survival of more primitive hematopoietic stem/progenitor cells, known to be the cells of origin of γ radiation-induced thymic lymphoma.[28,29] Furthermore, *Lck-Cre;Mcl-1*$^{fl/fl}$ mice present with a significant reduction of immature and mature T cells in the thymus, suggesting MCL-1 is critical for T-cell development (supplemental Figure 4A-B).[30]

We generated *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ and *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice to investigate whether loss of MCL-1 specifically in T-lymphoid cells (from the TN3 stage onward) could delay thymic lymphoma development in *p53*$^{-/-}$ mice. The *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ mice (median survival, 171 days) did not survive significantly longer compared with the *p53*$^{-/-}$*;Lck-Cre* controls (median survival, 161 days; *P* = .1216; Figure 3A), but we did observe a ∼50% reduction in thymic lymphoma incidence with a concomitant increase in sarcoma and certain other pathologies (Figure 3B). Thymi from tumor-bearing *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ mice were

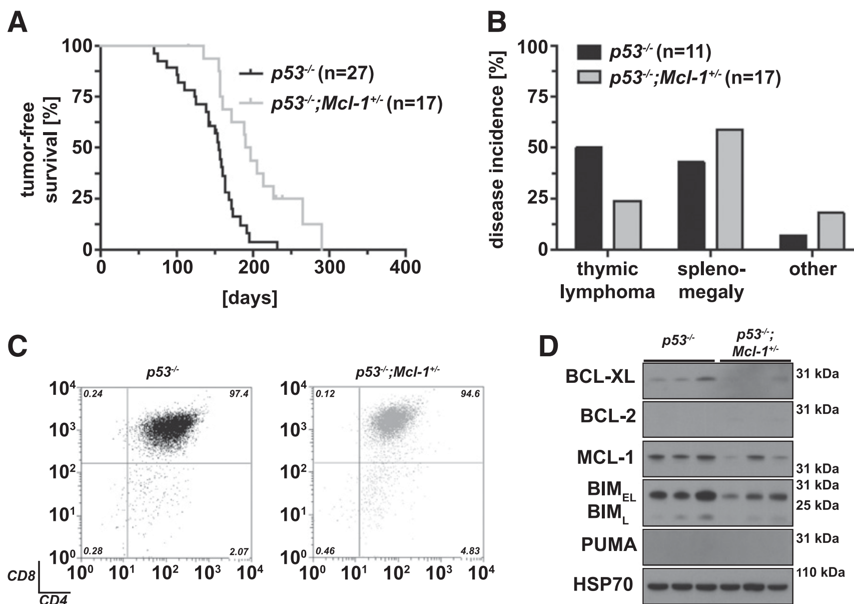

Figure 2. Loss of 1 allele of *Mcl-1* significantly delays thymic lymphoma development in *p53*$^{-/-}$ mice. (A) Tumor-free survival comparing *p53*$^{-/-}$ (control; n = 27) and *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice (n = 17; log-rank Mantel-Cox test, *P* = .0006). (B) Incidence in % of thymic lymphoma, splenomegaly, and other pathologies (eg, sarcoma) in *p53*$^{-/-}$ (n = 11) and *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice (n = 17). (C) Representative immunophenotyping of primary thymic lymphoma samples from *p53*$^{-/-}$ and *p53*$^{-/-}$*;Mcl-1*$^{+/-}$ mice (gated on PI$^-$ and TCRβ$^+$ large cells). (D) Analysis of the proteins indicated by western blotting of primary thymic lymphoma samples from mice of the indicated genotypes (representative of 7 samples each). Probing for HSP70 was used as a loading control.

