Critical B-lymphoid cell intrinsic role of endogenous MCL-1 in c-MYC-induced lymphomagenesis

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Evasion of apoptosis is critical for tumorigenesis, and sustained survival of nascent neoplastic cells may depend upon the endogenous levels of pro-survival BCL-2 family members. Indeed, previous studies using gene-targeted mice revealed that BCL-XL, but surprisingly not BCL-2, is critical for the development of c-MYC-induced pre-B/B lymphomas. However, it remains unclear whether another pro-survival BCL-2 relative contributes to their development. MCL-1 is an intriguing candidate, because it is required for cell survival during early B-lymphocyte differentiation. It is expressed abnormally high in several types of human B-cell lymphomas and is implicated in their resistance to chemotherapy. To test the B-cell intrinsic requirement for endogenous MCL-1 in lymphoma development, we conditionally deleted Mcl-1 in B-lymphoid cells of Eμ-Myc transgenic mice. We found that MCL-1 loss in early B-lymphoid progenitors delayed MYC-driven lymphomagenesis. Moreover, the lymphomas that arose when MCL-1 levels were diminished appeared to have been selected for reduced levels of BIM and/or increased levels of BCL-XL. These results underscore the importance of MCL-1 in lymphoma development and show that alterations in the levels of other cell death regulators can compensate for deficiencies in MCL-1 expression.

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However, it is still unclear whether BCL-XL is the sole pro-survival BCL-2 family member required for MYC-induced pre-B/B-lymphoma development. MCL-1 is of particular interest. Increases in MCL-1 gene copy number and concomitantly elevated MCL-1 protein are found in a substantial fraction of diverse cancer types. For a few cell lines derived from such cancers, MCL-1 knockdown by RNA interference was shown to cause apoptosis, demonstrating that MCL-1 is critical for their sustained survival. Similarly, acute myeloid leukemia (AML) cells driven by enforced expression of c-MYC or the MLL-ENL and MLL-AF9 fusion onco-proteins and c-MYC- or BCR-ABL-driven pre-B/B lymphomas were rapidly killed upon inducible genetic deletion or blockade of MCL-1.

MCL-1 is critical for the survival of rapidly proliferating hematopoietic progenitors and non-transformed pro-B/pre-B cells, the cells thought to be the origin of Eµ-Myc lymphoma. Therefore, we examined the role of MCL-1 in pre-B/B cell lymphoma development in Eµ-Myc transgenic mice by incorporating CD19-Cre or Rag1-Cre alleles to impose Mcl-1 gene deletion exclusively in the B-lymphoid compartment. We report that there was marked selection against Mcl-1 gene loss during c-MYC-driven lymphoma development and a delay in tumor onset. Moreover, the lymphomas that arose despite successful Mcl-1 recombination exhibited abnormally low levels of pro-apoptotic BIM and/or increased levels of pro-survival BCL-XL. These results show that MCL-1 is critical for c-MYC-driven pre-B/B-lymphoma development, and suggest that alterations in other core components of the apoptotic machinery can compensate for a reduction in MCL-1 levels.

Results

Impact of B-cell lineage-restricted deletion of Mcl-1 on MYC-driven lymphomagenesis. To explore the impact of B cell-restricted deletion of one or both allele(s) of Mcl-1 on c-MYC-driven lymphoma development, we generated Eµ-Myc mice with one or both Mcl-1 alleles flanked by loxP sites (hereafter called Mcl-1fl/fl or Mcl-1fl/+ respectively). Some cohorts also expressed the Cre recombinase selectively either from the common lymphoid progenitor stage (CLP), using a Rag-1-Cre transgene, or from the late pro-B cell stage onwards, using a CD19-Cre transgene. In our Mcl-1 gene-targeted mice, recombination of the Mcl-1fl allele subjugates a human CD4 reporter transgene to the Mcl-1fl promoter/enhancer elements. Hence, human CD4 (hCD4) expression, which is readily detectable by flow cytometric analysis using fluorochrome-labeled anti-human-CD4 antibodies, serves as a reporter of Mcl-1fl deletion.

