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Suppression of B cell lymphomagenesis by the BH3-only proteins Bmf and Bad

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Abstract

Oncogenic c-Myc is known to balance excessive proliferation by apoptosis that can be triggered by p53-dependent and p53-independent signaling networks. Here, we provide evidence that the "BH3-only" pro-apoptotic Bcl-2 family members Bmf and Bad are potent antagonists of c-Myc-driven B cell lymphomagenesis. Tumor formation was preceded by accumulation of preneoplastic pre-B and immature IgM^+ B cells in hematopoietic organs of *Eμ-myc/bmf*$^{-/-}$ mice, whereas *Eμ-myc/bad*$^{-/-}$ mice showed an increase of pre-B cells limited to the spleen. While loss of Bad had no impact on the tumor immunophenotype, Bmf-deficiency favored the development of IgM^+ B cell over pre-B cell tumors. This phenomenon was due to a strong protection of immature IgM^+ B cells from oncogene-driven apoptosis caused by loss of *bmf* and c-Myc-induced repression of Bmf expression in premalignant pre-B cells. Steady-state levels of B cell apoptosis were also reduced in the absence of Bad, in support of its role as a sentinel for trophic factor-deprivation. Loss of Bmf reduced the pressure to inactivate p53, whereas Bad-deficiency did not, identifying Bmf as a novel component of the p53-independent tumor suppressor pathway triggered by c-Myc.

Keywords

apoptosis; tumorigenesis; BH3-only proteins; c-Myc

Introduction

Defects in the 'Bcl-2 regulated' ('intrinsic' or 'mitochondrial') apoptosis pathway have been associated with cancer development, progression and drug-resistance. This apoptotic pathway is initiated when developmental cues, cytotoxic or oncogenic stress trigger activation of pro-apoptotic Bcl-2 family members of the BH3-only subgroup, such as Bim or Puma. This leads to activation of Bax/Bak proteins, the second pro-apoptotic subgroup of the Bcl-2 family, either by binding and neutralizing the function of Bcl-2-like pro-survival

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family members, including Bcl-2, Bcl-x_L and Mcl-1, that sequester these molecules, or by their direct interaction with Bax [1,2]. Subsequent oligomerization and pore formation by Bax and Bak causes mitochondrial outer membrane permeabilization (MOMP), allowing the release of apoptogenic molecules, including cytochrome c and smac/DIABLO, which promote activation of the proteolytic caspase cascade resulting in apoptotic cell death [1,2].

Eμ-Myc transgenic mice develop aggressive immature pre-B and IgM^+ B cell lymphomas and are a potent model to study the molecular basis of c-Myc-driven malignancies [3,4]. Disease pathogenesis in these mice resembles in certain aspects that of Burkitt lymphoma inasmuch that overexpression of c-Myc causes excessive proliferation of B cells, although of different developmental stages. This is initially balanced by massive apoptosis, until second genetic lesions, most commonly loss of p53 signaling or overexpression of Bcl-2 or Bcl-xL, blunts this response [5,6]. Loss of p53 impedes c-Myc-driven apoptosis by inefficient induction of effectors of the intrinsic apoptosis pathway such as Puma [7,8], whereas overexpression of Bcl-2 or Bcl-xL not only blocks the proapoptotic potential of Puma but also that of another critical p53-independent sentinel of oncogenic stress, i.e. Bim [9,10]. Consistently, loss of either BH3-only protein facilitates *c-myc*-driven lymphomagenesis in mice [10-12] and loss or decreased expression of Bim or Puma have been described in a number of human cancers [13]. Consistent with their role as tumor suppressors in c-Myc-induced oncogenesis, Bim and Puma were found silenced in a portion of human Burkitt lymphoma [11,14]. It is, however, currently unclear if also other BH3-only proteins can be engaged by c-Myc to prevent malignant transformation and if their absence may contribute to the pathogenesis of this disease.

Loss of the BH3-only protein Bmf in mice induces polyclonal B cell hyperplasia that is associated with decreased sensitivity of Bmf-deficient B cells to apoptosis-induction, although the physiological trigger during B cell development remains undefined [15]. Consistently, Bmf has been implicated in cell death induction of primary chronic lymphocytic B cell leukemia (B-CLL) in humans [16] but also other tumor entities such as oral and esophageal squamous cell carcinoma cells [17]. In contrast, mice deficient for Bad show normal lymphocyte development and number, but impaired B cell function [18]. Serum deprivation rendered Bad-proficient, but not Bad-deficient mouse embryonic fibroblasts (MEF) more susceptible to the effects of death receptor ligation and Bad deficient MEF were more resistant to the combined effect of IGF-1 withdrawal and etoposide treatment. Bad-deficient mice were also reported to develop diffuse large B-cell lymphomas, with an incidence of about 20%, albeit late in life (latency > 15 months) [18], but evidence for a role of Bad in human lymphoid malignancies is currently lacking [19]. However, loss-of-function mutations in the BH3-domain of Bad were reported in colon carcinoma patients and higher levels of Bad protein expression have been associated with better outcome in androgen dependent prostate cancer and in breast cancer [20-22]. The pro-apoptotic potential of Bad is thought to be regulated in part by phosphorylation, leading to its cytoplasmatic sequestration and inactivation, which can be mediated by the lipid-activated protein kinase AKT/PKB [23], central to a signaling pathway frequently hyperactivated in human cancers [24].

To investigate the contribution of the BH3-only proteins Bad and Bmf in c-Myc-driven B cell lymphomagenesis we crossed $bmf^{-/-}$ and $bad^{-/-}$ mice with transgenic mice expressing the *c-myc* oncogene under control of the *Eμ* heavy chain enhancer and compared it to the effects observed in response to loss of *bim,* a well established tumor suppressor in this model system of c-Myc-driven malignant disease.

Material and methods

Mice

All animal experiments were approved by the Austrian Ministry for Education, Research and Culture. The generation and genotyping of the *bmf*$^{-/-}$, *bim*$^{-/-}$, *bad*$^{-/-}$, and *Eμ-myc* transgenic mice have been described [4,15,18,25]. All mice used were on an inbred C57BL/6 genetic background.

Cell culture and reagents

FACS-sorted pre-B, immature and mature IgM^{+} B cells were cultured in DMEM (PAA) supplemented with 10% FCS (PAA), 250μM L-glutamine (Gibco) and 50μM 2-mercaptoethanol. Isolated lymphoma cells were cultured on supporting irradiated NIH-3T3 cells. Source of reagents: Etoposide, Dexamethasone, Paclitaxel, 5-aza-2′-deoxycytidine (all from Sigma-Aldrich), Bortezomib/Velcade® (M. Ausserlechner, Dept. of Pediatrics, Innsbruck), ABT-737 (Steve Elmore, Abbott Pharmaceuticals), or SAHA (R.W. Johnstone, Peter MacCallum Cancer Center, Melbourne). Daudi, Raji and Ramos BL cell lines were maintained in RPMI 1640 medium (PAA) supplemented with 10% FCS and 250μM L-glutamine.

