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

Anna Frenzel,[1] Verena Labi,[1] Waldemar Chmelewskij,[1] Christian Ploner,[2] Stephan Geley,[2] Heidelinde Fiegl,[3] Alexandar Tzankov,[4] and Andreas Villunger[1]

[1]Division of Developmental Immunology and [2]Division of Molecular Pathophysiology, Biocenter, Innsbruck Medical University, Innsbruck, Austria; [3]Department of Obstetrics and Gynecology, Innsbruck University Hospital, Innsbruck, Austria; and [4]Institute of Pathology, University of Basel, Basel, Switzerland

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 proapoptotic Bcl-2 family members Bcl-2 modifying factor (Bmf) and Bcl-2 antagonist of cell death (Bad) are potent antagonists of c-Myc–driven B-cell lymphomagenesis. Tumor formation was preceded by the accumulation of preneoplastic pre-B and immature immunoglobulin M–positive (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. Although the 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 caused by 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 also were 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. (Blood. 2010;115:995-1005)

Introduction

Defects in the B-cell lymphoma (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 or cytotoxic or oncogenic stress trigger activation of proapoptotic Bcl-2 family members of the BH3-only subgroup, such as Bcl-2 interacting mediator of cell death (Bim) or p53–up-regulated mediator of apoptosis (Puma). This action leads to the activation of Bax/Bak proteins, the second proapoptotic subgroup of the Bcl-2 family, either by binding and neutralizing the function of Bcl-2-like prosurvival family members, including Bcl-2, Bcl-xL, and Mcl-1, that sequester these molecules, or by their direct interaction with Bax.[1,2] Subsequent oligomerization and pore formation by Bax or Bak causes mitochondrial outer membrane permeabilization, allowing the release of apoptogenic molecules, including cytochrome c and second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low PI (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 immunoglobulin M-positive (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 excess 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, 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 several 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 whether other BH3-only proteins also can be engaged by c-Myc to prevent malignant transformation and whether their absence may contribute to the pathogenesis of this disease.

Loss of the BH3-only protein Bcl-2 modifying factor (Bmf) in mice induces polyclonal B-cell hyperplasia that is associated with decreased sensitivity of Bmf-deficient B cells to apoptosis induction, although the physiologic trigger during B-cell development remains undefined.[15] Consistently, Bmf has been implicated in cell death induction of primary B-cell chronic lymphocytic leukemia (CLL) in humans[16] but also other tumor entities such as oral and esophageal squamous cell carcinoma cells.[17] In contrast, mice deficient for Bcl-2 antagonist of cell death (Bad) display normal lymphocyte development and number but impaired B-cell function.[18] Serum deprivation rendered Bad-proficient but not Bad-deficient mouse embryonic fibroblasts more susceptible to the effects of death receptor ligation, and Bad-deficient mouse embryonic fibroblasts were more resistant to the combined effect of insulin growth factor-1 withdrawal and etoposide treatment. Bad-deficient mice also were reported to develop diffuse large B-cell

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lymphomas, with an incidence of approximately 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 proapoptotic 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 with the effects observed in response to loss of *bim*, a well-established tumor suppressor in this model system of c-Myc–driven malignant disease.

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

Fluorescence-activated cell-sorted (FACS)–sorted pre-B, immature, and mature IgM$^+$ B cells were cultured in Dulbecco modified Eagle medium (PAA) supplemented with 10% fetal calf serum (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, Department of Pediatrics, Innsbruck), ABT-737 (Steve Elmore, Abbott Pharmaceuticals), or suberoylanilide hydroxamic acid (SAHA; R. W. Johnstone, Peter MacCallum Cancer Centre, Melbourne, Australia). Daudi, Raji, and Ramos Burkitt lymphoma cell lines were maintained in RPMI 1640 medium (PAA) supplemented with 10% fetal calf serum 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–T-cell receptor-β (all eBioscience); and 53-6.7, anti-CD8 (all Becton Dickinson). Biotinylated antibodies were detected by the use of streptavidin-R-phycoerythrin (DAKO) or streptavidin-phycoerythrin-Cy7 (Becton Dickinson). HIB19, anti–human CD19 (eBioscience), was used for sorting B cells from peripheral blood. Sorting of cells was performed with the use of 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, Walter and Eliza Hall Institute, Melbourne, Australia). Equal loading of proteins was confirmed by probing filters with antibodies specific for β-actin (Sigma-Aldrich), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich), or mitogen-activated protein kinase (Cell Signaling). Horseradish peroxidase–conjugated sheep anti–rat Ig antibodies (Jackson ImmunoResearch Laboratories), rabbit anti–hamster antibodies (Southern Biotechnology), and goat anti–rabbit or rabbit anti–mouse antibodies (DAKO) served as secondary reagents, and the enhanced chemiluminiscence (Amersham) system was used for detection.

Cell viability assay

The percentage of viable cells in culture was determined by staining cell suspensions with 1 μg/mL 7-amino-actinomycin D (7-AAD; Sigma-Aldrich) plus fluorescein isothiocyanate-coupled annexin-V (Becton Dickinson) and analyzing the samples in a FACScan (Becton Dickinson).