We first compared the incidence and rate of pre-B/B-cell lymphoma development in Eµ-Myc, Eµ-Myc;CD19-Cre, Eµ-Myc;CD19-Cre;Mcl-1fl/+ and Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice (Figure 1a). The lymphoma-free survival of the control mice without Mcl-1 deletion (Eµ-Myc and Eµ-Myc;CD19-Cre) was similar: median survivals of 91 days and 117 days, respectively (Mantle–Cox Log-rank test P = 0.069, Figure 1a).
indicating proteins were determined by Western blot analysis in three lymphomas from each of the indicated genotypes. The protein from the indicated genotypes, using primers that recognize both the Mcl-1wt and Mcl-1fl alleles, or detect the Cre recombinase transgene or the Eµ-Myc transgene. (c) Flow cytometric analysis of human CD4 reporter expression in lymphomas that arose in Eµ-Myc (negative control), Eµ-Myc;CD19-Cre;Mcl-1fl/+ or Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice.

Figure 2: Expression of BCL-2 family members, p53, MDM2 and p19-ARF in lymphomas from Eµ-Myc mice with CD19-Cre-mediated deletion of Mcl-1. (a) The levels of the indicated proteins were determined by Western blot analysis in three lymphomas from each of the indicated genotypes. The protein from the Mcl-1fl allele is slightly larger than the wt MCL-1 protein indicated by arrows. Probing for HSP70 was used as a loading control. (b) Genotype analysis of FACS-sorted B220+ pre-B/B-lymphoma cells from mice of the indicated genotypes, using primers that recognize both the Mcl-1fl and Mcl-1wt alleles, or detect the Cre recombinase transgene or the Eµ-Myc transgene. (c) Flow cytometric analysis of human CD4 reporter expression in lymphomas that arose in Eµ-Myc (negative control), Eµ-Myc;CD19-Cre;Mcl-1fl/+ or Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice.

With one or two floxed Mcl-1 alleles, there was a modest (albeit not statistically significant) delay in lymphomagenesis compared with the Eµ-Myc;CD19-Cre animals: 130 and 123 days, respectively (P = 0.16 for both). Autopsy on the sick, lymphoma-burdened mice revealed that the Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice (P = 0.0172) had significantly less lymphoma cells in the blood than Eµ-Myc;CD19-Cre mice, but no such drop was found for the sick Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice. No significant differences between the genotypes appeared for spleen and lymph node weights (Figure 1b), or the numbers of erythrocytes and thrombocytes in the blood (Figure 1c).

Selection against MCL-1 loss. As constitutive or inducible loss of MCL-1 impairs the development as well as sustained expansion of many tumors, we tested whether the Mcl-1fl allele(s) had been recombined in the lymphomas that arose in the Eµ-Myc;CD19-Cre;Mcl-1fl/+ and Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice, or whether selection against Mcl-1 gene loss had occurred during their malignant transformation. Western blot analysis revealed that many lymphomas from Eµ-Myc;CD19-Cre;Mcl-1fl/+ (3/3 tested) and Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice (2/3 tested) retained MCL-1 expression, but its levels were significantly lower than in lymphomas from Eµ-Myc control mice (Figure 2a). The reduced MCL-1 protein expression appeared to be accompanied by a significant decrease in BIM protein expression in the Eµ-Myc;CD19-Cre;Mcl-1fl/+ and Eµ-Myc;CD19-Cre;Mcl-1fl/fl lymphomas tested. Also, BCL-XL appeared to be upregulated in the Eµ-Myc;CD19-Cre;Mcl-1fl/+ lymphomas, in which Mcl-1fl deletion was efficient (Figure 2a). Compared with Eµ-Myc;CD19-Cre control lymphomas, BCL-2 protein expression was comparable in the Eµ-Myc;CD19-Cre;Mcl-1fl/+ lymphomas and, curiously, was lower in the Eµ-Myc;CD19-Cre;Mcl-1fl/fl lymphomas that had retained their Mcl-1fl alleles (Figure 2a). Consistent with the Western blot results, PCR analysis of FACS-sorted primary lymphoma cells confirmed that some of the lymphoma cells arising in Eµ-Myc;CD19-Cre;Mcl-1fl/+ and Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice had not excised or only partially excised their Mcl-1fl alleles (Figure 2b).