Flow cytometric analysis and cell sorting

The monoclonal antibodies used, and their specificities, are as follows: RA3-6B2, anti-B220; R2/60, anti-CD43; II/41, anti-IgM; 11/26C, anti-IgD; MB19-1, anti-CD19; 53-7.3, anti-CD5; AA4.1, anti-CD93; D7 anti-Sca-1; GK1.5, anti-CD4; H57-597, anti-TCRβ (all eBioscience); 53-6.7, anti-CD8; (all Becton Dickinson). Biotinylated antibodies were detected using streptavidin-RPE (DAKO) or streptavidin-PE-Cy7 (Becton Dickinson). HIB19, anti-human CD19 (eBioscience) was used for sorting B cells from peripheral blood. Sorting of cells was performed using a FACSVantage cell sorter (Becton Dickinson). *In vivo* BrdU-labeling was performed as previously described [26].

Immunoblotting

Western blotting was performed as previously described [15]. Membranes were probed with rat anti-p19/ARF (5-C3-1), rabbit anti-p53 antiserum (FL-393) (Santa Cruz Biotechnology), monoclonal antibodies to Bcl-xL (54H6) (Cell Signaling), rabbit anti-Mcl-1 (Rockland), hamster anti-mouse Bcl-2 (3F11) and rat anti-mouse Bmf mAb (17A9) and rat anti-human Bmf (9G10) (a gift from A. Strasser). Equal loading of proteins was confirmed by probing filters with antibodies specific for β-actin (Sigma), GAPDH (Sigma) or MAPK (Cell Signaling). Horseradish peroxidase (HRP)-conjugated sheep anti-rat Ig antibodies (Jackson Research) rabbit anti-hamster antibodies (Southern Biotechnology) goat anti-rabbit or rabbit anti-mouse antibodies (DAKO) served as secondary reagents and the enhanced chemiluminiscence (ECL; Amersham) system was used for detection.

Cell viability assay

The percentage of viable cells in culture was determined by staining cell suspensions with 1μg/ml 7-AAD (Sigma) plus FITC-coupled Annexin-V (Beckton Dickinson) and analyzing the samples in a FACScan (Becton Dickinson).

Primary patient material

Material from Burkitt lymphoma cases (3, 8, 25, 35 and 45 years old male and one 18 years old female), diagnosed between 1991 and 2006 were collected from the tumor bank of the Institute of Pathology at the University Hospital of Basel. All cases fulfilled morphological and phenotypical criteria of Burkitt lymphoma and showed on revision *c-myc*

rearrangements as assessed by a dual-color, break-apart probe from Vysis/Abott, Downers Grove, IL, USA; order no. 05J91-001. Five tumors were of primary extra nodal origin (each one of tonsillar, epidural- and cubital origin, and two of ileo-coecal origin), while one was primary nodal. Retrieval of tissue was according to the regulations of the local institutional review board and data safety laws.

Lentiviral transduction

Lentiviral transduction of BL cell lines with expression vectors encoding BMF-specific shRNA was performed as previously described [27].

Bisulfite modification and BMF DNA methylation analysis and quantitative analysis of BMF mRNA levels

See supplemental information

Statistical analysis

Estimation of statistical differences between groups was carried out using the unpaired Student *t*-test or ANOVA analysis, where appropriate. Comparison of tumor onset was performed using a log-rank test and the χ^2-test was used for comparison of frequency distributions. P-values of <0.05 were considered to indicate statistically significant differences.

Results

Loss of Bmf or Bad accelerates the onset of lymphoma in Eμ-myc transgenic mice

To explore whether Bmf or Bad can act as tumor suppressors in oncogene-driven B cell lymphoma development, mice lacking the individual BH3-only proteins were crossed with Eμ-myc transgenic mice. For comparison, we also generated a cohort of mice expressing c-Myc on a *bim*$^{+/-}$ or *bim*$^{-/-}$ background [10]. Cohorts of Eμ-myc transgenic mice lacking one or both alleles of either Bad or Bmf were monitored until onset of overt disease. *Eμ-myc* mice lacking one allele of *bad* did not contract disease significantly faster than wild type (wt) *Eμ-myc* mice ($p=0.18$, Fig 1A), while *Eμ-myc/bad*$^{-/-}$ mice showed a shortened survival ($p<0.001$, Fig 1A). Loss of one allele of *bmf* lead to a slight acceleration of lymphoma onset as compared to *Eμ-myc* mice ($p<0.05$, Fig 1B), which was further enhanced by loss of the second *bmf* allele ($p<0.01$, Fig1B). Interestingly, loss of both alleles of *bmf* or *bad* accelerated c-Myc-driven tumorigenesis significantly less efficiently than loss of one allele of *bim* ($p<0.01$ and $p<0.0001$ respectively, Figs. 1A, B). Notably, *Eμ-myc* mice deficient for both, Bad and Bmf, did not contract disease earlier than single-mutant animals expressing the transgene (Fig. 1C). Taken together, this demonstrates that both Bmf and Bad possess tumor suppressor potential, but are overall less potent than Bim, at least in this disease model. Furthermore, Bmf and Bad may act in a redundant manner in this process, or at different stages of B cell development.

Loss of Bmf preferentially promotes development of IgM$^+$ tumors in Eμ-myc transgenic mice

Tumors developing in *Eμ-myc* mice normally have a CD19$^+$IgM$^-$ pre-B cell or an immature CD19$^+$IgM$^+$ B cell phenotype [4]. Consistent with previously published data, immunophenotyping of the lymphomas revealed a frequency of ~60% pre-B cell tumors in the wt *Eμ-myc* mice (16/28), ~30% of all cases (8/28) were immature IgM$^+$ B cell lymphomas and the remaining tumors displayed a mixed (pre-B/IgM$^+$) phenotype (Fig. 1D). In strong contrast, *Eμ-myc*/*bmf*$^{-/-}$ mice developed predominantly IgM$^+$ B cells lymphomas (20/30; 67%) and only 3/30 tumors (10%) were of pro/pre-B cell origin (Fig. 1D). The

tumor spectrum observed in the *Eμ-myc/bmf*$^{+/-}$ mice was intermediate between wt and *bmf*$^{-/-}$ *Eμ-myc* mice with 10/29 cases (34%) being pro/pre-B cell lymphomas, demonstrating a clear gene-dosage effect (Fig. 1D). Similar observations were made in *Eμ-myc* mice lacking *bim* whereas the immunophenotype of *bad*$^{+/-}$ and *bad*$^{-/-}$ lymphomas mirrored that of wt *Eμ-myc* mice (Fig. 1D). Further analysis revealed that the observed acceleration of tumorigenesis was mainly due to an earlier onset of IgM^{+} lymphomas in all genotypes tested (Fig. 1E), as previously noted in *Eμ-myc* mice lacking Bim [10]. Importantly, although not further reducing tumor latency, loss of Bad over Bmf again facilitated the development of pre-B tumors upon c-Myc overexpression (Fig. 1D). Together, this indicates that although Bmf and Bad are able to engage the same pro-survival molecules *in vitro* [30], the activities of both proteins are regulated differently during normal B cell maturation and/or in response to oncogenic stress *in vivo*.

