Anti-apoptotic A1 is not essential for lymphoma development in Eµ-Myc mice but helps sustain transplanted Eµ-Myc tumour cells

Mark Mensink1,2,3 · Natasha S. Anstee1,2,4 · Mikara Robati1 · Robyn L. Schenk1,2 · Marco J. Herold1,2 · Suzanne Cory1,2 · Cassandra J. Vandenberg1,2

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Abstract
The transcription factor c-MYC regulates a multiplicity of genes involved in cellular growth, proliferation, metabolism and DNA damage response and its overexpression is a hallmark of many tumours. Since MYC promotes apoptosis under conditions of stress, such as limited availability of nutrients or cytokines, MYC-driven cells are very much dependent on signals that inhibit cell death. Stress signals trigger apoptosis via the pathway regulated by opposing fractions of the BCL-2 protein family and previous genetic studies have shown that the development of B lymphoid tumours in Eµ-Myc mice is critically dependent on expression of pro-survival BCL-2 relatives MCL-1, BCL-W and, to a lesser extent, BCL-XL, but not BCL-2 itself, and that sustained growth of these lymphomas is dependent on MCL-1. Using recently developed mice that lack expression of all three functional pro-survival A1 genes, we show here that the kinetics of lymphoma development in Eµ-Myc mice and the competitive repopulation capacity of Eµ-Myc haemopoietic stem and progenitor cells is unaffected by the absence of A1. However, conditional loss of a single remaining functional A1 gene from transplanted A1-a−/− A1-b−/− A1-c−/− Eµ-Myc lymphomas slowed their expansion, significantly extending the life of the transplant recipients. Thus, A1 contributes to the survival of malignant Eµ-Myc-driven B lymphoid cells. These results strengthen the case for BFL-1, the human homologue of A1, being a valid target for drug development for MYC-driven tumours.

c-MYC (hereafter MYC), a basic helix–loop–helix leucine zipper transcription factor that regulates a multiplicity of genes involved in cell growth, proliferation and metabolism [1–3], has been implicated in the aetiology of many, perhaps all, human malignancies [4]. MYC levels are tightly regulated in normal cells but a wide range of mutations can override transcriptional and post-transcriptional controls and many provoke over-expression. High levels of MYC provoke apoptosis when cells experience stress from, for example, limited availability of cytokines or nutrients [5–7] and this imposes a brake on proliferation [8]. Consequently, anti-apoptotic mutations release that brake and synergise with MYC to promote malignant transformation [9–14].

MYC induces apoptosis via the pathway regulated by the BCL-2 family of proteins. BCL-2 and its closest homologues (BCL-XL, BCL-W, MCL-1 and A1/BFL-1) promote cell survival by neutralising pro-apoptotic relatives: BAX and BAK, and more distant relatives known as BH3-only proteins (for recent reviews see [15–17]). In healthy cells, BAX and BAK are primarily in an inactive monomeric state and any activated monomers are restrained by the pro-survival proteins. Stress conditions, such as deprivation of cytokines, oncogene expression or DNA damage, provoke up-regulation of pro-apoptotic BH3-only proteins, which bind tightly to the hydrophobic surface groove of pro-survival proteins via their BH3 domains, preventing them from interacting with other pro-survival proteins.
from inhibiting BAX and BAK. Certain BH3-only proteins (BIM, tBID, PUMA) can also bind transiently to the analogous surface groove of BAX and BAK, provoking a dramatic conformational change that prompts their homodimerisation [18–20]. BAX/BAK homo-dimers then aggregate to form homo-oligomeric pores, through which cytochrome c egresses to initiate sequential activation of caspases, which cleave hundreds of vital proteins, thereby dooming the cell.

Chromosome translocations found in Burkitt’s lymphoma and mouse plasmacytomas link the c-MYC gene to Ig gene loci [21], thereby subjugating MYC expression to
strong Ig gene enhancers. Transgenic Eμ-Myc mice developed to model such translocations [22, 23] have greatly advanced our understanding of MYC-driven lymphomagenesis. Overexpression of MYC promotes polyclonal expansion of highly proliferative non-malignant pre-B cells [22–24] that are highly susceptible to apoptosis [11] and progression to malignancy depends upon acquisition of additional synergistic somatic changes such as mutation of RAS [25] or of the p19ARF/p53 pathway [12]. Of note, lymphomagenesis in these mice is accelerated by overexpression of BCL-2 and other pro-survival homologues [10, 26, 27] or loss of BH3-only proteins BIM [13, 28], PUMA [14], BMF or BAD [29].