Flow cytometric analysis for the human CD4 reporter confirmed that most tumors arising in Eµ-Myc;CD19-Cre;Mcl-1fl/+ mice had efficiently excised their Mcl-1fl allele. However, only ~50~60% of the two floxed Mcl-1 alleles had been recombined in the lymphomas from the Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice (Figure 2c). This suggests that the remaining wild-type (wt) Mcl-1 allele in Eµ-Myc;CD19-Cre;Mcl-1fl/+ mice (Figure 2c). This suggests that the remaining wild-type (wt) Mcl-1 allele in Eµ-Myc;CD19-Cre;Mcl-1fl/+ mice is sufficient to sustain their survival during neoplastic transformation. In contrast, deletion of both Mcl-1fl alleles must impose a stress from which lymphoma-initiating B-lymphoid progenitors are unable to recover. Thus, lymphomas that arise in Eµ-Myc;CD19-Cre;Mcl-1fl/fl mice have potently selected against loss of both Mcl-1fl alleles and the stress caused by loss of one Mcl-1fl allele is partially relieved by adjustments in the levels of the BCL-XL and BIM proteins.
Overexpression of c-MYC causes selection bias against deletion of Mcl-1 alleles in pre-leukemic B-lymphoid cells. c-MYC promotes cell growth and cell proliferation, but under conditions of stress, such as nutrient or growth factor deprivation, high c-MYC levels predispose cells to undergo apoptosis.42–44 Pre-leukemic £µ-Myc mice exhibit increased numbers of pre-B cells in their bone marrow, spleen, lymph nodes and blood, but these cells are not transformed and consequently do not form tumors when transplanted into congenic recipient mice.37

Given that loss of one allele of Mcl-1 suffices to potently induce cell death in malignant £µ-Myc lymphomas,35 we hypothesized that loss of one Mcl-1 allele might also reduce the numbers of pre-leukemic pro-B, pre-B and/or slg^+ B cells (at 3–4 weeks of age) in £µ-Myc;CD19-Cre;Mcl-1^fl/+ mice compared with £µ-Myc and £µ-Myc;CD19-Cre control animals. The total bone marrow and lymph node cellularities of pre-leukemic £µ-Myc, £µ-Myc;CD19-Cre and £µ-Myc;CD19-Cre;Mcl-1^fl/+ mice were comparable to each other and to wt mice, but there was a notable increase, although not statistically significant, in the overall leukocyte numbers in the spleens of £µ-Myc and £µ-Myc;CD19-Cre mice compared with the £µ-Myc;CD19-Cre;Mcl-1^fl/+ animals and the wt controls (Figure 3a). As reported,37 £µ-Myc mice had more pre-B cells in their bone marrow than wt controls (Figure 3b). Interestingly, £µ-Myc;CD19-Cre mice had significantly fewer pre-B cells than £µ-Myc mice (Figure 3b; P = 0.0452), suggesting that the Cre recombinase imposes a cytotoxic stress on these cells. £µ-Myc;CD19-Cre;Mcl-1^fl/+ animals had even fewer pre-leukemic pre-B cells than the £µ-Myc;CD19-Cre animals, but this difference was not statistically significant (P = 0.10; Figure 3b), although the difference to the £µ-Myc mice was significant. There were no significant differences in the numbers of pro-B cells or slg^+ B cells in the bone marrow between mice of any of the genotypes examined (Figure 3b).