Primary patient material

Material from Burkitt lymphoma cases (3-, 8-, 25-, 35-, and 45-year-old male cases and an 18-year-old female case) 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 morphologic and phenotypical criteria of Burkitt lymphoma and showed on revision *c-myc* rearrangements as assessed by a dual-color, break-apart probe from Vysis/Abbott (order no. 05J91-001). Five tumors were of primary extra nodal origin (1 each of tonsillar, epidural, and cubital origin and 2 of ileo-coecal origin), whereas 1 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 Burkitt lymphoma 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 Methods for information (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Statistical analysis

Estimation of statistical differences between groups was carried out by use of the unpaired Student *t* test or analysis of variance, where appropriate. Comparison of tumor onset was performed by the use of a log-rank test, and the χ^2 test was used for comparison of frequency distributions. *P* values less than .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 ($P = .18$; Figure 1A), whereas *Eμ-myc/bad*$^{-/-}$ mice demonstrated a shortened survival ($P < .001$; Figure 1A). Loss of one allele of *bmf* lead to a slight acceleration of lymphoma onset compared with *Eμ-myc* mice ($P < .05$; Figure 1B), which was further enhanced by loss of the second *bmf* allele ($P < .01$; Figure 1B). Interestingly, loss of both

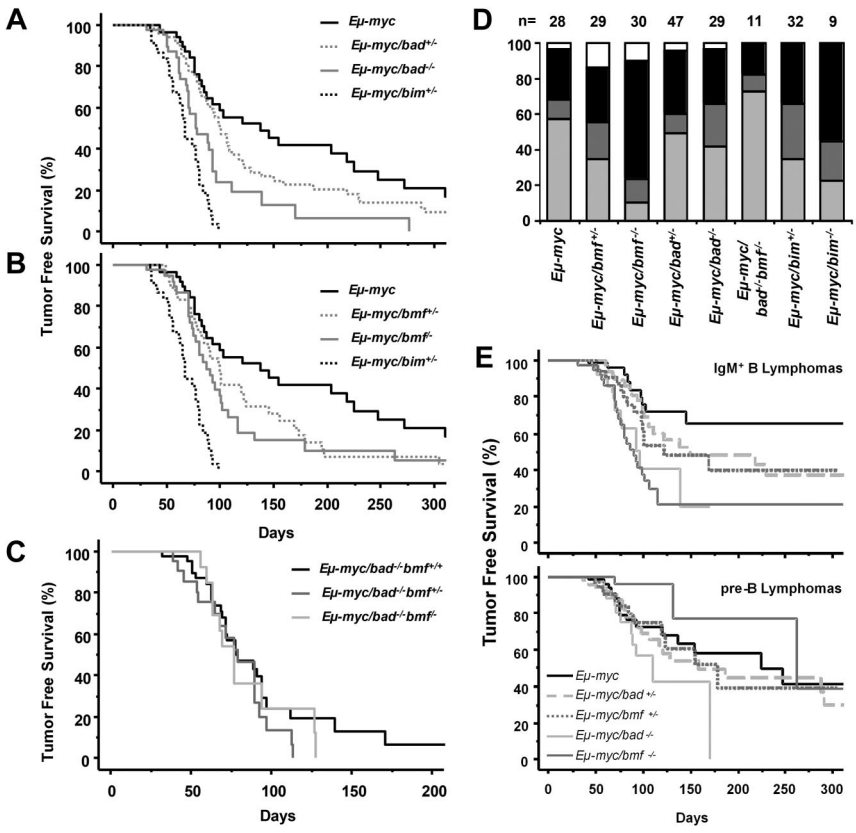

Figure 1. Loss of *bad* or *bmf* accelerates *c-myc*–induced lymphomagenesis. (A) Tumor-free survival of *Eμ-myc* (n = 29; median survival, 138 days), *Eμ-myc/bad*$^{+/-}$ (n = 47; median survival, 100 days), *Eμ-myc/bad*$^{-/-}$ (n = 29; median survival, 78 days), and *Eμ-myc/bim*$^{+/-}$ (n = 32; median survival, 67 days). Lymphomas occurred significantly earlier in *Eμ-myc/bad*$^{-/-}$ than in wt *Eμ-myc* animals ($P < .001$). (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*$^{+/-}$ ($P < .05$) and *Eμ-myc/bmf*$^{-/-}$ ($P < .01$) 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 = 11). (D) Distributions of pro/pre-B (light gray), mixed (dark gray), 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 with *Eμ-myc* animals ($P < .01$, χ^2 test). (E) Kaplan-Meier analysis of IgM$^+$ and mixed lymphomas (top) and of pre-B lymphomas (bottom) of *Eμ-myc* (black solid line), *Eμ-myc/bad*$^{+/-}$ (light gray, dashed line), *Eμ-myc/bad*$^{-/-}$ (light gray, solid line), *Eμ-myc/bmf*$^{+/-}$ (dark gray dotted line), and *Eμ-myc/bmf*$^{-/-}$ (dark gray solid line) mice. IgM$^+$ B lymphomas arose significantly earlier in *Eμ-myc/bmf*$^{-/-}$ ($P < .001$) and *Eμ-myc/bad*$^{-/-}$ ($P < .01$) than in wt *Eμ-myc* mice. Pre-B lymphomas were not significantly accelerated by loss of either *bad* or *bmf*.