Different cell types have a greater or lesser dependence on individual pro-survival family members, depending on the relative expression of other BCL-2 family members. Tumour cells are particularly dependent, because they express high levels of BH3-only proteins such as BIM due to the stresses cells are particularly dependent, because they express high individual pro-survival family members, depending on the expression of endogenous MCL-1 [32, 33], BCL-W [34] and, to a lesser extent, BCL-XL [32, 35] but not BCL-2 [36]. Mouse A1 mice were either B220+/sIg− (denoted by pro/preB), B220+/sIg+ (denoted by B) or a mixture of these phenotypes (denoted by mixed). No significant differences were found (Student’s T-test)
or kinetics (Fig. 1c) of lymphoma type. Furthermore, when sick mice were autopsied, there were no significant differences in the leukaemic burden in the blood and haemopoietic organs (Fig. 1d, Supplementary Figure S2b).

\( E\mu\)-Myc-driven tumour development is believed to initiate in the expanded pool of pro and pre-B cells in the bone marrow and spleen of young mice. [23] To ascertain whether loss of A1 had perturbed the premalignant phenotype, we analysed haemopoietic tissues of healthy young 4-week-old mice using immunostaining and flow cytometry. Consistent with the unchanged kinetics of tumour development, the \( A1^{+/+} \) \( E\mu\)-Myc and \( A1^{-/-} \) \( E\mu\)-Myc mice had comparable numbers of B220\(^+\) sIg\(^-\) cells in the bone marrow and spleen (Fig. 2, Supplementary Table S1). sIg\(^+\) B cells and all other major cell populations were also comparable. There were no notable differences between the \( A1^{+/+} \) and \( A1^{-/-} \) non-transgenic mice, as also reported elsewhere. [44]

Immature B lymphoid cells overexpressing MYC are highly susceptible to apoptosis [11–14]. To determine whether loss of A1 further increased their vulnerability, pro/pre-B cells (B220\(^+\)sIg\(^-\)) were isolated from bone marrow using flow cytometry and cultured in vitro without any added cytokines. Analysis over the 48 h-period showed no increase in susceptibility to apoptosis (Fig. 3a).

A1 expression was analysed in pre-malignant pro/pre-B and B cells from healthy young WT and \( E\mu\)-Myc mice by
western blot analysis (Fig. 3b). A1 was readily apparent in splenic B cells (B220+ slg+), being higher in the WT than the Eµ-Myc transgenic cells. However, it was not detectable in bone marrow pro/pre-B cells (B220+ slg-) of either genotype. These observations differ from those of Sochalska et al. [46] who, using the same antibody, detected A1 in pro-B as well as B cells and at higher levels in Eµ-Myc than WT populations. The reason for the difference is not clear but could be related to genetic background or other differences (see Discussion). The lack of significant A1 expression in pro/pre-B lymphoid cells in our Eµ-Myc mice would explain why A1+/+ and A1−/− Eµ-Myc pro/pre-B cells did not differ in their sensitivity to apoptosis (Fig. 3a). As previously reported [47], MCL-1 expression was elevated in the pre-leukaemic Eµ-Myc cells, particularly pro/pre-B cells, where MYC levels are higher than those in slg− B cells (Fig. 3b). Loss of A1 did not alter the level of MCL-1 or MYC within comparable cell populations.

To investigate whether changes in the expression of other BCL-2 family members or mutation of the p19ARF/p53 pathway might have compensated for the absence of A1 during lymphomagenesis, western blot analysis was performed. A1 protein was readily detected, at varying levels, in pro/pre-B, B and mixed lymphomas taken from lymph nodes of A1+/+ Eµ-Myc mice (Fig. 4a, b and Supplementary Figure S2c). As in Fig. 3b, however, neither of the control A1+/+ Eµ-Myc pre-malignant pro/pre-B samples (CD19+ cells from bone marrow) expressed A1 at detectable levels. Thus, either A1 levels increase in pro/pre-B cells during malignant transformation or its expression is dependent on the microenvironment.