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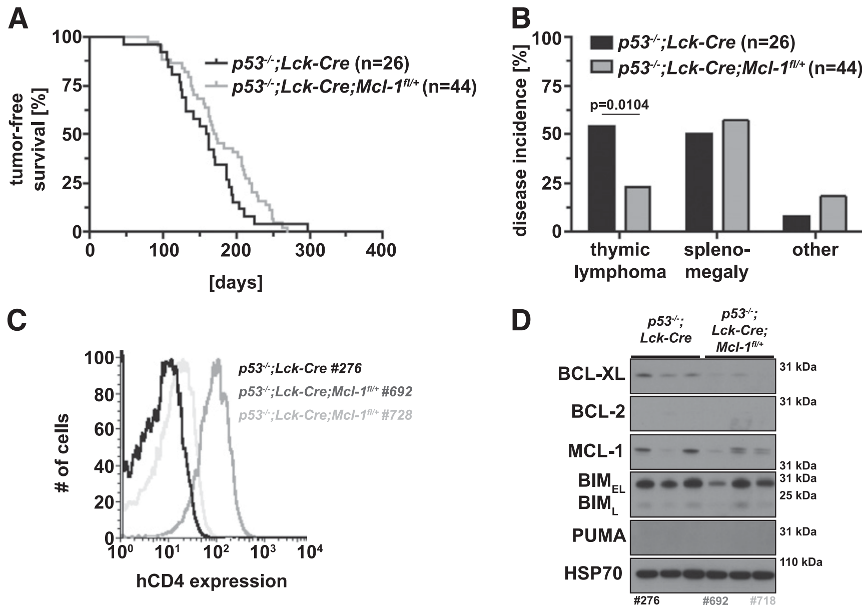

Figure 3. Impact of deletion of 1 allele of *Mcl-1* selectively in T-lymphoid cells on thymic lymphoma development in *p53*$^{-/-}$ mice. (A) Tumor-free survival of *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ mice (n = 44) in comparison with control mice (*p53*$^{-/-}$*;Lck-Cre;* n = 26; log-rank Mantel-Cox test, *P* = .1216). (B) Flow cytometric analysis of *Mcl-1*^{fl} recombination in 2 independent primary thymic lymphoma samples (TCRβ^{+}CD4^{+}CD8^{+}) from *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ mice compared with a *p53*$^{-/-}$*;Lck-Cre* thymic lymphoma (negative control) by immunofluorescent staining for the human CD4 reporter. (C) Incidence in % of thymic lymphoma, splenomegaly, sarcoma, or other pathologies in the cohorts of *p53*$^{-/-}$*; Lck-Cre* (n = 26) and *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ mice (n = 44). (D) Analysis of the proteins indicated by western blotting of primary thymic lymphoma samples from mice of the indicated genotypes (representative of 6-10 samples each). Probing for HSP70 was used as a loading control. The numbers below western blots indicate the tumor samples also tested in B.

significantly smaller, but spleen weights were comparable to those of sick *p53*$^{-/-}$*;Lck-Cre* controls (p_{thymus} = .0045; p_{spleen} = .6639; supplemental Figure 4A-B). To investigate whether the floxed *Mcl-1* allele had been recombined in the *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ lymphomas, we took advantage of the human CD4 (hCD4) reporter engineered into the *Mcl-1* gene targeting construct that is only expressed on *Mcl-1*^{fl} recombination.[20,31] Approximately 50% of *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ lymphomas expressed substantial levels of hCD4 and hence had recombined the floxed *Mcl-1* allele (see tumor 692 in Figure 3C). The others were hCD4^{-} (see tumor 728 in Figure 3C) and thus had been selected against loss of even a single *Mcl-1* allele. Accordingly, western blotting showed that tumor 692 expressed only low levels of MCL-1 whereas tumor 728 had higher levels of MCL-1 (note that the MCL-1 protein encoded by the *Mcl-1*^{fl} allele runs with slightly higher molecular weight than MCL-1 protein encoded by the wild-type allele[26]; Figure 3D). The hCD4-positive lymphomas expressing low levels of MCL-1 appeared to be selected for reduced levels of proapoptotic BIM. There were no consistent differences in the expression of BCL-XL, BCL-2, or PUMA between the different lymphomas (Figure 3D).

Because thymic lymphoma development in *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/+}$ mice was not markedly delayed compared with control *p53*$^{-/-}$*; Lck-Cre* mice, we hypothesized that complete loss of MCL-1 in T-lymphoid cells might have more pronounced impact on lymphoma development in *p53*$^{-/-}$ mice. We therefore generated *p53*$^{-/-}$*; Lck-Cre;Mcl-1*$^{fl/fl}$ mice. Remarkably, their survival (median survival, 149 days) was comparable to that of *p53*$^{-/-}$*;Lck-Cre* controls (median survival, 161 days; *P* = .8292; Figure 4A). Compared with *p53*$^{-/-}$*; Lck-Cre* controls, the *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice had a reduced incidence of thymic lymphoma and splenomegaly but