alleles of *bmf* or *bad* accelerated c-Myc–driven tumorigenesis significantly less efficiently than loss of one allele of *bim* ($P < .01$ and $P < .001$, respectively; Figure 1A-B). Notably, *Eμ-myc* mice deficient for both Bad and Bmf did not contract disease earlier than single-mutant animals expressing the transgene (Figure 1C). Taken together, this finding 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 approximately 60% pre-B–cell tumors in the wt *Eμ-myc* mice (16 of 28), approximately 30% of all cases (8 of 28) were immature IgM$^+$ B-cell lymphomas, and the remaining tumors displayed a mixed (pre-B/IgM$^+$) phenotype (Figure 1D). In strong contrast, *Eμ-myc/bmf*$^{-/-}$ mice developed predominantly IgM$^+$ B-cell lymphomas (20 of 30; 67%), and only 3 of 30 tumors (10%) were of pro/pre-B–cell origin (Figure 1D). The tumor spectrum observed in the *Eμ-myc/bmf*$^{+/-}$ mice was intermediate between wt and *bmf*$^{-/-}$ *Eμ-myc* mice with 10 of 29 cases (34%) being pro/pre-B–cell lymphomas, demonstrating a clear gene-dosage effect (Figure 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 (Figure 1D). Further analysis revealed that the observed acceleration of tumorigenesis was mainly the result of an earlier onset of IgM$^+$ lymphomas in all genotypes tested (Figure 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 (Figure 1D). Together, this finding indicates that although Bmf and Bad are able to engage the same prosurvival 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 of 30 and 4 of 29, respectively, vs 1 of 30 *Eμ-myc* mice; Figure 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 toward a role for Bmf in apoptosis of early hematopoietic progenitors.

Bmf-deficient Eμ-myc transgenic mice bear greater 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 with the mean white blood cell count of diseased *Eμ-myc* mice, we observed a greater than 4-fold increase in the number of circulating leukocytes in diseased transgenic mice lacking Bmf ($P < .001$; Figure 2A). In addition, *Eμ-myc/bmf*$^{-/-}$ mice had a significantly more pronounced splenomegaly than wt or Bad-deficient tumor mice (Figure 2B). Taken together, the 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 (Figure 2A-B).

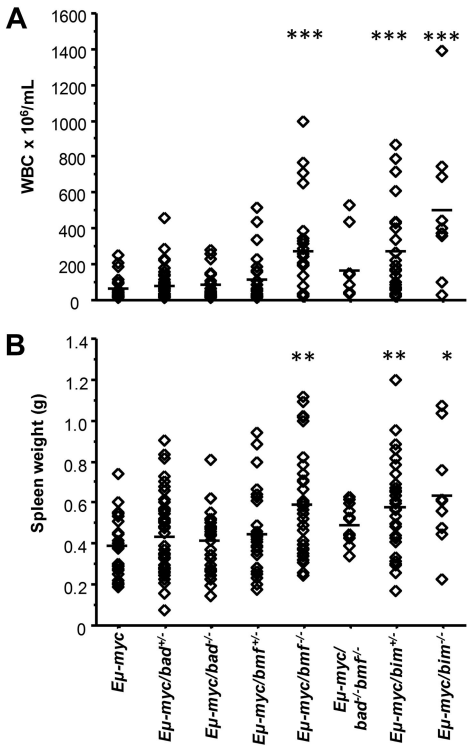

Figure 2. Loss of *bmf* enhances the severity of *Eμ-myc* lymphomas. (A) Numbers of total leukocytes in the blood of moribund mice of the indicated genotypes. The leukocyte counts were significantly greater in $bmf^{-/-}$ *Eμ-myc* mice than in wt *Eμ-myc* mice (mean leukocyte count in *Eμ-myc*/$bmf^{-/-}$ mice was $263 \pm 251 \times 10^6$/mL vs $58 \pm 67 \times 10^6$/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 ± 0.253 g in *Eμ-myc*/$bmf^{-/-}$ vs 0.395 ± 0.140 g in *Eμ-myc* or 0.428 ± 0.160 g in *Eμ-myc*/$bad^{-/-}$). Diamonds represent individual spleen weights in mice of the indicated genotypes with bars indicating the corresponding means. $^*P < .01$, $^{**}P < .001$, $^{***}P < .001$ compared with *Eμ-myc*.