Interestingly, *Eμ-myc/bmf*$^{-/-}$ and *Eμ-myc/bmf*$^{+/-}$ mice also showed an increased frequency of lymphomas that lacked expression of the B cell markers CD19 and IgM, but expressed B220, CD4, CD5, AA4.1 and Sca-1 (3/30 and 4/29, respectively, vs. 1/30 *Eμ-myc* mice, Fig. 1D). The immunophenotype of these lymphomas resembles that of lymphomas observed in *Eμ-myc/Eμ-bcl-2* and *Eμ-myc/Eμ-bcl-x* double-transgenic mice [31,32], pointing towards a role for Bmf in apoptosis of early hematopoietic progenitors.

Bmf deficient Eμ-myc transgenic mice bear higher tumor load

A closer evaluation of the hematopoietic compartment of diseased mice revealed that loss of Bmf favored the development of leukemia in *Eμ-myc* transgenic mice. Compared to the mean white blood cell count of diseased *Eμ-myc* mice we observed a >4-fold increase in the number of circulating leukocytes in diseased transgenic mice lacking Bmf ($p<0.0001$) (Fig. 2A). In addition, *Eμ-myc/bmf*$^{-/-}$ mice had a significantly more pronounced splenomegaly than wt or Bad-deficient tumor mice (Fig. 2B). Taken together loss of Bmf significantly increased the tumor load of ill mice and favored development of IgM^{+} leukemia, similar to findings made previously in *Eμ-myc/bim*$^{-/-}$ mice [10] and recapitulated here (Figs. 2A, B).

Loss of Bmf or Bad causes an accumulation of B cells in pre-leukemic mice

Young, healthy *Eμ-myc* transgenic mice display an expanded population of pre-B cells and immature IgM^{+} transitional (T1) B cells, caused by c-Myc-driven proliferation, leading to the accumulation of these cells in secondary lymphoid organs. In contrast, the number of mature B cells was reduced in *Eμ-myc* mice (Fig. 3A), as previously reported [4]. The expansion of these premalignant cells is dampened for a limited period of time by c-Myc-induced apoptosis. While the increased numbers of pro/pre-B cells in the bone marrow were comparable between *Eμ-myc, Eμ-myc/bmf*$^{-/-}$ and *Eμ-myc/bad*$^{-/-}$ mice, we observed that the total B cell number in the spleens was significantly increased in *Eμ-myc* mice lacking Bad or Bmf (Fig. 3A). Interestingly, *Eμ-myc/bad*$^{-/-}$ mice showed increased pre-B cell numbers in the spleen when compared to *Eμ-myc mice*, while loss of Bmf favored the accumulation of more mature T1 B cells in *Eμ-myc* transgenic animals (Fig. 3A). Most striking, however, loss of Bmf caused an up to 10-fold increase in B cells of all differentiation stages in peripheral blood of premalignant *Eμ-myc* transgenic mice ($7.3\pm3.5\times10^{6}$/ml vs. $75.5\pm8.0\times10^{6}$/ml), whereas loss of Bad had no such effect (Fig. 3A). This suggested that loss of either BH3-only protein facilitated B cell survival upon oncogenic stress, albeit at different developmental stages leading to the accumulation of the *Eμ-myc* transgenic B cells in premalignant animals.

To confirm our hypothesis, we isolated pre-B cells from the bone marrow as well as immature and mature B cells from the spleens of premalignant *Eμ-myc* transgenic mice either deficient or proficient for Bad or Bmf and assessed cell survival after *in vitro* culture

in the absence of supporting cytokines. *Eμ-myc* transgenic pre-B and B cells died very rapidly, when compared to non-transgenic cells *in vitro*. Loss of Bad or Bmf did confer some minor protection to non-transgenic pre-B cells in culture ($bad^{-/-}$ $p<0.05$ and $bmf^{-/-}$ $p<0.001$ compared to *wt* at 48h) but mature B cells of both genotypes died as fast as wt cells (Fig. 3B). Notably, Bad deficiency failed to confer protection to any of the subsets from *Eμ-myc* transgenic mice. However, while Bmf-deficient pre-B cells were not protected from c-Myc-induced apoptosis, both immature and mature B cells lacking *bmf* survived oncogenic stress significantly better than their wt counterparts. In fact, the immature *Eμ-myc*-transgenic B cells were most efficiently protected from c-Myc-driven apoptosis by loss of Bmf and survived almost as well as non-transgenic B cells (Fig. 3B, lower panel). This contrasts observations made in $bim^{-/-}$ mice where loss of Bim protected pre-B and B cells potently from spontaneous and c-Myc-induced apoptosis [10].

Although the cells from *Eμ-myc*/$bad^{-/-}$ mice did not survive better than those from wt *Eμ-myc* mice when cultured *in vitro*, loss of Bad led to an accumulation of premalignant B cells in young *Eμ-myc* transgenic mice (Fig. 3A,B). This could be due to the absence of signals *in vitro* that would otherwise activate Bad in *Eμ-myc* transgenic cells to limit transformation *in vivo*. We therefore quantified the rate of steady-state levels of B cell apoptosis in spleens and lymph nodes of these mice by immediate Annexin-V/PI staining. Consistent with our hypothesis, steady-state levels of apoptosis of pre-B and B cells were lower in *Eμ-myc* mice lacking Bad when compared to *Eμ-myc* controls (Fig. 3C). In addition *in vivo* BrdU incorporation studies confirmed that the rates of proliferation did not differ between any of the cell and genotypes analyzed (Fig. 3D). It remains possible, however, that the survival advantage of Bad-deficient B cells observed in vivo is not cell-autonomous and that non cell-autonomous effects also contribute to tumor formation in $bmf^{-/-}$ mice. Once established, Bad-deficient tumors grow in wt and Bad-deficient hosts with equal kinetics, as do Bmf-deficient tumors in wt or Bmf-deficient hosts (suppl. Fig. 1), arguing against non cell-autonomous effects.

Loss of Bmf reduces, but does not eliminate, the pressure to lose p53 function

Due to the strong pro-apoptotic drive of c-Myc, tumors that develop in *Eμ-myc* mice frequently show aberrations in the p19ARF/Mdm2/p53 pathway [6]. Western blotting for p53 and p19ARF, where high levels of the protein are indicative for non-functional p53 due to absence of the p53-induced negative feedback-loop on ARF expression, was used to investigate the status of this pathway in lymphomas lacking Bad or Bmf. High levels of ARF were detected in 9/38 (24%) of wt *Eμ-myc*; 8/24 (33%) of $bad^{+/-}$ *Eμ-myc* and 5/22 (23%) of $bad^{-/-}$*Eμ-myc* lymphomas (Fig. 4A). In contrast, only 2/22 (9%) $bmf^{+/-}$, 3/27 (11%) $bmf^{-/-}$ and 0/7 $bad^{-/-}bmf^{-/-}$ *Eμ-myc* lymphomas, respectively, were deficient of functional p53 in this type of analysis (Fig. 4A and not shown). Consistent with previous findings [10], 0/6 $bim^{-/-}$ and only 1/9 $bim^{+/-}$ lymphomas analyzed showed increased ARF levels (not shown).