MCL-1 was clearly present in all tumours (16/16) (Fig. 4) but, perhaps counter-intuitively, was lower in the A1−/− than the A1+/+ tumours. BCL-2 was readily detectable in 15/16 tumours, at variable levels, and low in all pre-leukaemic samples. BCL-XL was low in all samples except for two A1+/+ Eµ-Myc B lymphomas. Overall, there was no consistent difference in the expression pattern for A1 positive vs. A1-negative cells, for either the pro-survival proteins or for BH3-only proteins PUMA and BIM.

About 30% of Eµ-Myc tumours carry mutations in the p53 pathway [12, 13]. Consistent with this, p53 and/or high p19ARF was evident in 2 of the 16 lymphomas analysed (Fig. 4), one being A1−/− and the other A1+/−.

Taken together, these observations indicate that absence of endogenous A1 has no impact on the kinetics or phenotype of tumour development in Eµ-Myc mice. Although no consistent compensatory changes were detected, this does not rule out the possibility that individual tumours have compensated for the absence of A1 by modulating the expression of different pro- and anti-apoptosis genes.

Loss of A1 confers no disadvantage during competitive repopulation

We next compared the competitive ability of A1+/+ and A1−/− haemopoietic stem and progenitor cells (HSPCs) from Eµ-Myc mice in a bone marrow reconstitution assay. We used UBC-GFP/Eµ-Myc as competitor bone marrow cells mixed in a 1:1 ratio with either A1+/+ Eµ-Myc or A1−/− Eµ-Myc bone marrow cells. The donor cells, which were all Ly5.2, were transplanted into lethally irradiated Ly5.1 recipients and blood was analysed by flow cytometry 6 weeks later. Fig. 5 shows that loss of A1 did not impair the haemopoietic repopulation capacity of Eµ-Myc HSPCs:
those from \( A^{1/-} \) Myc mice were as competitive as those from \( A^{1+/+} \) Myc mice, as both genotypes (GFP\(^{-}\)) constituted \(~50\%\) of the Ly5.2\(^{+}\) cells in all cell populations analysed, including pre-B and B cells.

**Impact of loss of A1 in transplanted lymphomas**

Finally, to ascertain whether A1 plays a role in supporting expansion of fully malignant MYC-driven cells in vivo, we undertook conditional deletion in transplanted Eµ-Myc tumours, the strategy used to establish the essential role of MCL-1 for the survival of Eµ-Myc lymphoma cells in vivo [32]. To do so, we first generated Eµ-Myc lymphomas carrying floxed \( A^{1/-} \) alleles and an inducible Cre gene, by crossing Eµ-Myc mice with \( A^{1/-} A^{1-b}^{0/0} A^{1-d}^{-/-} \) (hereafter \( A^{100} \)) mice [44] and Rosa26CreERT2 mice (hereafter CreERT2) [48], which express Cre recombinase that is inactive in the absence of tamoxifen (see Materials and Methods).

When we compared A1 expression levels in premalignant B lymphoid cells from healthy young 4-week-old mice (Supplementary Figure S3a), perhaps not surprisingly, A1 expression was substantially less in the premalignant B cells of \( A^{100} \) (ie \( A^{1-a}^{--} A^{1-b}^{0/0} A^{1-d}^{--} \)) Eµ-MycCreERT2 mice compared to those from \( A^{1+/+} \) Eµ-MycCreERT2 mice and the pro/pre-B cells did not detectably express A1, consistent with the data presented above for premalignant Eµ-Myc pro/pre-B cells (Figs 3b, 4).