As some lymphomas that arose in £µ-Myc;CD19-Cre;Mcl-1^fl/+ mice had been selected for retention of their Mcl-1^fl allele, we hypothesized that there may be potent selection against loss of the Mcl-1^fl allele already in the pre-leukemic state. To examine this, we stained spleen cells from pre-leukemic £µ-Myc;CD19-Cre;Mcl-1^fl/+ mice as well as those from £µ-Myc, CD19-Cre;Mcl-1^fl/+ and wt animals with antibodies against B220 (B-cell marker) and hCD4 (reporter for £µ-Myc) (Figure 3c). As expected, the B-lymphoid cells from £µ-Myc and wt mice did not express hCD4. The B-lymphoid cells from CD19-Cre;Mcl-1^fl/+ mice were composed of two
distinct populations, one negative (~60%) and the other positive (~40%) for hCD4 (Figure 3c). This demonstrates that some B-lymphoid cells in these animals were able to delete the Mcl-1fl allele, although the efficiency was not very high. This is consistent with the previously reported 46 relatively poor recombination efficiency of the CD19-Cre deletion strain that we used in our experiments. Interestingly, in the Eµ-Myc;CD19-Cre;Mcl-1fl/+ mice only ~20% of the pre-leukemic B-lymphoid cells were hCD4+ (Figure 3c).

These findings reveal that deregulated c-MYC expression exerts potent selection against loss of one Mcl-1fl allele in B-lymphoid cells, whereas loss of one Mcl-1 allele is more readily tolerated in normal B-lymphoid cells.

Efficient deletion of Mcl-1fl allele(s) in B-lymphoid progenitors using the Rag1-Cre transgene substantially delays lymphomagenesis in Eµ-Myc mice. As CD19-Cre-mediated deletion of Mcl-1fl alleles was rather inefficient, we wanted to test whether deleting Mcl-1fl allele(s) more efficiently and at an earlier stage in B-cell development would have a greater impact in our lymphoma model. For this we employed the Rag1-Cre knockin allele, which was reported to recombine floxed genes with very high efficiency at the CLP stage. 46, 47 Lymphoma onset was slightly delayed from the CLP stage. 46, 47 Lymphoma onset was slightly delayed in the Eµ-Myc;Rag1-Cre mice compared with the Eµ-Myc control animals. Although this difference was not significant (P = 0.06), this indicates that the Rag1-Cre transgene exerts some toxicity on B-lymphoid cells undergoing neoplastic transformation. Remarkably, the median lymphoma-free survival of Eµ-Myc;Rag1-Cre;Mcl-1fl/+ mice (346 days) was far longer than in control Eµ-Myc (91 days) and Eµ-Myc;Rag1-Cre mice (129 days, P** = 0.003, Figure 4a), clearly demonstrating the importance of MCL-1 in c-MYC-induced lymphomagenesis.

The lymphoma-burdened, sick Eµ-Myc;Rag1-Cre;Mcl-1fl/+ mice showed significantly lower lymph node weights (*P = 0.031) and lymphocyte numbers in the peripheral blood (*P = 0.046) than sick Eµ-Myc;Rag1-Cre mice (Figures 4b and c). No significant differences were found in the spleen weights or in the numbers of erythrocytes and thrombocytes in the blood.

The marked delay in lymphoma development seen in the Eµ-Myc;Rag1-Cre;Mcl-1fl/+ mice suggested that Rag1-Cre was considerably more efficient in Mcl-1fl deletion than CD19-Cre. To test this hypothesis, we analyzed lymphoma cells from Eµ-Myc;Rag1-Cre and Eµ-Myc;Rag1-Cre;Mcl-1fl/+ mice for hCD4 expression (Figure 5a). Strikingly, the selection against cells expressing the hCD4 reporter (i.e. selection against cells that had deleted the Mcl-1fl allele) was clearly more potent in Eµ-Myc;Rag1-Cre;Mcl-1fl/+ lymphoma cells than in those from the Eµ-Myc;CD19-Cre;Mcl-1fl/+ mice (compare data in Figures 2c and 5a). In the absence of oncogenic stress, Rag1-Cre;Mcl-1fl/+ mice efficiently deleted one Mcl-1 allele in B-lymphoid cells, but interestingly, there was potent selection against loss of both Mcl-1 alleles even without c-MYC overexpression (Figure 5b). These results reveal that non-
transformed B-lymphoid cells can tolerate loss of one but not loss of both Mcl-1 alleles, whereas cells with deregulated c-MYC expression (both pre-leukemic cells undergoing transformation as well as malignant lymphomas) cannot tolerate even loss of a single allele. Unfortunately we were unable to generate \( E\mu\text{-Myc;Rag-1-Cre;Mcl-1}^{fl/\text{a}} \) mice due to issues with infertility.