As c-Myc-driven transformation is facilitated either by inactivation of the p53 pathway or by overexpression of Bcl-2 or Bcl-xL [5] we tested whether up-regulation of a pro-survival Bcl-2 family member was preferred over loss of p53 during the transformation of *Eμ-myc*/$bmf^{-/-}$ B cells. Therefore, lymphomas derived from mice of the different genotypes were analyzed by Western blot for the expression of Bcl-2, Bcl-xL and Mcl-1. The three proteins were expressed in variable levels among the lymphomas, independent of their immunophenotype, but we failed to detect increased frequencies of overexpression of pro-survival Bcl-2 proteins in Bmf-deficient over wt or Bad-deficient c-Myc-driven lymphomas (Fig. 4B and data not shown).

Deregulated expression of Bmf in the presence of c-Myc

Myc is known to regulate the expression of several members of the Bcl-2 family. In premalignant *Eμ-myc* transgenic pre-B and B cells Bcl-2 and Bcl-xL are repressed [5], while Bim and Puma proteins are induced [10-12]. To test whether the expression of Bmf or Bad were also deregulated on an *Eμ-myc* transgenic background, premalignant pre-B and B cells from wt and *Eμ-myc* mice were FACS-sorted and subjected to Western blot analysis. Surprisingly, although different isoforms of Bmf were expressed at high levels in wt pre-B cells [15], they were barely detectable in the *Eμ-myc* transgenic pre-B cells, whereas the protein levels were similar in IgM^+ wt and *Eμ-myc* B cells (Fig. 5A). This observation might also explain why loss of Bmf favored the development of immature IgM^+ over pre-B tumors. Consistent with the pattern of expression in the premalignant cells, Bmf levels were also very low or lacking in all pre-B cell tumors analyzed and was detected in 8/10 IgM^+ tumors (Fig. 5B,C). Notably, loss of Bmf expression was also observed in 1/6 IgM^+ *Eμ-myc/bmf*$^{+/-}$ tumors tested (Fig. 5C), suggesting that its loss or silencing may be a recurrent event in Myc-driven B cell lymphomagenesis. In contrast, both isoforms described for Bad [18] were present in wt and c-Myc transgenic premalignant cells (Fig. 5A) and one or the other isoform was expressed in *Eμ-myc* tumor samples (Fig. 5B). Tumors arising in *Eμ-myc/bad*$^{+/-}$ animals tested positive for Bad protein expression in 9/9 cases analyzed suggesting retention of the second allele (Fig. 5C). Furthermore, we aimed to investigate whether the Bad protein found in premalignant *Eμ-myc* transgenic B cells was hypo-phosphorylated, indicative of its activation [33]. However, the weak signal that we obtained using different antibody against phospho-Bad was also observed in cell extracts from Bad-deficient mice (not shown).

Loss of Bim, but not loss of Bad or Bmf confers drug-resistance phenotypes in c-myc dependent lymphomas

Activation of Bmf or Bad has been reported to be required for cell death induced by certain anticancer agents, including inhibitors of histone-deacetylases or tyrosine-kinases as well as glucocorticoids [15,17,34]. Therefore, we investigated if absence of Bad or Bmf would confer drug-resistance phenotypes to *c-myc*-dependent lymphomas. Freshly harvested tumor samples were cultivated for 24h in the absence or presence of graded doses of the glucocorticoid dexamethasone, the DNA-damaging drug etoposide, the HDAC-inhibitor SAHA, the proteasome inhibitor bortezomib, the microtubule-stabilizing agent paclitaxel, or the BH3-mimetic ABT-737. Surprisingly, neither loss of Bmf nor Bad conferred significant drug-resistance *in vitro*. This observation was independent of the immunophenotypes of the investigated tumor samples (not shown). In contrast, loss of Bim delayed tumor cell apoptosis triggered by etoposide, dexamethasone, paclitaxel, or, as reported before, SAHA [35], but not cell death caused by proteasome inhibition or treatment with ABT-737 (Fig. 6).

Low-level expression of BMF in Burkitt lymphoma

In human B-cell chronic lymphocytic leukemia (CLL), Bmf is expressed at significant levels and further induced upon serum-deprivation [16,36]. Furthermore, gene chip analysis demonstrated presence of *BMF* mRNA in acute lymphoblastic leukemia (ALL) [37], suggesting that BMF protein is expressed in human tumors that are the histogenetic equivalent to the lymphomas that arise in *Eμ-myc* transgenic mice. However, ALL and CLL usually do not associate with Myc-overexpression. In order to assess whether deregulated c-Myc might correlate with Bmf expression, we quantified its protein levels in three frequently studied Burkitt lymphoma (BL) cell lines i.e. Daudi, Ramos and Raji, as well as in six biopsy samples from patients diagnosed with BL. We also assessed expression of Bad as well as Bim, since the latter protein is reportedly lost or inactivated frequently in human BL [14]. Protein lysates from FACS-sorted $CD19^+$ B cells derived from the peripheral blood

of healthy donors, known to express significant levels of *BMF* mRNA [16], were included for comparison. While Bad and Bim protein were found expressed at comparable levels in all samples analyzed, Bmf isoforms were only expressed at significant levels in healthy $CD19^+$ B cells, but barely detectable in the three cell lines and primary tumor tissues (Fig. 7A). Since *BMF* contains a predicted CpG island (http://cpgislands.usc.edu/cpg.aspx) in its promoter region between position −688 to +492 in relation to the predicted transcription start site that may subject it to methylation-dependent silencing [17], we investigated if inhibition of DNA-methyltransferases by addition of 5′-aza-2′-deoxycytidine would suffice to restore Bmf expression and apoptosis in BL cell lines. Indeed, mRNA levels and protein were significantly induced in the BL lines (Fig. 7B, suppl. Fig. 2), suggesting that demethylation directly triggers transcription of the BMF gene. However, Methylight-PCR analysis covering seven methylation-sensitive CpGs failed to reveal evidence for direct promoter methylation in all cell lines, a finding confirmed by bisulfite sequencing, covering 43 additional putative methylation-sensitive sites in the promoter region and exon 1 of the tree cell lines (not shown). In search of other conditions that could induce Bmf in BL cells, we also found Bmf induction accompanied by cell death in the three cell lines after SAHA treatment or serum deprivation (Fig 7C and suppl. Fig. 3). Lentiviral knock-down of Bmf in Ramos cells had no significant effect on apoptosis induced by SAHA (Fig 7D), demonstrating that Bmf alone is not rate-limiting for apoptosis induction under these conditions, similar to our findings in *Eμ-myc* tumors derived from Bmf-deficient mice (Fig. 6). Knock down of Bmf could however delay serum deprivation-induced cell death in these cells (Fig 7D), indicating that at least under certain conditions Bmf can be decisive in the regulation of cell death of Burkitt lymphoma cells.