Loss of Bmf or Bad causes an accumulation of B cells in preleukemic 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 (Figure 3A), as previously reported.[4] The expansion of these premalignant cells is dampened for a limited period of time by c-Myc–induced apoptosis. Although the increased numbers of pro/pre-B cells in the bone marrow were comparable among *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 (Figure 3A). Interestingly, *Eμ-myc*/$bad^{-/-}$ mice showed increased pre-B–cell numbers in the spleen compared with *Eμ-myc mice*, whereas loss of Bmf favored the accumulation of more mature T1 B cells in *Eμ-myc* transgenic animals (Figure 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 ($7.3 \pm 3.5 \times 10^6$/mL vs $75.5 \pm 8.0 \times 10^6$/mL), whereas loss of Bad had no such effect (Figure 3A). This finding 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 compared with nontransgenic cells in vitro. Loss of Bad or Bmf did confer some minor protection to nontransgenic pre-B cells in culture ($bad^{-/-}$, $P < .05$, and $bmf^{-/-}$, $P < .001$, compared with *wt* at 48 hours) but more mature B cells of both genotypes died as fast as wt cells (Figure 3B). Notably, Bad deficiency failed to confer protection to any of the subsets from *Eμ-myc* transgenic mice. However, whereas 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 nontransgenic B cells (Figure 3B bottom). This finding 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 (Figure 3A). This accumulation could be attributable 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/propidium iodide staining. Consistent with our hypothesis, steady-state levels of apoptosis of pre-B and B cells were lower in *Eμ-myc* mice lacking Bad compared with *Eμ-myc* controls (Figure 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 (Figure 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 (supplemental Figure 1), arguing against non–cell-autonomous effects.

Loss of Bmf reduces the pressure to lose p53 function

Because of the strong proapoptotic 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 nonfunctional p53 because of the absence of a 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 (24%) of 38 of wt *Eμ-myc*; 8 (33%) of 24 of $bad^{+/-}$*Eμ-myc*, and 5 (23%) of 22 of $bad^{-/-}$*Eμ-myc* lymphomas (Figure 4A). In contrast, only 2 (9%) of 22 $bmf^{+/-}$, 3 (11%) of 27 $bmf^{-/-}$, and 0 of 7 $bad^{-/-}bmf^{-/-}$ *Eμ-myc* lymphomas, respectively, were deficient of functional p53 in this type of analysis (Figure 4A and not shown). Consistent with previous findings,[10] 0 of 6 $bim^{-/-}$ and only 1 of 9 $bim^{+/-}$ lymphomas analyzed showed increased ARF levels (not shown).