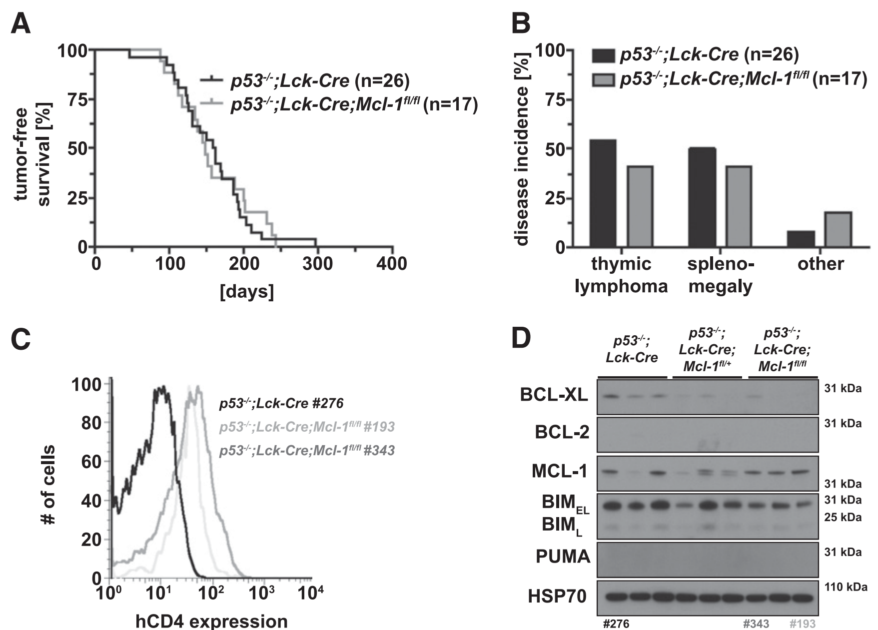

Figure 4. Impact of deletion of both alleles of *Mcl-1* selectively in T-lymphoid cells on thymic lymphoma development in *p53*$^{-/-}$ mice. (A) Tumor-free survival of *p53*$^{-/-}$*;Lck-Cre* (n = 26) and *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice (n = 17; log-rank Mantel-Cox test, *P* = .8292). (B) Flow cytometric analysis of *Mcl-1*^{fl} recombination in primary thymic lymphoma samples from *p53*$^{-/-}$*;Lck-Cre* (negative control) and *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice by immunofluorescent staining for the human CD4 (hCD4) reporter. (C) Incidence in % of thymic lymphoma, splenomegaly, sarcoma, or other pathologies in cohorts of *p53*$^{-/-}$*;Lck-Cre* (n = 26) and *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice (n = 17). (D) Analysis of the proteins indicated by western blotting of primary thymic lymphoma samples from mice of the indicated genotypes (representative of 6-10 samples each). Probing for HSP70 was used as a loading control. The numbers below western blots indicate the tumor samples also tested in B. Lanes 1 to 6 are the same image shown in Figure 3D and are presented here for comparison.

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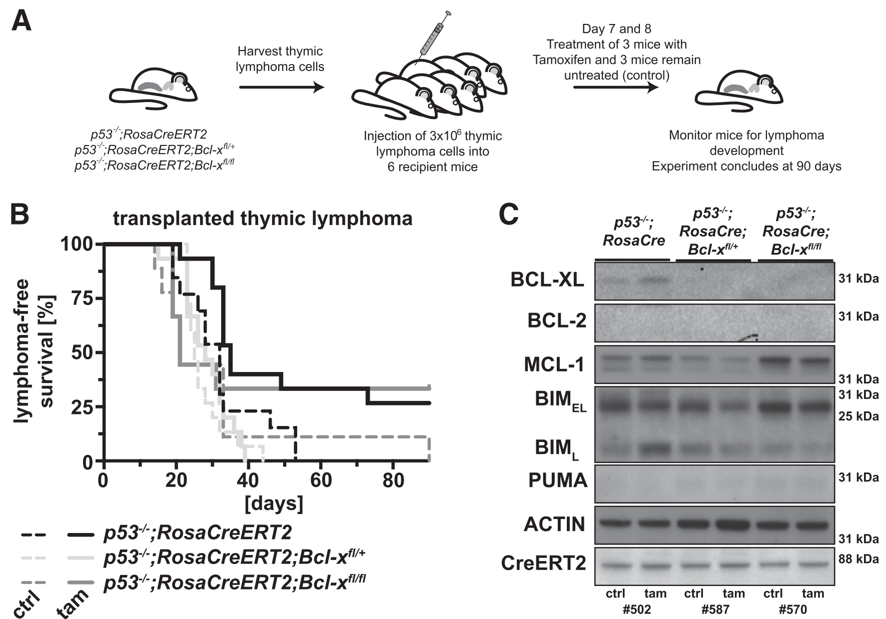