Discussion

Using the *Eμ-myc* transgenic mouse model of B cell lymphomagenesis we found that the BH3-only proteins Bad and Bmf are so far unrecognized antagonists of *c-myc*-driven tumor formation. Our findings extend the list of BH3-only proteins that can act as tumor suppressors in this disease model, next to Bim and Puma [10-12]. Notably, other members of this family i.e. Noxa or Bid do play only redundant or no role in regulating Myc-induced lymphomagenesis [[12,13] and R.W. Johnstone, pers. communication], highlighting the importance of understanding the contribution of individual Bcl-2 family proteins to oncogene-driven transformation.

Strikingly, loss of Bmf, but not Bad, caused a strong shift in the observed tumor spectrum and *Eμ-myc bmf*$^{-/-}$ animals presented with heavily increased tumor load and leukemia-like phenotype (Fig. 1,2). Onset of disease was preceded by an increased accumulation of premalignant pre-B- and immature B cells in different lymphoid organs in the absence of Bad or Bmf, exceeding numbers observed in *Eμ-myc* transgenic mice (Fig. 3A), which was not due to differences in proliferation capacity between genotypes (Fig. 3C). This indicated that loss of either BH3-only protein enhanced the survival of pre-leukemic cells in the presence of oncogenic c-Myc, increasing the pool of cells that can acquire a second oncogenic lesion that overcomes c-Myc-induced apoptosis, or that loss of Bmf or Bad may represent such secondary lesion, allowing transformation. We believe that loss of Bmf constitutes a genetic lesion that directly facilitates transformation by blocking c-Myc-driven B cell apoptosis (Fig. 3B). This is supported by our observations that although the size of the population “at-risk” for a second lesion is increased in the absence of Bmf up to ten-fold (Fig. 3A), the frequency of tumors that inactivate the p53-pathway actually dropped (Fig. 4A). This was not simply due to the shift towards the development of IgM^+ lymphomas, seen when Bmf is absent (Fig. 1D), since p53-inactivation occurs as frequently in IgM^+ as in pre-B *wt Eμ-myc* lymphomas (3/17 in pro/pre-B lymphomas and 3/13 in IgM^+ lymphomas tested). While a preference of tumors to lose ARF over p53 in the absence of Bmf can

currently not be excluded it is intriguing that some tumors arising in *Eμ-myc* and *Eμ-myc/bmf*$^{+/-}$ mice lose Bmf protein expression (Fig. 5B,C). The molecular basis of this phenomenon, however, awaits detailed investigation. Reduced selection pressure against p53 has also been reported in *Eμ-myc* tumors lacking *bim* or *bax,* but not *noxa*, while studies on *puma* revealed contradictory results[10-12,38]. Since neither Bim nor Bmf are regulated by p53 directly, they presumably act in the same Bax-dependent, but p53-independent apoptosis pathways, engaged by c-Myc.

Notably, *Eμ-myc/bmf*$^{-/-}$ mice developed mainly IgM$^+$ lymphomas correlating with the fact that loss of Bmf protected immature IgM$^+$ B cells most potently from c-Myc-induced apoptosis (Fig. 3B). The degree of protection provided by loss of Bmf to pre-B cells was minor and in line with the observation that c-Myc overexpressing wt pre-B cells showed a strong reduction of Bmf protein expression (Fig. 5A). A similar tumor immunophenotype was observed in *Eμ-myc* mice lacking Bim, as previously suggested by others [10] or in mice deficient for the tyrosine kinases Btk and Tec, that both trigger maturation and proliferation of developing pre-B cells and B cells after successful (pre)-BCR rearrangement [39]. In *btk*$^{-/-}$*tec*$^{-/-}$ double-knockout mice, pre-B cell development is essentially completely blocked, but efficiently restored by introduction of the *Eμ-myc* transgene. Interestingly, ~75% of tumors developing in these mice express IgM on their surface [39]. It is unclear why IgM$^+$ tumors preferentially develop in these mice, but maybe Btk and Tec-dependent maturation signals are required to maintain BH3-only protein expression and checkpoint function in developing pre-B cells.

Surprisingly, although loss of Bad also facilitated *c-myc*-driven lymphomagenesis, it did not cause a shift in tumor spectrum or promote a leukemia phenotype, as did loss of Bmf or Bim (Figs.1,2). Furthermore, loss of Bad preferentially facilitated the accumulation of premalignant pre-B cells (Fig. 3A), suggesting that it limits the survival of B cell precursors upon oncogenic stress only in a very narrow developmental window. Alternatively, loss of Bad may facilitate tumor formation by allowing the survival of cells under conditions where trophic factors are limiting, such as during rapid c-Myc-driven proliferation, as previously also suggested by others [40].

This effect may not even need to be B cell autonomous. Regardless of the mechanism, this increases the number of cells “at-risk” for secondary oncogenic lesions in the absence of Bad. Consistently, we did not observe a change in the percentage of *bad*$^{-/-}$ tumors that had inactivated the p53 pathway. Also, mice lacking Bmf and Bad simultaneously did not contract disease significantly earlier than single knockout mice expressing the *Eμ-myc* transgene, but in contrast to Bmf-deficient *Eμ-myc* mice, they developed pre-B as well as IgM$^+$ B-cell lymphomas again (Fig. 1,2). It is interesting to note that loss of one allele of *bim* appears even more potent in accelerating tumorigenesis than loss of both alleles of *bmf* or *bad* (Fig. 1). This probably relates to the fact that loss of Bim potently protected both pre-B and B cells alike from death induced by Myc-overexpression [10] whereas loss of Bmf could only protect IgM$^+$ B cells and Bad-deficiency appeared to delay Myc-driven B cell death only poorly, as suggested by our combined in vitro and in vivo results (Fig. 2). Also, Bim-levels are induced upon c-Myc overexpression [10], while neither Bad nor Bmf levels were found increased in pre-malignant B cells or tumors, suggesting an auxiliary and more cell type restricted role for these two proteins in Myc-induced killing. The broader efficacy of Bim may also be related to the fact that it can neutralize all Bcl-2 prosurvival homologues with comparable efficiency and/or its potential to activate Bax directly, while Bad and Bmf appear to bind and neutralize Bfl1/A1 and Mcl1 inefficiently and cannot trigger direct Bax activation [1,30].

Along that line, our screen for drug-resistance in Bad- or Bmf-deficient *c-myc*-driven lymphomas, in contrast to those lacking Bim, did not reveal any resistant phenotypes although a number of drugs have been tested that depend at least in part on Bad or Bmf for killing untransformed cells [15,18]. Notably, in contrast to observations in squamous cell carcinomas and primary lymphocytes [15,17], but consistent with studies in CLL [41] and our own findings in BL-lines, loss of Bmf did not confer drug-resistance to HDAC-inhibition in vitro (Fig. 7; suppl. Fig.3), suggesting redundancy with other BH3-only proteins. Nonetheless, it will be interesting to see if loss of Bmf may affect the efficacy of such anticancer drugs or combinatorial treatment *in vivo*. Of note, tumor cells of all genotypes were equally responsive to the BH3-mimetic ABT-737, but killing was only achieved when high concentrations of the drug were applied, in line with recent findings from Whitecross and colleagues [43]. This observation may be related to the fact that all lymphomas expressed significant levels of Mcl-1 (Fig. 4B), rendering tumor cells more resistant to this drug [44]. Our results also indicate that the reported drug-resistance observed in *Eμ-myc* driven lymphomas expressing myr-AKT is presumably not due to repression of Bad function, but may depend on additional anti-apoptotic effects exerted by the AKT-pathway, e.g. repression of Bim and/or Puma [45,46].