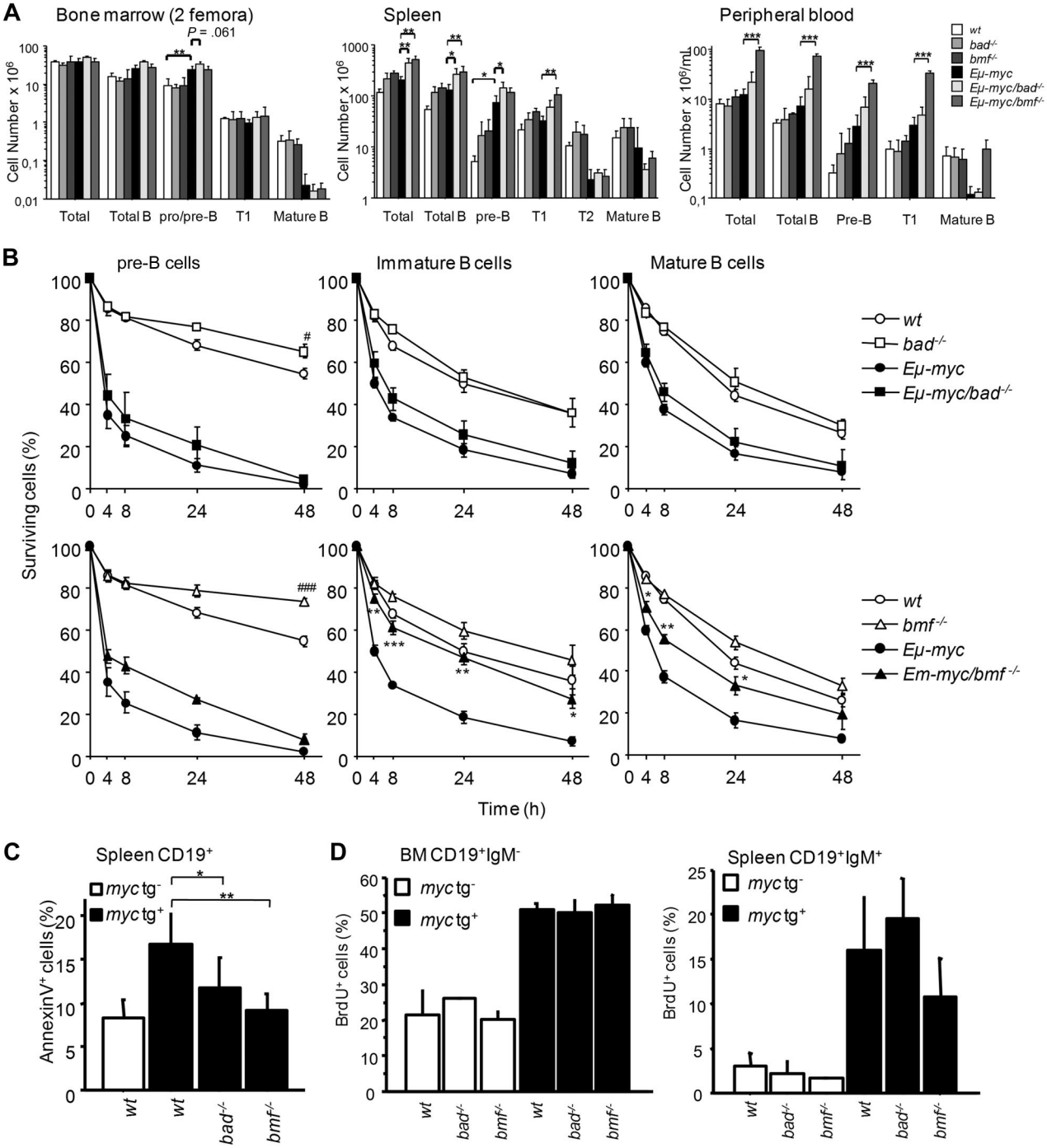

Figure 3. Loss of Bmf enhances the survival of premalignant *Eμ-myc* B lymphocytes. For analysis of preleukemic mice, absence of transplantable tumor cells was confirmed by injecting 2×10^6 spleen cells into wt C57BL/6 recipients followed for at least 2 months. (A) Cell number and B-cell subset composition 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 to 4 mice per genotype. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. 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 48 hours ex vivo. Percentages of surviving cells were determined by annexin-V/propidium iodide staining. Data represent means ± SEM from 3 to 4 independent experiments for each genotype. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ compared with *Eμ-myc*, $\#P < .05$, $\#\#\#P < .001$ compared with wt. (C) Freshly isolated splenocytes from 4-week-old mice of the indicated genotypes were immediately stained with anti-CD19-phycoerythrin together with fluorescein isothiocyanate -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. $^*P < .05$, $^{**}P < .01$. (D) At 4 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.

Because 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 prosurvival 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

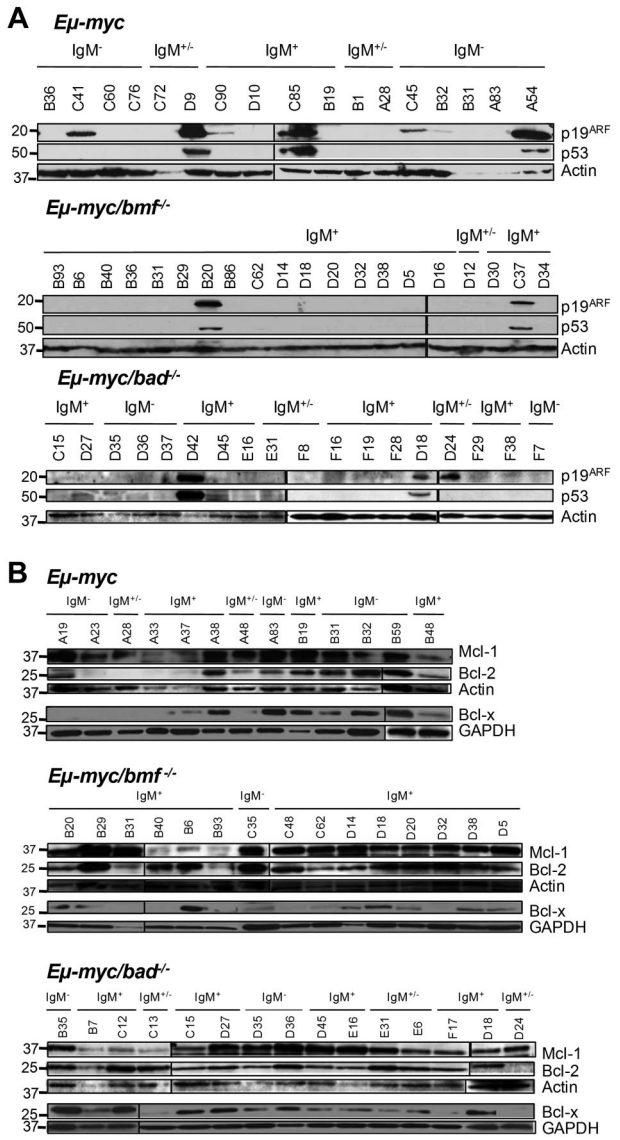

Figure 4. Loss of Bmf but not Bad reduces the pressure to lose p53. (A) Representative Western blot analysis of $p19^{ARF}$ and p53 expression as well as (B) Bcl-2, Bcl-x, and Mcl-1 expression in lymphoma lysates derived from wt, *bmf*$^{-/-}$ and *bad*$^{-/-}$ *Eμ-myc* mice. Membranes were reprobed with the use of anti-actin or anti-GAPDH antibodies as a loading control.