Figure 5. Acute loss of BCL-XL has no impact on the sustained expansion of *p53*$^{-/-}$ thymic lymphomas in mice. (A) Schematic of the experimental protocol (also used for experiments shown in Figure 6). Lymphomas were harvested from *p53*$^{-/-}$*;RosaCreERT2* (control), *p53*$^{-/-}$*;RosaCreERT2;Bcl-x*$^{fl/+}$, *p53*$^{-/-}$*;RosaCreERT2;Bcl-x*$^{fl/fl}$, *p53*$^{-/-}$*;RosaCreERT2;Mcl-1*$^{fl/+}$, or *p53*$^{-/-}$*;RosaCreERT2;Mcl-1*$^{fl/fl}$ mice (all Ly5.2$^+$). Lymphoma cells (3 × 10^6; 3-6 independent primary lymphoma samples per genotype tested) were injected into 6 C57BL/6-Ly5.1$^+$ recipient mice each. Three recipient mice were treated by oral gavage with 4 mg of tamoxifen on days 7 and 8 after lymphoma cell transplantation, and the other 3 recipients were left untreated and served as controls. These mice were then monitored for lymphoma development. The experiment was concluded 90 days after cell transplantation. (B) Lymphoma-free survival of the mice that had been transplanted with lymphoma cells of the genotypes indicated and treated with either tamoxifen (solid lines; to delete the floxed *Bcl-x* alleles) or had been left untreated (dashed lines; negative control). (C) Analysis of the proteins indicated by western blotting of transplanted thymic lymphoma samples of the indicated genotypes (representative of 3 samples each) that had grown in recipients treated with tamoxifen (tam) or in recipients that had been left untreated (ctrl). Probing for actin was used as a loading control.

presented more frequently with sarcoma or certain other pathologies (Figure 4B). Postmortem analysis of sick tumor-bearing mice did not reveal any significant differences between the 2 genotypes in terms of thymus or spleen weights (supplemental Figure 4A-B). Lymphomas from *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice had a similar immunophenotype to that of tumors from *p53*$^{-/-}$ or *p53*$^{-/-}$*;Lck-Cre* mice (6/6; TCRβ$^+$CD4$^+$CD8$^+$; data not shown). All *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ lymphomas expressed intermediate levels of the hCD4 reporter (Figure 4C), indicating that ≥1 floxed *Mcl-1* allele had been recombined. Western blot analysis showed that MCL-1 was maintained at readily detectable levels in *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ lymphomas (5/6 lymphomas), comparable to the levels of MCL-1 in *p53*$^{-/-}$*;Lck-Cre* control lymphomas (5/5 lymphomas, representative blots shown; Figure 4D). This shows that one *Mcl-1*^{fl} allele had not been recombined in these lymphomas. There were no consistent differences in the levels of BCL-2 or PUMA between tumors from *p53*$^{-/-}$*;Lck-Cre* and *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ mice, although we noted a small decrease in BCL-XL and BIM protein levels in the latter (Figure 4D).

These findings reveal that during thymic lymphoma development driven by loss of p53 potent selection against loss of MCL-1 operates at the TN3 differentiation stage or later.

Inducible loss of BCL-XL has no impact on the sustained expansion of *p53*$^{-/-}$ lymphomas in mice

Although BCL-XL is not essential for thymic lymphoma development in *p53*$^{-/-}$ mice (Figure 1), we wondered whether it might be required for the sustained survival and expansion of these lymphomas. To examine this, we generated *p53*$^{-/-}$*;Bcl-x*$^{fl/+}$ and *p53*$^{-/-}$*;Bcl-x*$^{fl/fl}$ mice that also contained the *RosaCreERT2* transgene to enable acute deletion of *Bcl-x* in established tumors. This transgene is expressed in all cell types and encodes a conditional Cre-recombinase ER fusion protein (CreERT2) that can be activated by tamoxifen. We examined 3 to 6 *p53*$^{-/-}$*;Bcl-x*$^{fl/+}$ and *p53*$^{-/-}$*;Bcl-x*$^{fl/fl}$ and (control) *p53*$^{-/-}$ lymphomas (all C57BL/6-Ly5.2). These were each injected into 6 C57BL/6-Ly5.1 recipients. On days 7 and 8, 3 of these recipients were treated with tamoxifen to activate the latent CreERT2 recombinase to delete the *Bcl-x*^{fl} alleles, whereas the remainder were left untreated (Figure 5A). These recipients were monitored for lymphoma growth over a 90-day period.