Lymphomas arose with comparable kinetics in each of the Eµ-Myc genotypes: \( A^{1+/+} \) Eµ-Myc, \( A^{1+/+} \) Eµ-MycCreERT2, \( A^{100} \) Eµ-MycCreERT2 and \( A^{100} \) Eµ-MycCreERT2 (all Ly5.2\(^{+}\)) (Supplementary Figure S3b). Multiple lymphomas of each genotype were transplanted intravenously into non-irradiated C57BL/6-Ly5.1 recipients (\( 3 \times 10^{6} \) cells into each of 6 recipients per tumour). On days 5 and 6, three of the recipients were treated with tamoxifen by oral gavage and three received vehicle, then survival was monitored until ethical endpoint (Fig. 6a, Supplementary Figure S3c). Notably, mice transplanted with lymphomas carrying floxed \( A^{1-b} \) alleles that were subsequently treated with tamoxifen (red) had a median survival of 6–7 days.
Fig. 5 A1−/− Eμ-Myc haemopoietic stem and progenitor cells are as competitive as A1+/+ Eμ-Myc cells in reconstituting haemopoiesis. Bone marrow competitive reconstitution experiments were set up using UBC-GFP/Eμ-Myc as the competitor. For analysis these can be distinguished by GFP expression from the A1+/+ Eμ-Myc and A1−/− Eμ-Myc test bone marrow cells, and by Ly5.2 expression from the Ly5.1 recipients. For each experiment UBC-GFP/Eμ-Myc cells were mixed in a 1:1 ratio with either A1+/+ Eμ-Myc or A1−/− Eμ-Myc cells and a total of 3 × 10⁶ cells transplanted into those lethally irradiated Ly5.1 recipients. Six weeks post reconstitution, mice were bled and analysed for GFP, Ly5.2, IgM, IgD, CD4, CD8, Mac1 and Gr1 expression by flow cytometry. The data shown here represents mean ± SEM of 6 independent experiments. Data plotted is % GFP of total Ly5.2+, so is a comparison of the test bone marrow vs only UBC-GFP/Eμ-Myc (Ly5.1 cells have been excluded) longer than those treated with vehicle (A1ββ+ Eμ-Myc-CreERT2, P = 0.0019 and A1ββ Eμ-MycCreERT2, P = 0.0075). The implication is that loss of A1 had impaired the expansion of the tumour cells by enhancing apoptosis.

To ascertain whether tamoxifen had indeed induced deletion of the floxed A1-b gene, the tumours that eventually killed the recipients were analysed by PCR. For all tumours analysed (n = 30), A1-b floxed allele(s) were efficiently deleted by tamoxifen activation of CreERT2 (e.g. Fig. 6b).

Discussion

The development of lymphomas in Eμ-Myc mice is dependent on expression of BCL-2 family members MCL-1 [33], BCL-XL [35] and BCL-W [34] but not BCL-2 [36]. This dependence on pro-survival BCL-2 family members is attributed to the increased susceptibility to apoptosis of developing B lymphoid cells in Eμ-Myc mice [11, 12, 49].

Recently, Sochalska et al. [46] reported that knockdown of all A1 genes did not alter the kinetics of Eμ-Myc lymphomagenesis but, finding that pre-B cells with reduced A1 levels were underrepresented in haemopoietic organs and that the tumours that arose had escaped A1 knockdown, suggested a vital role for A1 in the development of Eμ-Myc lymphomas.

We found no evidence of a requirement for A1 during lymphoma development in Eμ-Myc mice. There was no difference in the onset of tumour-induced morbidity in A1 nullizygous vs. WT Eμ-Myc mice (Fig. 1) and neither was there any alteration in the number of premalignant B lymphoid cells (Fig. 2) or in the repopulating capacity of A1+/ Eμ-Myc haemopoietic stem and progenitor cells (Fig. 5). It is not surprising that premalignant cell numbers were not altered by A1 deletion as we were unable to detect A1 expression in pre-B cells from either WT or Eμ-Myc mice (Fig. 3b). In contrast, A1 expression was readily detectable in pro/pre B lymphoma cells of A1+/ Eμ-Myc mice (Fig. 4), consistent with the selective pressure observed by Sochalska et al. to maintain A1 during lymphomagenesis [46].