blot for the expression of Bcl-2, Bcl-xL, and Mcl-1. The 3 proteins were expressed in variable levels among the lymphomas, independent of their immunophenotype, but we failed to detect increased frequencies of overexpression of prosurvival Bcl-2 proteins in Bmf-deficient over wt or Bad-deficient c-Myc–driven lymphomas (Figure 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] whereas Bim and Puma proteins are induced.[10-12] To test whether the expression of Bmf or Bad also were 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 (Figure 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 of 10 IgM^+ tumors (Figure 5B-C). Notably, loss of Bmf expression also was observed in 1 of 6 IgM^+ *Eμ-myc/bmf*$^{+/-}$ tumors tested (Figure 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 (Figure 5A), and one or the other isoform was expressed in *Eμ-myc* tumor samples (Figure 5B). Tumors arising in *Eμ-myc/bad*$^{+/-}$ animals tested positive for Bad protein expression in 9 of 9 cases analyzed suggesting retention of the second allele (Figure 5C). Furthermore, we aimed to investigate whether the Bad protein found in premalignant *Eμ-myc* transgenic B cells was hypophosphorylated, indicative of its activation.[33] However, the weak signal that we obtained using different antibody against phospho-Bad also was 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 whether the absence of Bad or Bmf would confer drug-resistance phenotypes to *c-myc*–dependent lymphomas. Freshly harvested tumor samples were cultivated for 24 hours 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 (Figure 6).

Low-level expression of BMF in Burkitt lymphoma

In human B-CLL, Bmf is expressed at significant levels and further induced upon serum-deprivation.[16,36] Furthermore, gene chip analysis demonstrated the presence of *BMF* mRNA in acute lymphoblastic leukemia,[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, acute lymphoblastic leukemia and CLL usually do not associate with Myc overexpression. To assess whether deregulated c-Myc might correlate with Bmf expression, we quantified its protein levels in 3 frequently studied Burkitt lymphoma cell lines, Daudi, Ramos, and Raji, as well as in 6 biopsy samples from patients diagnosed with Burkitt lymphoma. We also assessed expression of Bad as well as Bim because the latter protein is reportedly lost or inactivated frequently in human Burkitt lymphoma.[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. Although Bad and Bim protein were found expressed at comparable levels in all samples analyzed, Bmf

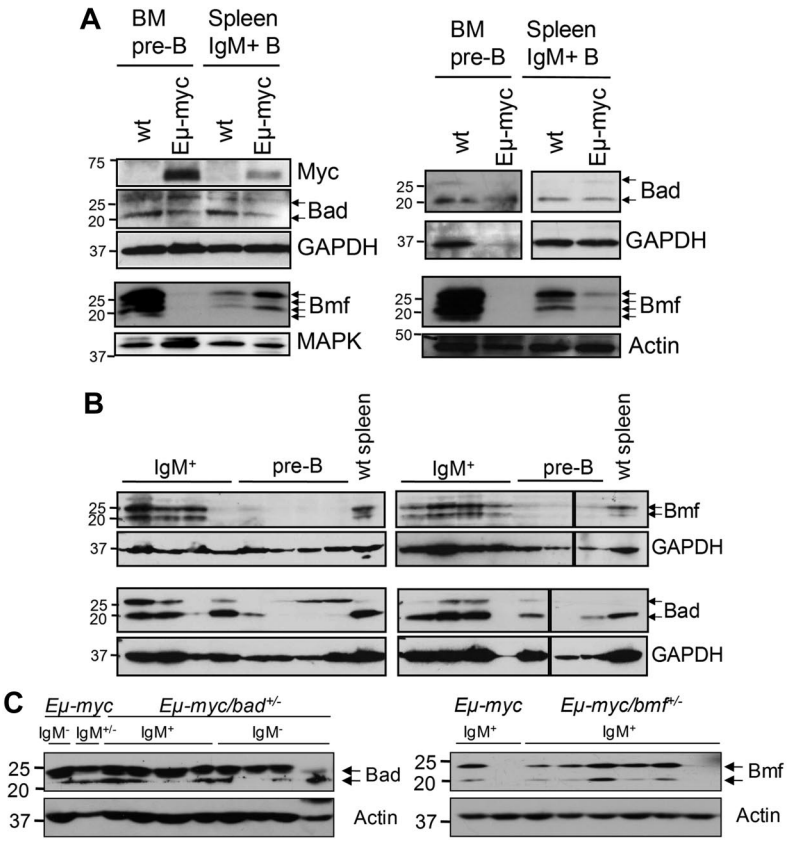

Figure 5. Expression of Bmf and Bad in (pre-)malignant *Eμ-myc* and wt B lymphocytes. Wt and *Eμ-myc* FACS-sorted pre-B cells from the bone marrow and IgM$^+$ B cells from the spleens of 4-week-old mice were analyzed for expression of (A) c-Myc, Bmf, or Bad. Representative blots from 2 independent cell sorts are shown. Membranes were reprobed by the use of 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 by the use of 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 by the use of anti-actin antibody as a loading control.