Collectively, these data show that Mcl-1 is essential for the survival of MYC overexpressing pre-leukemic B-lymphoid cells undergoing neoplastic transformation. Therefore, B-lymphoid-restricted loss of one allele of Mcl-1 can substantially delay pre-B/B-lymphoma development in \( E\mu\text{-Myc} \) mice.

Discussion

Evasion of cell death is considered an essential requirement for the development of cancer. Impaired apoptosis in cancer cells (particularly in hematological malignancies) often results from deregulated expression of pro-survival or pro-apoptotic members of the BCL-2 protein family. In cells undergoing neoplastic transformation, apoptosis can be triggered by stress conditions induced by newly acquired oncogenic mutations (e.g. deregulated c-MYC expression) or by limiting availability of nutrients or growth factors from the tumor micro-environment. Regardless of the trigger that activates apoptosis signaling, evasion of cell death is essential for a population of nascent neoplastic cells to expand and sub-clones to acquire additional oncogenic lesions that cooperate with the initiating oncogenic mutation(s) to promote emergence of malignant cells.

Although BCL-2 overexpression greatly accelerates lymphomagenesis in \( E\mu\text{-Myc} \) transgenic mice, endogenous BCL-2 is dispensable for MYC-driven lymphoma development. In contrast, BCL-XL was found to be essential for the survival of both normal as well as c-MYC over-expressing B-cell progenitors and its loss therefore inhibited lymphoma development in \( E\mu\text{-Myc} \) mice. Here we show that MCL-1 is also critical for c-MYC-driven lymphoma development.

We employed two Cre transgenic strains to delete Mcl-1 either at the late pro-B cell (CD19-Cre) or the CLP stage (Rag-1-Cre). Surprisingly, we found that lymphoma development in the \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) and \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) mice was only slightly slower than in the control \( E\mu\text{-Myc;CD19-Cre} \) mice. The difference to the \( E\mu\text{-Myc} \) mice was statistically significant but the difference to the \( E\mu\text{-Myc;CD19-Cre} \) mice was not, probably because constitutive Cre activity imposes a slight toxicity in B-lymphoid cells, as previously observed in other cell types. Interestingly, in young, pre-leukemic \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) mice considerably fewer B-lymphoid cells had deleted their Mcl-1 allele (detected as human CD4) than in the \( CD19-Cre; Mcl-1^{fl/\text{a}} \) animals. This demonstrates that deregulated c-MYC expression renders nascent neoplastic cells exquisitely dependent on an adequate MCL-1 protein level (i.e., provided by both Mcl-1 alleles) for their survival. This selection against pre-leukemic B-lymphoid cells that had deleted their Mcl-1 allele(s) explains why some pre-B/B-lymphomas arising in \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) and \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) mice had been selected against loss of their Mcl-1 allele(s). Thus, cells retaining their full MCL-1 complement had an advantage in progressing through further steps of neoplastic transformation. Moreover, lymphomas that arose in \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) and \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) mice despite loss of one Mcl-1 allele appeared to have undergone selection for upregulation of BCL-XL and/or a reduction in pro-apoptotic BIM. This in turn suggests that keeping BIM in check constitutes a major function for MCL-1 in B-lymphoid cells undergoing transformation.