Mice that were injected with *p53*$^{-/-}$*;RosaCreERT2* thymic lymphoma cells and then treated with tamoxifen (median survival, 35 days) survived slightly longer than their untreated counterparts (median survival, 32 days), demonstrating that Cre-recombinase induction has only minor cytotoxic impact on these lymphomas in vivo. Tamoxifen-treated mice bearing *p53*$^{-/-}$*;RosaCreERT2;Bcl-x*$^{fl/+}$ (median survival, 28 days) or *p53*$^{-/-}$*;RosaCreERT2;Bcl-x*$^{fl/fl}$ lymphomas (median survival, 21 days) did not survive significantly longer than the tamoxifen-treated mice bearing control *p53*$^{-/-}$*;RosaCreERT2* lymphoma (median survival, 35 days; Figure 5B). Postmortem examination revealed that transplanted lymphoma cells consistently infiltrated organs in the abdominal cavity, including spleen, liver, pancreas, and ovaries, whereas only a small proportion engrafted into the thymus. There were no consistent differences in thymus, spleen, and liver weights or organ infiltration between mice bearing lymphomas of the different genotypes (also regardless of whether recipients had been left untreated or treated with tamoxifen; supplemental Figure 5A-C and histological data not shown).

Western blot analysis of thymic lymphomas that had expanded in tamoxifen-treated or control recipient mice showed similar levels of CreERT2, demonstrating no selection against expression of this latent recombinase. The reduction in BCL-XL levels in *p53*$^{-/-}$*;RosaCreERT2;Bcl-x*$^{fl/+}$ and *p53*$^{-/-}$*;RosaCreERT2;Bcl-x*$^{fl/fl}$ lymphomas appeared to be compensated by an increase in MCL-1 and a decrease in BIM (Figure 5C). These results demonstrate that BCL-XL is not required for the sustained survival and expansion of p53-deficient thymic lymphoma cells.

Inducible loss of MCL-1 abrogates the growth of *p53*$^{-/-}$ lymphomas in mice

Because MCL-1 appears critical for the development of lymphoma elicited by loss of p53 (Figure 2), we examined the impact of acute loss of MCL-1 on the growth of *p53*$^{-/-}$ lymphomas in mice using the

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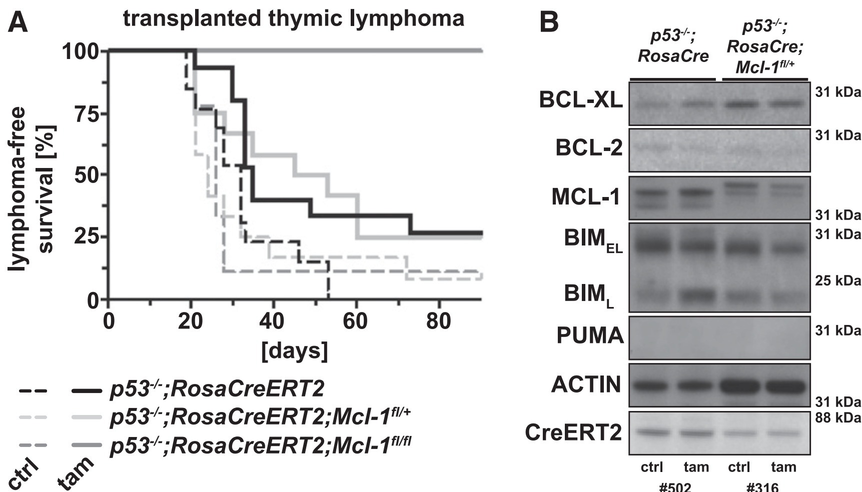