isoforms were only expressed at significant levels in healthy CD19$^+$ B cells but barely detectable in the 3 cell lines and primary tumor tissues (Figure 7A). Because *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 whether inhibition of DNA-methyltransferases by the addition of 5′-aza-2′-deoxycytidine would suffice to restore Bmf expression and apoptosis in Burkitt lymphoma cell lines. Indeed, mRNA levels and protein were significantly induced in the Burkitt lymphoma lines (Figure 7B; supplemental Figure 2), suggesting that demethylation directly triggers transcription of the *BMF* gene. However, MethyLight polymerase chain reaction analysis[28,29] covering 7 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 of the tree cell lines (not shown). In search of other conditions that could induce Bmf in Burkitt lymphoma cells, we also found Bmf induction accompanied by cell death in the 3 cell lines after SAHA treatment or serum deprivation (Figure 7C; supplemental Figure 3). Lentiviral knockdown of BMF in Ramos cells had no significant effect on apoptosis induced by SAHA (Figure 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 (Figure 6). However, knockdown of BMF could delay serum deprivation-induced cell death in these cells (Figure 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 ie Noxa or Bid do play only redundant or no role in regulating Myc-induced lymphomagenesis (R. W. Johnstone, oral communication, Janury 2009),[12,13] 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 (Figures 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 (Figure 3A), which was not caused by differences in proliferation capacity between genotypes (Figure 3C). This finding indicated that loss of either BH3-only protein enhanced the survival of preleukemic 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 (Figure 3B). This belief is supported by our observations that although the size of the population "at-risk" for a second lesion

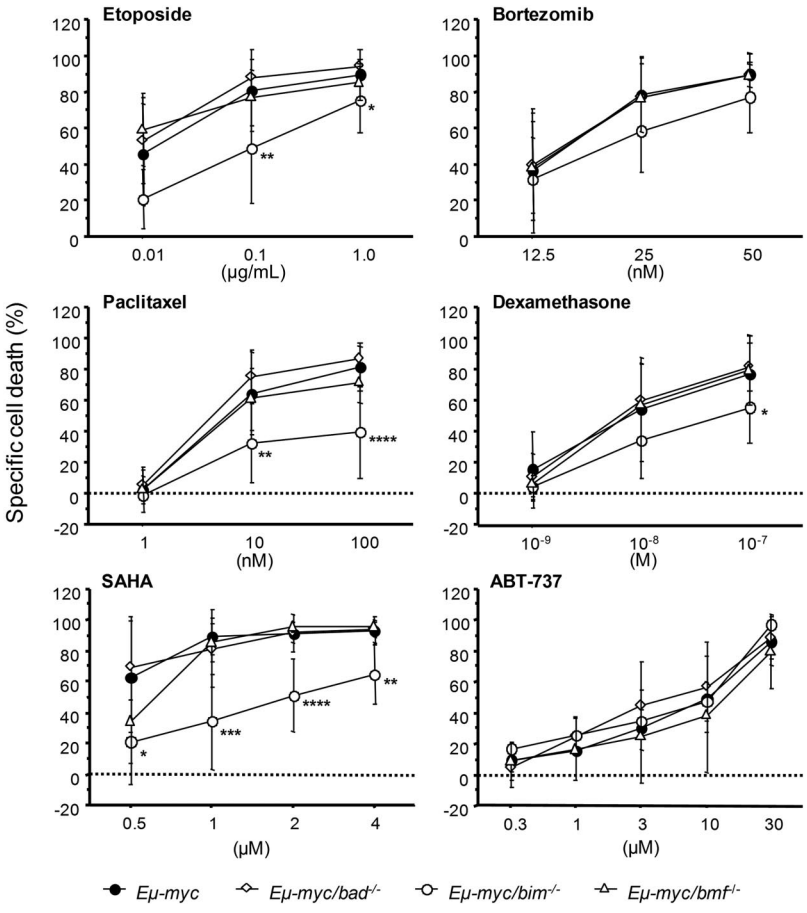

Figure 6. Loss of Bim but not Bad or Bmf protects *Eμ-myc* lymphomas from drug-induced apoptosis in vitro. Freshly isolated *Eμ-myc* lymphoma cells were cultured on supporting irradiated NIH-3T3 cells in the presence of chemotherapeutic agents at the indicated concentrations for 24 hours. Cell death 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 or more animals/genotype. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .001$.

is increased in the absence of Bmf up to 10-fold (Figure 3A), the frequency of tumors that inactivate the p53 pathway actually dropped (Figure 4A). This decrease was not simply attributable to the shift toward the development of IgM^+ lymphomas, observed when Bmf is absent (Figure 1D), because p53 inactivation occurs as frequently in IgM^+ as in pre-B *wt Eμ-myc* lymphomas (3 of 17 in pro/pre-B lymphomas and 3 of 13 in IgM^+ lymphomas tested). Although 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 (Figure 5B-C). The molecular basis of this phenomenon, however, awaits detailed investigation. Reduced selection pressure against p53 also has been reported in *Eμ-myc* tumors lacking *bim* or *bax* but not *noxa*, whereas studies on *puma* revealed contradictory results.[10-12,38] Because 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 (Figure 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 (Figure 5A). A similar tumor immunophenotype was observed in *Eμ-myc* mice lacking Bim, as previously suggested by Egle et al[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-)B–cell receptor 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, approximately 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 (Figures 1-2). Furthermore, loss of Bad preferentially facilitated the accumulation of premalignant pre-B cells (Figure 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 (Figures 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