Lymphoma-free survival was extended to a much greater extent in \( E\mu\text{-Myc;Rag-1-Cre;Mcl-1}^{fl/\text{a}} \) mice compared with the \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) and \( E\mu\text{-Myc;CD19-Cre;Mcl-1}^{fl/\text{a}} \) animals. This may indicate that loss of one Mcl-1 allele at the earlier CLP stage of lymphoid cell development (i.e., when
Rag1-Cre but not CD19-Cre is expressed) is more efficient in killing incipient neoplastic cells and therefore more efficient in delaying lymphoma development compared with Mcl-1<sup>−/−</sup> deletion at the later pro-B-cell stage (when CD19-Cre expression commences). Alternatively, the Rag1-Cre transgene may simply be more efficient than the CD19-Cre transgene; the latter would therefore more readily allow escape of B-lymphoid cells that had failed to excise Mcl-1<sup>−/−</sup>. In conclusion, our findings demonstrate that MCL-1 is critical for the survival of c-MYC overexpressing lymphoma-initiating cells and hence for development of lymphoma. MCL-1 appears to be more important than BCL-XL because loss of one Mcl-1 allele substantially delayed lymphoma development in Eμ-Myc;Rag1-Cre;Mcl1<sup>fl/fl</sup> mice, whereas loss of one Bclx allele had only minor impact. Loss of BIM-restored lymphoma development in mice with an Eμ-Myc; Bclx<sup>−/−</sup> lymphoid system and many pre-B/B lymphomas that arose in Eμ-Myc;CD19-Cre;Mcl1<sup>fl/fl</sup> or Eμ-Myc;CD19-Cre; Mcl1<sup>−/−</sup> mice despite loss of one Mcl-1 allele appeared to have undergone selection for low levels of BIM. This suggests that BIM is the critical pro-apoptotic BH3-only protein activated in response to oncogenic stress to kill Eμ-Myc pre-leukemic B-lymphoid cells to suppress progression to malignant lymphoma. These results and the observation that loss of even a single allele of Mcl-1 efficiently kills malignant Eμ-Myc lymphoma cells<sup>41</sup> provide further impetus to develop MCL-1 specific inhibitors (e.g. BH3 mimetics) for cancer therapy.<sup>50,51</sup> Materials and methods Experimental mice. All experiments with mice were conducted according to the guidelines of The Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. Eμ-Myc transgenic mice (generated on a mixed C57BL/6x6JL background and then backcrossed for >30 generations onto a C57BL/6 background) expressing the c-Myc transgene under control of the immunoglobulin heavy chain gene enhancer Eμ have been previously reported.<sup>35</sup> The Mcl-1<sup>−/−</sup> mice were generated on a C57BL/6 background using C57BL/6-derived ES cells. The Rag1-Cre<sup>Cre/Cre</sup> and CD19-Cre<sup>Cre/Cre</sup> mice were generated on a mixed C57BL/6x129SV genetic background using 129SV-derived ES cells and then backcrossed onto a C57BL/6 background for >20 generations before commencement of our studies. Genotyping. Genotyping was performed as previously reported.<sup>36</sup> Oligonucleotide sequences for genotyping of these alleles will be provided on request. Analysis of lymphoma-burdened mice. Eμ-Myc transgenic mice were examined daily by animal technicians for signs of malignant disease. Mice were sacrificed when declared unwell by the animal technicians. Signs of disease included splenomegaly, lymphadenopathy, hind-limb paralysis, hunched stature, weight loss and labored breathing (indicative of lymphoma growth in the thymus). Sick mice were euthanized, tissues removed, weighed and then used for flow cytometry and histological analyses and tissue culture. Western blot analysis. Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein lysates (30 μg protein) mixed with 4x Laemmli buffer were loaded onto a 10% Bis-Tris gel (Life Technologies, Scoresby, VIC, Australia) and electrophoresis was conducted according to the manufacturer’s instructions. Proteins were transferred onto nitrocellulose membranes using the iBlot system (Life Technologies, Scoresby, VIC, Australia). Nitrocellulose membranes were blocked for 2 h using 5% skim milk powder dissolved in phosphate-buffered saline supplemented with 0.5% Tween-20. Western blots were probed with the following monoclonal or polyclonal antibodies: rabbit anti-mouse MCL-1 (19Ca-15), hamster anti-mouse BCL-2 (3F11), mouse anti-mouse