Figure 6. Acute loss of both alleles of *Mcl-1* significantly prolongs survival of mice bearing *p53*$^{-/-}$ lymphomas. This experiment was conducted as described in Figure 5A. (A) Lymphoma-free survival of the mice that had been transplanted with lymphoma cells of the genotypes indicated and treated with either tamoxifen (solid line to delete the floxed *Mcl-1* alleles) or that had been left untreated (dashed line; negative control). (B) Analysis of the proteins indicated by western blotting of transplanted thymic lymphoma samples of the indicated genotypes (representative of 3 samples each) that had grown in recipients treated with tamoxifen (tam) or recipients that had been left untreated (ctrl). Probing for actin was used as a loading control. Lanes 1 and 2 are the same samples as shown in lanes 1 and 2 of Figure 5C and are presented here for comparison.

protocol outlined above and depicted in Figure 5A. Mice transplanted with *p53*$^{-/-}$*;RosaCreERT2;Mcl-1*$^{fl/+}$ lymphomas did not survive significantly longer (median survival, 49 days) after tamoxifen treatment (to delete 1 *Mcl-1*^{fl} allele) compared with tamoxifen-treated recipients bearing *p53*$^{-/-}$*;RosaCreERT2* lymphomas (median survival, 35 days; P = .9831). In contrast, acute loss of both *Mcl-1*^{fl} alleles in *p53*$^{-/-}$*; RosaCreERT2;Mcl-1*$^{fl/fl}$ lymphomas significantly (P = .0002) prolonged the survival of mice transplanted with such tumors (Figure 6A). Remarkably, none (0/9 recipients, 3 independent thymic lymphomas) of the tamoxifen-treated recipients developed signs of lymphoma within 90 days following tumor transplantation, whereas all untreated control recipients bearing the same tumors rapidly developed lymphoma (median latency, 26 days; Figure 6A).

Mice transplanted with *p53*$^{-/-}$*;RosaCreERT2* or *p53*$^{-/-}$*; RosaCreERT2;Mcl-1*$^{fl/+}$ lymphomas had similar thymus, spleen, and liver weights and similar lymphoma dissemination in the abdomen regardless of whether they had been treated with tamoxifen (to delete 1 *Mcl-1* allele in the latter tumors) or had been left untreated (supplemental Figure 6A-C; data not shown). In contrast, thymus (P = .0077) and spleen (P = .0347) but not liver weights (P = .1016) of mice transplanted with *p53*$^{-/-}$*;RosaCreERT2;Mcl-1*$^{fl/fl}$ lymphomas treated with tamoxifen were significantly lower compared with those of untreated control recipients transplanted with the same lymphomas (supplemental Figure 6A-C). Notably, internal organs (eg, ovaries and pancreas) of tamoxifen-treated mice transplanted with *p53*$^{-/-}$*;RosaCreERT2;Mcl-1*$^{fl/fl}$ lymphomas were of healthy appearance at the end point of the study (90-day lymphoma-free survival).

Western blot analysis showed that *p53*$^{-/-}$*;RosaCreERT2;Mcl-1*$^{fl/+}$ lymphomas that were isolated from recipients that had been treated with tamoxifen had slightly lower levels of MCL-1 compared with the same lymphoma collected from untreated control recipients (Figure 6B). This indicates that *p53*$^{-/-}$ lymphomas can survive and expand with a minor reduction in MCL-1 but not in the complete absence of this pro-survival protein. Collectively, these results demonstrate that MCL-1 is essential for the sustained survival and expansion of *p53*$^{-/-}$ thymic lymphomas.

Discussion

Mutations in *p53* are found in ~50% of sporadic cancers in humans and inheritance of heterozygous mutations in *p53* results in the Li-Fraumeni cancer predisposition syndrome. Due to the critical role of p53 in DNA damage-induced apoptosis,[32-34] tumors-bearing mutations in *p53* are refractory to diverse anticancer therapeutics that elicit DNA lesions. With the intent to identify cancer vulnerabilities, we performed genetic studies to determine which pro-survival BCL-2 family member is critical for the development of lymphomas from nascent neoplastic cells in *p53*$^{-/-}$ mice and which one is essential for the sustained expansion of such malignant tumors. For this we used the p53-deficient (*p53*$^{-/-}$) mouse model; these animals are highly predisposed to develop thymic lymphoma (~80% incidence by ~150 days in *p53*$^{-/-}$ mice), sarcoma, and certain other cancers with a 100% cancer-related mortality by ~300 days.[5,6,35]