Differences found in the aspects of the two studies may reflect major differences in the experimental systems. We used germline deleted A1 knockout mice (A1−/− A1-b−/− A1-d−/−) [44] and, during their lengthy stepwise derivation, these mice may well have adapted to lack of A1. In contrast, Sochalska et al. used haemopoietic-specific mosaic expression of transgenic A1 shRNA to constitutively knock down all three functional A1 genes [46]. Furthermore, while the (VV-A1) mice generated by Sochalska et al. had been bred to C57BL/6 mice, residual genetic differences also seem possible, as the line was originally generated and maintained in a C57BL/6 x CBA F1 background [41]. Since A1 expression levels vary with the activation status of B cells [43, 50] differences in the pathogen load within the animal facilities may also have played a role. In this regard we note that the impaired early T-cell differentiation, B cell homoeostasis and granulopoiesis reported for constitutive A1 knockdown [41] were not observed in the A1 knockout mice derived in our animal facility [44].

The importance of individual pro-survival BCL-2 family members varies at different stages of Eμ-Myc lymphomagenesis. While BCL-XL1 expression helps prevent apoptosis during the development of Eμ-Myc lymphomas [35], it is dispensable for the sustained growth of fully malignant lymphoma cells in transplant recipients [32]. In contrast, MCL-1 is essential for Eμ-Myc lymphomas at both stages [32, 33]. Cognisant of such differences, we also assessed the requirement for A1 during the expansion of established Eμ-Myc lymphoma cells and observed a significant delay in tumour progression of transplanted A1ββ Eμ-Myc lymphoma cells following activation of Cre-mediated deletion (P = 0.0075, Fig. 6). In view of the possibility of adaptation to the lower level of A1 in A1−/− A1-bββ A1-c−/− Eμ-Myc mice (Supplementary Figure S3a), the impact of A1 loss might be even greater should it be possible to simultaneously delete all three functional alleles. Similarly, inhibition of BFL-1, the sole A1 analogue in humans, may have substantial impact.

In summary, taken together with the study by Sochalska et al. [46], our data suggest that, like MCL-1, BFL-1 is a potential target for the treatment of MYC-driven human tumours. A BFL-1-specific BH3 mimetic should be a useful avenue to pursue.
Fig. 6 Conditional deletion of floxed A1-b gene in transplanted Eµ-Myc lymphomas enhances survival of transplanted mice. a Survival curves of mice transplanted with Eµ-Myc-driven lymphomas and then treated with tamoxifen or vehicle. Each individual (Ly5.2+) tumour from female primary mice (Supplementary Figure S3b) was transplanted intravenously into 6 female Ly5.1+ recipients (3 × 10⁶ cells/recipient), three of which received tamoxifen (200 mg/kg body weight) on d5 and d6 post-transplantation and three received vehicle alone. Mice transplanted with A1+/+ Eµ-Myc (n = 7 independent primary tumours) or A1+/+ Eµ-MycCreERT2 tumours (n = 7) showed no significant difference in survival between tamoxifen and vehicle treatment. However, mice transplanted with A1fl/+ Eµ-MycCreERT2 tumours (n = 8) survived significantly longer following tamoxifen treatment (median survival 14 d for vehicle-treated mice vs. 21 d for tamoxifen-treated mice; P = 0.0019, log-rank test), as did mice transplanted with A1fl/fl Eµ-MycCreERT2 tumours (n = 12) (median survival of 17 d for vehicle-treated mice vs. 23 d for tamoxifen-treated mice; P = 0.0075, log-rank test). Data for A1-1 and A1-2 cohorts have been pooled. (see Supplementary Figure S3C for the data divided into the individual A1 lines). b Analysis of lymphomas arising in the lymph nodes of transplant recipients after treatment with tamoxifen or vehicle. DNA was purified from sorted Ly5.2+ lymphoma cells (to exclude any recipient cells, which would not have floxed A1 alleles), after which PCR was performed to confirm the presence of Eµ-Myc, RosaCreERT2, and to determine which A1-b alleles were present (A1-d was also analysed as a control). The number of the primary tumour that was transplanted is indicated above the lanes, and whether the recipient had been treated with tamoxifen or vehicle is indicated as + or − respectively. The survival (in days) of recipients post transplantation is indicated below the blot. In all, 30 tumours were analysed by PCR (11 A1fl/+ Eµ-Myc CreERT2 and 19 A1fl/fl Eµ-Myc CreERT2 lymphomas) and in every case deletion of the floxed A1b alleles was very efficient following tamoxifen treatment.
since BFL-1 is overexpressed in a variety of haemopoietic and other malignancies: ALL, CLL, AML, DLBCL, melanoma, stomach and colon cancers and breast cancer [51].