BCL-XL (BD Pharmingen, BD BioSciences, San Jose, CA, USA; 2F12), rabbit anti-mouse BIM (Stressgen, 9292), rabbit anti-mouse PUMA (Ab-27669, Abcam, Melbourne, Victoria, Australia), mouse anti-HSP70 (R Anderson, Peter McCallum Cancer Centre; loading control), rabbit anti-mouse p53 (Leica Biosystems, Mount Waverley, Victoria, Australia; CMS) and rat anti-mouse p19-ARF (Rockland Immunoc benchers, Pottstown, PA, USA; 5.C3.1), overnight at 4°C. Blots were washed three times in phosphate-buffered saline supplemented with 0.5% Tween-20. The blots were then incubated for 1 h at room temperature with secondary HRP-conjugated antibodies against mouse, rat, hamster or rabbit IgG and again washed before exposure to the Amersham ECL reaction and developing on an autoradiograph Hyperfilm (GE Healthcare, Parramatta, NSW, Australia). Lymphoma and pre-leukemic analysis by flow cytometry. Lymphoid organs were harvested from lymphoma-burdened mice and single-cell suspensions prepared using forceps. Cells (5 x 10<sup>5</sup>) were resuspended in buffered saline supplemented with 10% FCS and 2% normal rat serum and stained for 30 min at 4°C with rat monoclonal antibodies to B220 (RA3-682, The Walter and Eliza Hall Institute (WEHI)), ckit (ACK4, WEHI), IgM (5.1.7, WEHI) and IgD (11-26, WEHI; all produced and conjugated with fluorochromes in our laboratory) and mouse monoclonal antibody to human CD4 (BD Pharmingen #555347, RPA-T4). Statistical analysis. Kaplan–Meier mouse survival curves were generated and analyzed with GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). Mouse cohorts were compared using the log-rank Mantel–Cox test. P values of <0.05 were considered significant. In vitro cell survival, blood cell counts, organ weights and RNA levels were plotted and analyzed with GraphPad Prism using two-tailed student’s t-test comparing two groups with each other. Error bars are presented as standard error of mean (± s.e.m.). Conflict of Interest SG, ARDD, GLK, PB, JMA and AS are employed by The Walter and Eliza Hall Institute, which receives milestone payments from Genentech and AbbVie for the development of ABT-199 for cancer therapy. Acknowledgements. We thank Prof S Cory for mice; Dr. LA O’Reilly for discussions; L Reid, J Mansheim, N Iannarella, S Allan, C D’Alessandro and G Siciliano for expert animal care; B Helbert for genotyping; J Corbin for automated blood analysis. This work was supported by grants and fellowships from the Cancer Council of Victoria (SG, ARDD), Leukaemia Foundation Australia (SG), the Lady Tata Memorial Trust (SG), Cure Brain Cancer Australia (AS), the National Health and Medical Research Council (Program Grant #1016701, NHMRC Australia Fellowship 1020363 to AS, Project Grant #1086291 to GLK), the Leukemia and Lymphoma Society (SOCR Grant #7001-03 to AS), Kay Kendall Leukemia Fund Interim Fellowship KKL331 (GLK), Cancer Council Victoria Grant-In-Aid #1086157 (GLK), Melbourne International Research and the Melbourne International Fee Remission Scholarship (University of Melbourne, SG) and Cancer Therapeutics CRC Top-up Scholarship (SG, ARDD), The estate of Anthony (Toni) Redstone OAM, University of Melbourne International Research and International Fee Remission Scholarships (SG), Australian Postgraduate Award (ARDD), and the operational infrastructure grants through the Australian Government IRIISS and the Victorian State Government OIS. 1. 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In vitro cell survival, blood cell counts, organ weights and RNA levels were plotted and analyzed with GraphPad Prism using two-tailed student’s t-test comparing two groups with each other. Error bars are presented as standard error of mean (± s.e.m.). Conflict of Interest SG, ARDD, GLK, PB, JMA and AS are employed by The Walter and Eliza Hall Institute, which receives milestone payments from Genentech and AbbVie for the development of ABT-199 for cancer therapy. Acknowledgements. We thank Prof S Cory for mice; Dr. LA O’Reilly for discussions; L Reid, J Mansheim, N Iannarella, S Allan, C D’Alessandro and G Siciliano for expert animal care; B Helbert for genotyping; J Corbin for automated blood analysis. 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