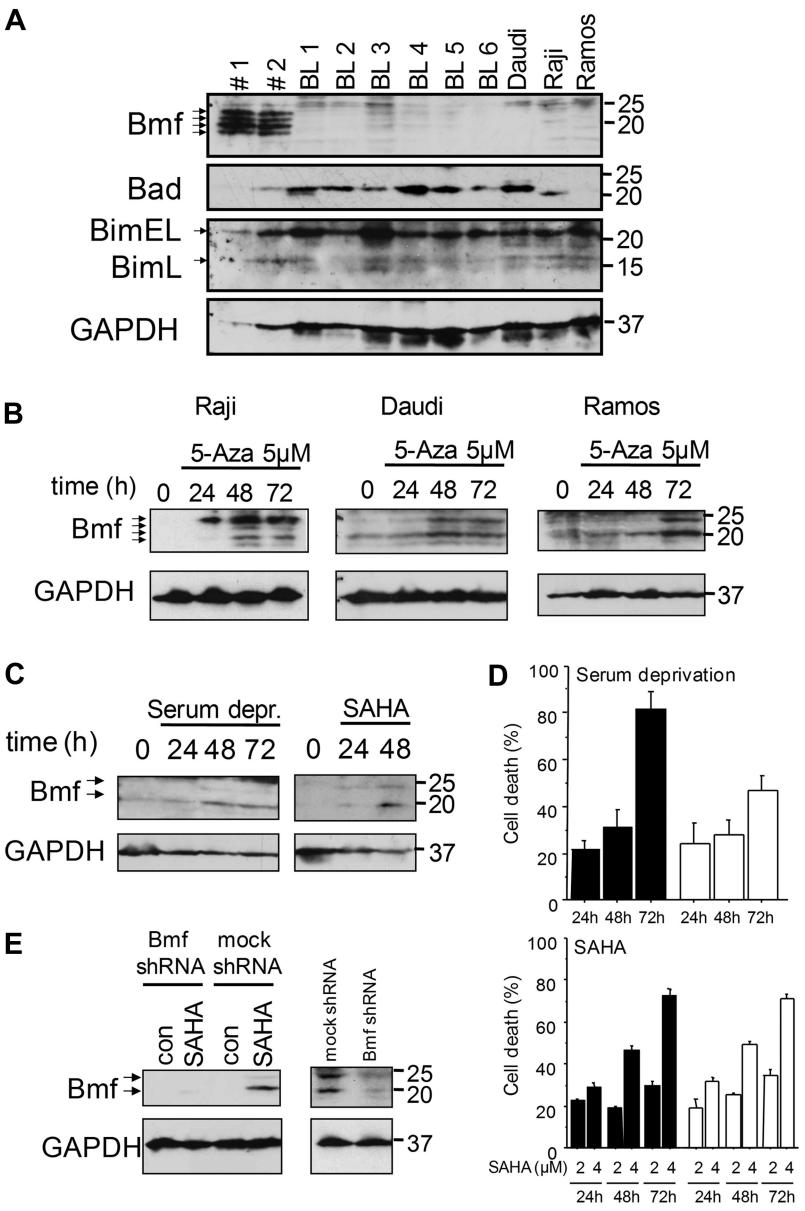

Figure 7. Bmf expression is absent in Burkitt lymphoma cells but can be restored upon demethylation. (A) Western blot analysis of Bmf, Bim, and Bad in CD19$^+$ cells derived from peripheral blood of healthy donors (#1 and #2), primary Burkitt lymphoma samples (BL1-BL6), and Burkitt lymphoma cell lines Daudi, Raji, and Ramos. (B) Western blot analysis of Bmf levels in Burkitt lymphoma 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) or cells deprived of serum for 48 hours (right).

alleles of *bmf* or *bad* (Figure 1). This finding 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 (Figure 2). Also, Bim levels are induced upon c-Myc overexpression,[10] whereas neither Bad nor Bmf levels were found increased in premalignant B cells or tumors, suggesting an auxiliary and more cell type restricted role for both 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, whereas Bad and Bmf appear to bind and neutralize Bfl1/A1 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 several 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 Burkitt lymphoma lines, the loss of Bmf did not confer drug resistance to histone deacetylase inhibition in vitro (Figure 7; supplemental Figure 3), suggesting redundancy with other BH3-only proteins. Nonetheless, it will be interesting to see whether 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,[42] but killing was only achieved when high concentrations of the drug were applied, in line with recent findings from Whitecross et al.[43] This observation may be related to the fact that all lymphomas expressed significant levels of Mcl-1 (Figure 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 the result of repression of Bad function but may depend on additional antiapoptotic effects exerted by the AKT-pathway, for example, repression of Bim and/or Puma.[45,46]

Finally, because we failed to find evidence for direct regulation of BMF gene expression by c-Myc in promoter reporter studies or

promoter methylation in human Burkitt lymphoma 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, for instance, 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.

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Authorship

Contribution: A.F. designed research, performed experiments, analyzed data, and wrote the paper, V.L., W.C., C.P., and H.F. performed experiments; S.G. provided reagents; A.T. provided tumor samples; and A.V. designed research, analyzed data, and wrote the paper.

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The current address for A.F. is Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden.

Correspondence: Prof Andreas Villunger, PhD, Division of Developmental Immunology, Biocenter, Innsbruck Medical University, Fritz-Pregl-Str 3, A-6020 Innsbruck, Austria; e-mail: andreas.villunger@i-med.ac.at.

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