Loss of BCL-XL from the early TN3 T-cell progenitor stage (TCRβ^-CD3$^-$CD4$^-$CD8$^-$CD25$^+$CD44$^+$, using *Lck-Cre* to delete the floxed *Bcl-x* alleles) onward had no impact on thymic lymphoma development driven by loss of p53. This is consistent with our previous observation that combined inhibition of BCL-2, BCL-XL, and BCL-W with the BH3 mimetic ABT-737 did not delay tumor development in *p53*$^{-/-}$ mice,[36] although antagonism of BCL-XL function was most likely not complete in that study. These results demonstrate that BCL-XL is dispensable for the survival of nascent neoplastic cells that give rise to thymic lymphoma (usually TCRβ^+CD4$^+$CD8$^+$), although this pro-survival BCL-2 family member is critical for the survival of nontransformed TCR$^+$CD4$^+$CD8$^+$ immature thymocytes.[37] This suggests that the thymic lymphoma initiating cells in *p53*$^{-/-}$ mice are probably found in a more immature progenitor population (see below for further discussion).

Interestingly, loss of even a single *Mcl-1* allele substantially reduced the incidence and delayed the onset of thymic lymphoma in *p53*$^{-/-}$ mice. Thus, the malignant lymphoma cells may be highly dependent on MCL-1 or the tumor initiating cells in *p53*$^{-/-}$ mice may rely on MCL-1 for their survival. The tumor initiating cells in *p53*$^{-/-}$ mice have not yet been identified, but in γ radiation-induced thymic lymphoma, the cell of origin is an immature progenitor (Lin$^-$Sca-1$^+$c-Kit$^+$ cells or common lymphoid progenitor cells) in the bone marrow.[38] Our data indicate that the cell of origin of lymphoma in *p53*$^{-/-}$ mice is probably not a hematopoietic stem/progenitor cell but a T-lineage committed progenitor. This would explain the potent selection against loss of MCL-1 during lymphoma development that occurred in all *p53*$^{-/-}$*;Lck-Cre;Mcl-1*$^{fl/fl}$ and in ~50% of *p53*$^{-/-}$*; Lck-Cre;Mcl-1*$^{fl/+}$ mice; note that *Lck-Cre* becomes competent at recombining floxed genes at the TN3 TCRβ^-CD3$^-$CD4$^-$CD8$^-$ CD25$^+$CD44$^+$ T cell-committed progenitor stage. Notably, these

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cells require MCL-1 (but not BCL-XL) for their survival[39] and are rapidly proliferating and thus subject to replication errors, a potential source of oncogenic lesions that could cooperate with loss of p53 in lymphomagenesis.

Previous studies showed that MCL-1 is critical for the development of AML driven by deregulated c-MYC[19] or the MLL-ENL and AML-ETO fusion oncogenes[20] and BCR-ABL-driven pre-B lymphoma development.[40] Conversely, BCL-XL is required for c-MYC-driven pre-B/B lymphoma development.[16] Thus, there may be a cell type-specific and/or oncogenic lesion-specific dependency on distinct pro-survival BCL-2 family members for the development of different hematopoietic malignancies.

Interestingly, the thymic lymphomas in $p53^{-/-}$;$Mcl\text{-}1^{+/-}$, $p53^{-/-}$;*Lck-Cre*;$Mcl\text{-}1^{fl/+}$ and $p53^{-/-}$;*Lck-Cre*;$Mcl\text{-}1^{fl/fl}$ mice that expressed relatively low levels of MCL-1 (in the latter 2 due to recombination of 1 $Mcl\text{-}1^{fl}$ allele) generally had markedly reduced levels of proapoptotic BIM compared with the lymphomas of the same genotypes that had higher MCL-1 levels. This apparent selection for reduced BIM levels in tumors containing low MCL-1 levels suggests that preventing BIM-mediated apoptosis could be the crucial role of MCL-1 in the lymphoma initiating cells in $p53^{-/-}$ mice. How BIM is activated in lymphoma initiating cells is unclear but could be due to oncogene activation, replication stress or limited availability of growth factors, all of which initiate apoptosis in a BIM-dependent manner.[41,42]