Materials and methods

Mice

Experimental protocols involved in the use of mice were conducted according to the guidelines of the Animal Ethics Committee of the Walter and Eliza Hall Institute (WEHI). All mice were on a C57BL/6 background and bred at WEHI. To generate A1 \(^{+/−}\) Eμ-Myc mice, Eμ-Myc transgenic males [22] were crossed with two strains of A1 \(^{−/−}\) females (A1-1 and A1-2) [44], and offspring were interbred separately. A1 \(^{+/−}\) Eμ-MycCreERT2 mice were generated by interbreeding Eμ-Myc transgenic males with Rosa26-CreERT2 females [48] and A1 \(^{+/−}\) (A1-α\(^+/−\)/A1-b\(^+/−\)/A1-c\(^+/−\)) females (A1-1 and A1-2) followed by interbreeding of offspring. Genotyping was performed as previously described [44]. Tg(UBC-GFP) 30Scha/J female mice [52] were bred with Eμ-Myc transgenic males to produce double transgenic offspring. Cohorts of mice were aged to ethical end point or euthanased at 28–30 days of age for premalignant analysis. Ethical endpoint was determined independently by trained animal technicians; criteria included splenomegaly, lymphadenopathy, hind-limb paralysis, hunched stature, weight loss, laboured breathing.

Tumour analysis

Mice were euthanased according to the guidelines of the Institute’s Animal Ethics Committee. Haemopoietic organs (spleen, inguinal lymph nodes, axillary lymph nodes, brachial lymph nodes, mesenteric lymph node and thymus) were weighed and a peripheral blood sample was collected by either eye bleed or heart bleed. Blood counts and composition were determined using an ADVIA 2120 haematology analyser (Siemens, Erlangen, Germany). Cell suspensions were prepared from lymphomas and cryopreserved. Lymphomas were immunophenotyped by staining suspensions were prepared from lymphomas and cryopreserved (Roche, Basel, Switzerland). Protein concentration was determined using an ADVIA 2120 analyser (Siemens). Red blood cells were removed using 0.168 M ammonium chloride. Single cell suspensions were prepared from haemopoietic tissues and cell counts enumerated on a CASY Cell Counter (Scharfe System GmbH, Reutlingen, Germany). Cell composition was determined by immunostaining and flow cytometry (LSR II flow cytometer, BD Biosciences), using FlowJo software and the following fluorochrome-labelled surface marker-specific monoclonal antibodies produced in house: α-CD8-PE (clone YTS169); α-CD4-APC (clone H129.19); α-TCRβ-FITC (clone H57–597); α-B220-PE (clone RA3–6B2); α-IgM-FITC (clone 5.1); α-IgD-FITC (clone 11–26 C); α-IgD-PE; α-CD43-APC (clone S7); α-Mac1-PE (clone M1/70); α-Gr1-APC (clone RB6–8C5); α-Thy1-PE (clone T24/31); α-Ter119-APC.

Pro/pre-B cell survival assay

Bone marrow cells (10 \(^{×}\) 10\(^6\)) from 4 week-old mice were stained by incubating with α-B220-PE, α-IgM-FITC and α-IgD-FITC, then washed and resuspended in 4 μg/mL propidium iodide (PI). Viable pro/pre-B cells (B220+IgM+IgD+PI–) were isolated by flow cytometry then cultured at 1 × 10\(^6\) cells/mL in high-glucose Dulbecco’s Modified Eagle’s medium supplemented with 10% foetal calf serum, 50 μM 2-ME and 100 μM asparaginase without additional cytokines to observe spontaneous death. Cell viability was determined at 0, 4, 8, 24, 48 and 72 h by staining with annexin-V-FITC and 4 μg/mL PI followed by analysis on an LSR II flow cytometer.