Finally, since we failed to find evidence for direct regulation of BMF gene expression by c-Myc in promoter reporter studies or promoter methylation in human BL-lines, we speculate that oncogenic signals, such as the one provided by c-Myc, can down-modulate Bmf expression in mice and men by alternative means, e.g. by induction of miRNAs [47]. Notably, miR-125b and miR-221 were recently shown to bind to the 3′UTR of the BMF mRNA in human glioma and hepatocellular carcinoma cell lines, respectively [48,49]. The relevance of Bmf levels for lymphoma formation and/or progression driven by aberrant expression of c-Myc in humans remains to be investigated in full detail.

Supplementary Material

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Acknowledgments

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Abbreviations

Bcl-2	B cell lymphoma 2
BH	Bcl-2 homology
BL	Burkitt lymphoma
Bmf	Bcl-2 modifying factor
Bad	Bcl-2 antagonist of cell death
Bim	Bcl-2 interacting mediator of cell death
Puma	p53-upregulated mediator of apoptosis
HDAC	histone deacetylase
SAHA	suberoylanilide hydroxamic acid

PI	propidium iodide
MEF	mouse embryonic fibroblasts

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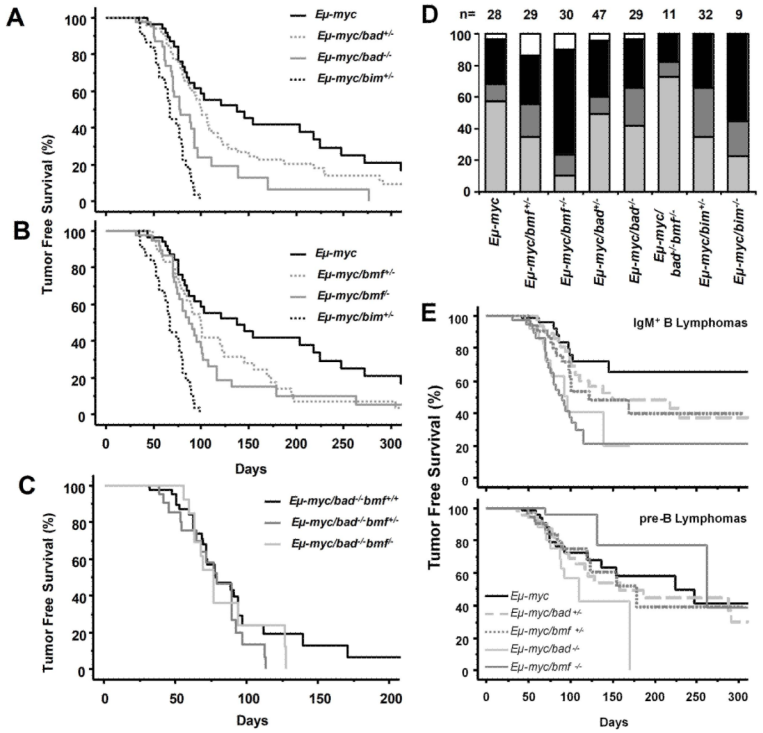

Figure 1. Loss of *bad* or *bmf* accelerates *c-myc*-induced lymphomagenesis

(A) Tumor free survival of *Eμ-myc* (n=29, median survival 138 days), *Eμ-myc/bad*$^{+/-}$ (n=47, median survival 100 days), *Eμ-myc/bad*$^{-/-}$ (n=29, median survival 78 days) and *Eμ-myc/bim*$^{+/-}$ (n=32, median survival 67 days). Lymphomas occurred significantly earlier in *Eμ-myc/bad*$^{-/-}$ than in wt *Eμ-myc* animals ($p<0.001$). **(B)** Tumor free survival of *Eμ-myc, Eμ-myc/bmf*$^{+/-}$ (n=30, median survival 100 days), *Eμ-myc/bmf*$^{-/-}$ (n=30, median survival 87 days) and *Eμ-myc/bim*$^{+/-}$. Lymphomas occurred significantly earlier in *Eμ-myc/bmf*$^{+/-}$ ($p<0.05$) and *Eμ-myc/bmf*$^{-/-}$ ($p<0.01$) than in wt *Eμ-myc* animals. **(C)** Tumor free survival of *Eμ-myc/bad*$^{-/-}$*bmf*$^{+/+}$, *Eμ-myc/bad*$^{-/-}$*bmf*$^{+/-}$ (n=16) and *Eμ-myc/bad*$^{-/-}$*bmf*$^{-/-}$ *mice* (n=8). **(D)** Distributions of pro/pre-B (light grey), mixed (dark grey), IgM$^+$ (black) and B220$^+$CD4$^+$ (white) lymphomas occurring in mice of the indicated genotypes. The distribution of lymphoma phenotypes was significantly different in *Eμ-myc/bmf*$^{-/-}$ compared to *Eμ-myc* animals ($p<0.01$, χ^2-test). **(E)** Kaplan-Meier analysis of IgM$^+$ and mixed lymphomas (upper panel) and of pre-B lymphomas (lower panel) of *Eμ-myc* (black solid line), *Eμ-myc/bad*$^{+/-}$ (light grey, dashed line), *Eμ-myc/bad*$^{-/-}$ (light grey, solid line), *Eμ-myc/bmf*$^{+/-}$ (dark grey dotted line) and *Eμ-myc/bmf*$^{-/-}$ (dark grey solid line) mice. IgM$^+$ B lymphomas arose significantly earlier in *Eμ-myc/bmf*$^{-/-}$ ($p<0.0001$) and *Eμ-myc/bad*$^{-/-}$ ($p<0.01$) than in wt *Eμ-myc* mice. Pre-B lymphomas were not significantly accelerated by loss of either *bad* or *bmf*.