The reduction in the incidence of thymic lymphoma in $p53^{-/-}$ mice with constitutive loss of 1 allele of *Mcl-1* or T cell-restricted loss of 1 or both *Mcl-1* alleles was accompanied by a concomitant increase in the incidence of sarcoma, a tumor that develops more slowly than the lymphomas in $p53^{-/-}$ mice.[5,6] The latter 2 strains only allow investigation of the impact of T cell-specific loss of MCL-1. In these mice the precursors of the sarcomas are unaffected by $Mcl\text{-}1^{fl}$ recombination, and sarcoma may thus occur at a higher frequency because the mice survive longer due to the delay in thymic lymphoma development. However, in the $p53^{-/-}$;$Mcl\text{-}1^{+/-}$ mice, all cells including those giving rise to sarcoma, lacked 1 allele of *Mcl-1*. In these mice, we observed a 2.6-fold increase in sarcoma accounting for a similar-fold decrease in thymic lymphoma incidence. This indicates that MCL-1 may be less critical for sarcoma development than for lymphomagenesis. Perhaps 1 or more other pro-survival BCL-2 family members guarantee the survival of the cells undergoing neoplastic changes to become sarcomas. Alternatively, evasion of apoptosis may be less critical for sarcoma development than for lymphomagenesis. In accordance with this hypothesis, it was shown that, although restoration of p53 caused apoptosis in $p53^{-/-}$ thymic lymphomas, this caused cellular senescence in $p53^{-/-}$ sarcomas.[43]

For the development of novel therapeutic strategies, it is essential to know which pro-survival BCL-2 family member is critical for the sustained survival and expansion of a particular tumor. This can be investigated by using specific drugs (if they exist, such as ABT-199 to inhibit BCL-2) or by conditional deletion of pro-survival *Bcl-2* family genes in malignant tumors by using the tamoxifen-regulated CreERT2 recombinase. By performing tumor transplant experiments and then inducing acute deletion of either *Mcl-1* or *Bcl-x* floxed alleles, we found that MCL-1 but not BCL-XL is critical for sustained survival and expansion of p53-deficient thymic lymphomas. This is reminiscent of previous reports, which showed that MCL-1 is also critical for the sustained growth of AML driven by various fusion oncogenes,[19,20] as well as c-MYC-driven pre-B/B lymphoma.[31] Collectively, these observations indicate that the transformed state driven by diverse oncogenic lesions (eg, deregulated MYC, loss of p53, and MLL-ENL) renders a broad range of malignant hematopoietic cells highly dependent on MCL-1. The reasons for this are presently not clear. Differences in expression of different pro-survival BCL-2 family members does not appear to be the answer, because the aforementioned tumors all express readily detectable levels of not only MCL-1 but also other pro-survival BCL-2 family members. These considerations are important to decide which cancers should be treated with which type of BH3 mimetic drugs. Although BCL-2 inhibition alone (using ABT-199) shows promise in clinical trials of chronic lymphocytic leukemia (CLL),[44] our data here suggest that MCL-1 blockade (if this does not cause unacceptable collateral damage) may be the treatment of choice for T-cell lymphomas, particularly those containing defects in the p53 pathway.

Acknowledgments

The authors thank Drs M. J. Herold, G. L. Kelly, and D. H. Gray for advice, P. Bouillet and L. A. O'Reilly for reagents, J. Mansheim, C. Gatt, L. Reid, S. Allan, K. Landells, and G. Siciliano for expert animal care, B. Helbert, H. Ierino, K. Mackwell, and C. Young for genotyping, and J. Corbin for automated blood analysis.

This work was supported by grants and fellowships from the Cancer Council of Victoria (postdoctoral fellowship to S.G., Sydney Parker Smith postdoctoral research fellowship to A.R.D.D., and postgraduate research scholarship to L.J.V.), the Lady Tata Memorial Trust (postdoctoral research award to S.G.), National Health and Medical Research Council (program grant 1016701; National Health and Medical Research Council fellowship 1020363 (to A.S.), the Leukemia and Lymphoma Society (Specialized Centres of Research grant 7001-03, to A.S.), University of Melbourne International Research Scholarship (to S.G.), University of Melbourne International Fee Remission scholarship (to S.G.), Australian Postgraduate award (to A.R.D.D.), Cancer Therapeutics CRC Top-up scholarship (to S.G. and A.R.D.D.), and the operational infrastructure grants through the Australian Government Independent Research Institutes Infrastructure Support Scheme and the Victorian State Government Operational Infrastructure Support.

Authorship

Contribution: S.G. conceived ideas, planned and conducted the majority of experiments, and wrote the manuscript; A.R.D.D. and L.J.V. helped with some experiments and manuscript writing; and A.S. provided experimental ideas and intellectual guidance and helped with manuscript writing.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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