Western blot analysis

Western blots were performed according to standard procedures using protein lysates prepared from cryopreserved cell pellets using RIPA buffer (300 mM NaCl, 2% IGEPAL CA-630, 1% deoxycholic acid, 0.2% SDS, 100 mM Tris-HCl pH 8.0) containing complete ULTRA protease inhibitors (Roche, Basel, Switzerland). Protein concentration was determined by Bradford assay. Samples (15–20 μg total protein) were run on NuPAGE Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes with an iBlot (Life Technologies) according to the manufacturer’s protocol. Membranes were subsequently probed with the following antibodies: A1 (clone 6D6, WEHI mAb lab), BCL-2 (clone 7, BD Biosciences), BCL-X\(_L\) (clone 44, BD Biosciences), MCL-1 (clone 19C4–15, WEHI mAb lab), PUMA (polyclonal, Abcam), BIM (clone 3C5, WEHI mAb lab), p53 (FL-393, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p19ARF (p19ARF exon 2, Rockland,
Gilbertsville, PA, USA), c-MYC (D84C12, Cell Signaling Technology, Danvers, MA, USA) and β-ACTIN (clone AC-74, Sigma-Aldrich). Blots were visualised using LuminataTM Forte western HRP substrate (Merck-Millipore) on a ChemiDoc Touch (Bio-Rad, Hercules, CA, USA) and analysed using Image Lab software (Bio-Rad).

Haemopoietic competitive reconstitution

Bone marrow was collected from 4-week-old female UBC-GFP/My-Myc, A1+/+ My-Myc and A1−/− My-Myc mice and resuspended in phosphate buffered saline to 15 × 10^6/mL. UBC-GFP/My-Myc cells were mixed at a 1:1 ratio with A1+/+ My-Myc or A1−/− My-Myc cells and 3 × 10^6 cells were injected into lethally irradiated (2 × 5.5 Gy spaced by 2 h) C57BL/6-Ly5.1 mice. For each competitive bone marrow mixture, 3 recipient mice were used. To prevent infections, transplanted animals were initially provided with water containing neomycin (Sigma). Six weeks later, when their haemopoietic system had re-established, blood was collected from the retro-orbital plexus for ADVIA and FACS analysis.

Conditional A1 deletion in transplanted lymphomas

Lymphomas originating from A1+/+ My-Myc, A1+/+ My-MycCreERT2, A1+/+ My-MycCreERT2 and A1−/− My-MycCreERT2 female mice were cryopreserved as cell suspensions for later use. Tumour cells were thawed and resuspended at 15 × 10^6 cells/mL in PBS, then 3 × 10^6 cells (200 μL) injected into the tail veins of 6 female C57BL/6-Ly5.1 recipient mice (unirradiated). On d5 and d6 post-transplantation, 3 out of 6 mice were treated with 200 mg tamoxifen/kg body weight (Sigma-Aldrich) in peanut oil/10% ethanol by oral gavage, while the remaining 3 mice received only vehicle (peanut oil/10% ethanol). Any transplantations deemed unsuccessful (i.e. the vehicle-treated mice did not all become sick at a similar time) were excluded from analysis. When transplanted mice reached ethical endpoint, they were euthanased and their lymphomas cryopreserved as single-cell suspensions for later use. Lymphoma cells were thawed and stained with α-Ly5.1-APC (A20.1) and α-Ly5.2-PE (S-450-15.2). Viable donor-derived (Ly5.2+PI+) tumour cells were purified by flow cytometry and DNA was isolated using a DNeasy Qiagen kit and analysed for A1-b, A1-d, My-Myc and Rosa26CreER genes by PCR and gel electrophoresis.

Statistical analysis

GraphPad Prism (GraphPad Software Inc.) was used to graph and statistically analyse data. For analysis of Kaplan–Meier mouse survival curves, significance was determined using the log-rank (Mantel-Cox) test. Ordinary one-way ANOVA with Tukey’s multiple comparisons test or Student’s T-test was used for statistical analysis; P values <0.05 were considered to be statistically significant.

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Author contributions

CJV and SC conceived the studies, planned experiments, analysed the data and wrote the manuscript. MM, NSA, MR and CJV performed the experiments and analysed the data. RLS and MJH provided the mice and intellectual input.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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