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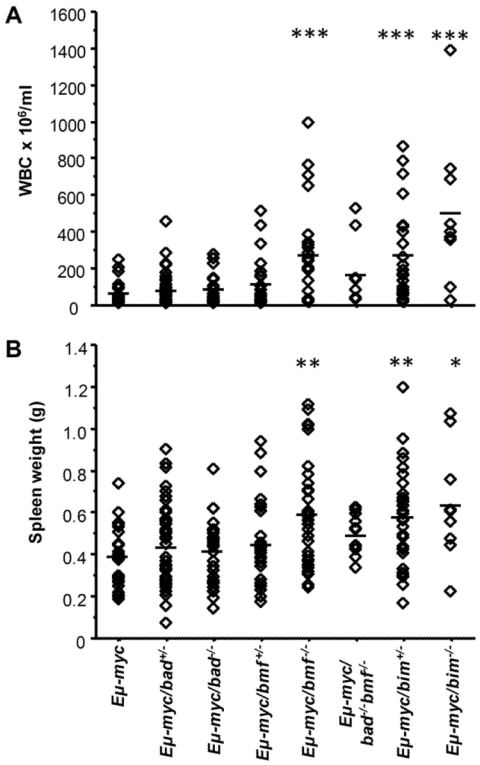

Figure 2. Loss of *bmf* enhances the severity of *Eμ-myc* lymphomas

(A) Numbers of total leukocytes in the blood of moribund mice of the indicated genotypes. The leukocyte counts were significantly higher $bmf^{-/-}$ *Eμ-myc* mice than in wt *Eμ-myc* mice (mean leukocyte count in *Eμ-myc/*$bmf^{-/-}$ mice was $263\pm251\times10^6$/ml versus $58\pm67\times10^6$/ml in *Eμ-myc* mice). Diamonds represent individual blood count of mice and bars the corresponding means **(B)** Spleen weights of moribund mice of the indicated genotypes. Loss of *bmf* but not loss of *bad,* lead to a significant increase in spleen size in moribund mice (mean spleen weight 0.599±0.253g in *Eμ-myc/*$bmf^{-/-}$, *vs.* 0.395±0.140g in *Eμ-myc* or 0.428±0.160g in *Eμ-myc/*$bad^{-/-}$). Diamonds represent individual spleen weights in mice of the indicated genotypes with bars indicating the corresponding means. * $p<0.01$, ** $p<0.001$, *** $p<0.0001$ compared to *Eμ-myc*.

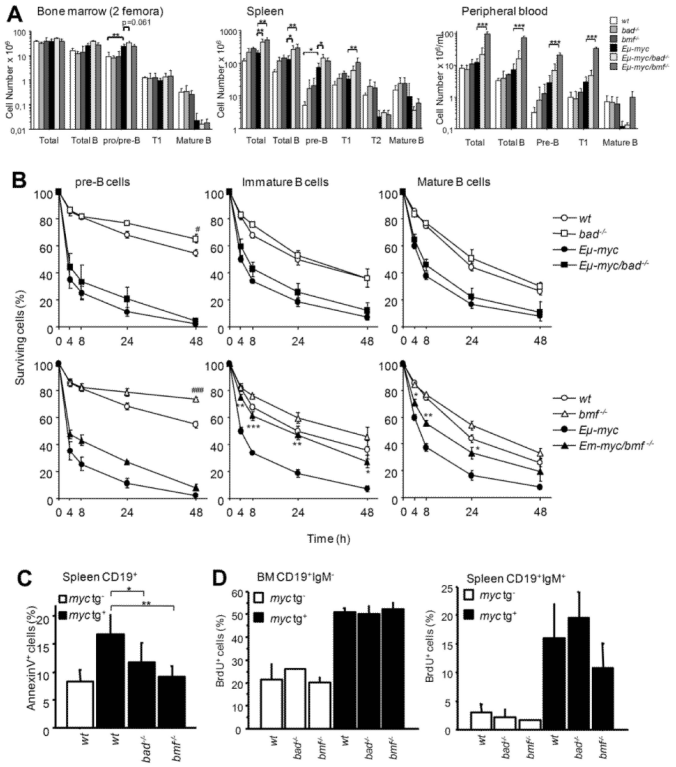

Figure 3. Loss of Bmf enhances the survival of premalignant *Eμ-myc* B lymphocytes

For analysis of pre-leukemic cells, lack of transplantable tumor cells was confirmed by injecting 2×10^6 spleen cells into wt C57BL/6 recipients followed for at least 2 months. **(A)** Cell number and B cell subset composition determined by cell counting and flow cytometric analysis of bone marrow (2 *femora*), spleen and blood from 4-week-old mice of the indicated genotypes. Data represent means ± SD from 3-4 mice per genotype. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Total B cells ($CD19^+$), Pro/pre-B ($CD19^+IgM^-CD43^-$), T1 ($IgM^{high}CD21^+$), T2 ($IgM^{high}CD21^+CD23^+$) and mature (IgM^+D^+) B cells. **(B)** Pre-B cells ($CD19^+IgM^-CD43^-$) sorted from bone marrow and immature ($IgM^{high}IgD^{low}$) as well as mature B cells (IgD^{high}) sorted from spleens from 4-week-old mice of the indicated genotypes, were cultured up to 48h *ex-vivo*. Percentages of surviving cells were determined by Annexin-V/PI staining. Data represent means ± SEM from 3-4 independent experiments for each genotype. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to *Eμ-myc*, $^{\#}$ $p<0.05$, $^{\#\#\#}$ $p<0.001$ compared to wt. **(C)** Freshly isolated splenocytes from 4-week-old mice of the indicated genotypes were immediately stained with anti-CD19-PE together with FITC-Annexin-V plus 7-AAD and analyzed by flow cytometry. Percentages of apoptotic cells in the $CD19^+$ gate were determined. Data represent means ± SD from 3 independent experiments for each genotype. * $p<0.05$, ** $p<0.01$. (**D**) Four hours after *in vivo* labeling, the percentage of $BrdU^+$ $CD19^+IgM^-$ pro/pre-B cells in the bone marrow and of mature $BrdU^+CD19^+IgM^+$ B cells in the spleen was evaluated by combined cell-surface and intracellular antigen staining. Data represent means ± SD from 2 experiments for each genotype.

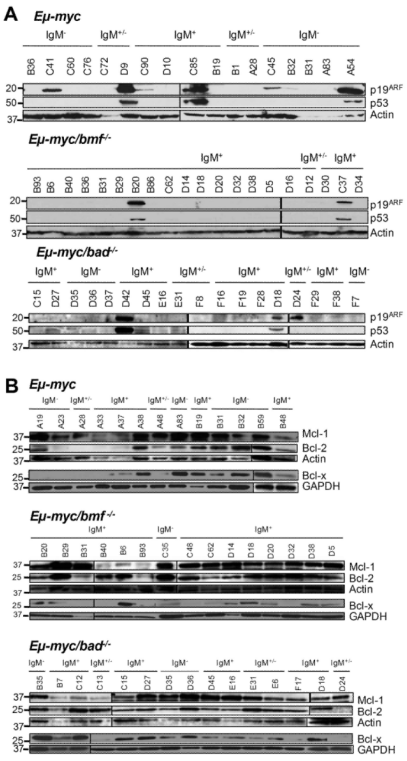

Figure 4. Loss of Bmf, but not Bad, reduces the pressure to lose p53

(A) Representative Western blot analysis of p19/ARF and p53 expression as well as **(B)** Bcl-2, Bcl-x and Mcl-1 expression in lymphoma lysates derived from wt, *bmf*$^{-/-}$ and *bad*$^{-/-}$ *Eμ-myc* mice. Membranes were reprobed using anti-actin or anti-GAPDH antibodies as a loading control.

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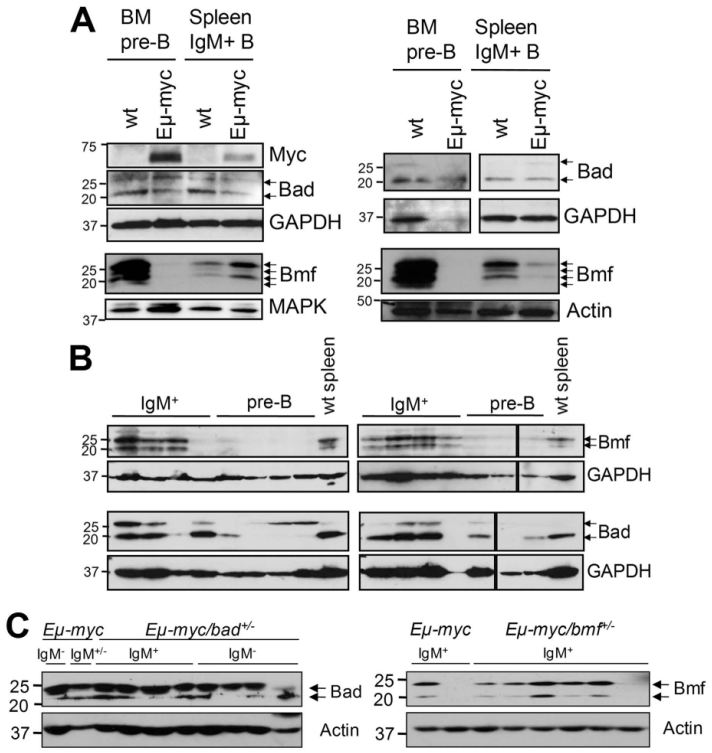

Figure 5. Expression of Bmf and Bad in (pre)-malignant *Eμ-myc* and wt B lymphocytes

Wt and *Eμ-myc* FACS-sorted pre-B cells from the bone marrow and IgM$^+$ B cells from the spleens of 4-week-old mice were analyzed for expression of **(A)** c-Myc, Bmf or Bad. Representative blots from two independent cell sorts are shown. Membranes were reprobed using anti-MAPK, anti-actin as a loading control. **(B)** Representative immunoblots assessing expression of Bmf and Bad in *Eμ-myc*-driven tumors. Membranes were reprobed using anti-GAPDH antibody as a loading control. **(C)** Representative immunoblots assessing LOH in *Eμ-myc*-driven tumors derived from *bmf*$^{+/-}$ or *bad*$^{+/-}$ animals. Membranes were reprobed using anti-actin antibody as a loading control.

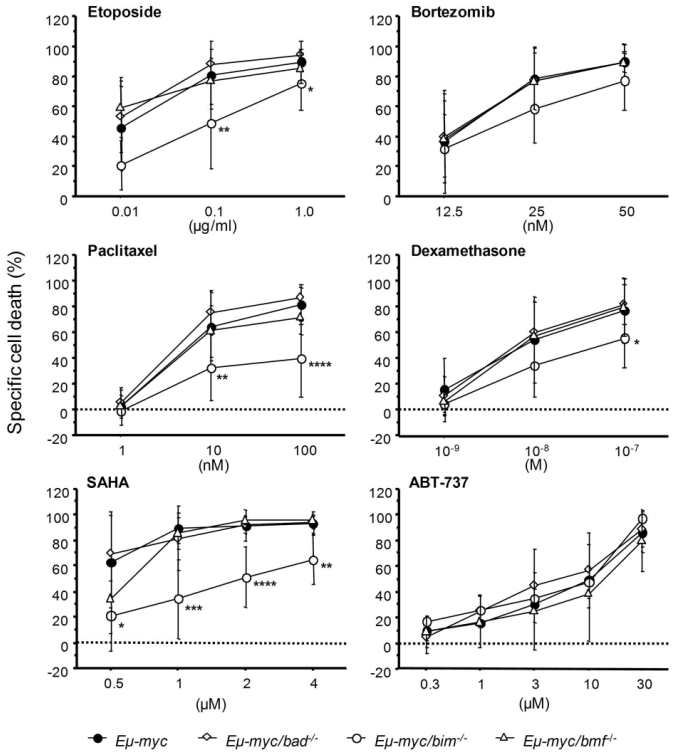

Figure 6. Loss of Bim but not Bad or Bmf protects *Eμ-myc* lymphomas from drug-induced apoptosis *in vitro*

Freshly isolated *Eμ-myc* lymphoma cells were cultured on supporting irradiated NIH-3T3 cells in the presence of chemotherapeutic agents at the indicated concentrations for 24h. Cell death was determined by AnnexinV/7-AAD staining in $CD19^+$ tumor cells. Specific cell death relative to cells cultured without the addition of any drugs was calculated. Values represent means ± SD of 4 animals/genotype. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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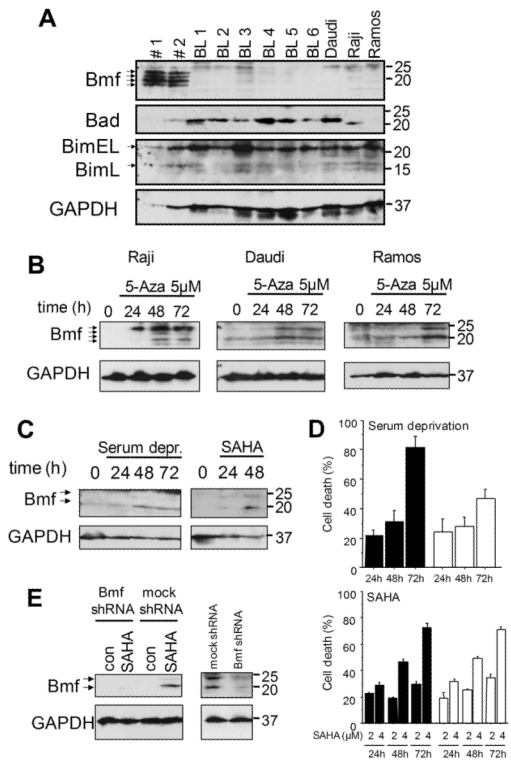

Figure 7. Bmf expression is absent in Burkitt lymphoma cells but can be restored upon demethylation

(A) Western blot analysis of Bmf, Bim and Bad in CD19^{+} cells derived from peripheral blood of healthy donors (#1 and #2), primary Burkitt lymphoma samples (BL1-BL6) and BL cell lines Daudi, Raji and Ramos. **(B)** Western blot analysis of Bmf levels in BL cell lines after inhibition of DNA-methyltransferases with 5-aza-2′-deoxycytidine (5μM) for the indicated times. **(C)** Western blot analysis of Bmf levels in Ramos cells after serum deprivation or inhibition of with SAHA (2μM) for the indicated times. **(D)** Cell death determined by AnnexinV/7-AAD staining in Ramos cells expressing either an shRNA against Bmf (white bars) or an unspecific shRNA (black bars) after serum deprivation or treatment with 2 or 4μM SAHA for the indicated times. Values represent mean±SE of 3 independent experiments. **(E)** Efficiency of knock down was confirmed by Western blot analysis of Bmf levels in cells treated with 2μM SAHA for 48 hours (left panel) or cells deprived of serum for 48 hours (right panel).