Research Article

Induction of Apoptosis in Plasma Cells by B Lymphocyte–Induced Maturation Protein-1 Knockdown

Fan-Ru Lin,[1,2] Hui-Kai Kuo,[1] Hsia-Yuan Ying,[1] Fu-Hung Yang,[1,2] and Kuo-I Lin[1,2]

[1]Genomics Research Center, Academia Sinica; [2]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan

Abstract

B lymphocyte–induced maturation protein-1 (Blimp-1) is a transcriptional repressor that plays an important role during plasmacytic differentiation and is expressed in normal and transformed plasma cells. We here investigated the importance of continuous Blimp-1 expression. We found that knockdown of Blimp-1 expression by lentiviral vector-delivered short hairpin RNA causes apoptosis in multiple myeloma cell lines and plasmacytoma cells, indicating that continued expression of Blimp-1 is required for cell survival. We examined the mechanism underlying Blimp-1 knockdown-mediated apoptosis and found that the Blimp-1 knockdown neither reversed the phenotypic markers of plasma cells nor caused cell cycle arrest. Instead, our results show that knockdown of Blimp-1 induced the proapoptotic protein Bim, reduced the antiapoptotic protein Mcl-1, and activated caspase-9 and caspase-3. We further link apoptosis in transformed plasma cells mediated by proteasome inhibitors, the effective therapeutic agent for multiple myeloma patients, with reduced expression of Blimp-1. Lastly, we show that Blimp-1–dependent cell survival may act downstream of IFN regulatory factor 4 (IRF4) because IRF4 knockdown leads to down-regulation of Blimp-1 and apoptosis in multiple myeloma cells and plasmacytoma cells. Together, our data suggest that Blimp-1 ensures the survival of transformed plasma cells. [Cancer Res 2007;67(24):11914–23]

Introduction

B lymphocyte–induced maturation protein-1 (Blimp-1) is a master regulator crucial for inducing plasmacytic differentiation (1, 2). It is a transcriptional repressor that directly turns off the expression of several genes, including *c-MYC* (3, 4), *CIITA* (5), *ID3* (6), *SPI-B* (6), and *PAX-5* (7), through interaction with histone deacetylase (HDAC) family proteins (8), Groucho (9), a histone methyltransferase, G9a (10), and an arginine-specific histone methyltransferase, Prmt5 (11). Blimp-1 is present in plasma cells formed in primary and secondary immune responses to either thymus-dependent or thymus-independent antigens (12). Blimp-1 is also expressed in transformed plasma cells (6, 13). Recent results showing that Blimp-1 is required for maintenance of long-lived plasma cells suggest that Blimp-1 regulates a reversible gene expression program and that this protein has potential applications in the therapeutic management of plasma cell–related diseases (14).

Plasma cell tumors in human are classified as multiple myeloma, solitary bone plasmacytoma, or extramedullary plasmacytoma (15). The mechanism(s) of tumorigenesis for multiple myeloma is not clear. Some genetic modifications have been reported to occur during tumor progression [e.g., translocation of genes such as *CCND1, CCND3, FGFR3*, and *MAF* to immunoglobulin regulatory regions (16) and rearrangement of *c-MYC* to a nonimmunoglobulin locus (16)]. Most of the tumor progression takes place in bone marrow where the interaction between multiple myeloma cells and host bone marrow regulates tumor cell growth, survival, and migration (17). Bcl-xL, Bcl-2, and Mcl-1 showed increased levels of expression in multiple myeloma cells, although Mcl-1, rather than Bcl-2 and Bcl-xL, is the main player in promoting multiple myeloma cell survival (18, 19). Previous microarray cluster analysis results revealed that many of the Blimp-1 targets involved in regulating B-cell identity and germinal center function, and in inducing Ig secretion, are regulated normally by Blimp-1 in multiple myeloma, whereas genes controlling proliferation, such as c-MYC, are not subjected to Blimp-1 repression (6), suggesting a role for Blimp-1 in maintaining plasma cells.

Here, we sought to determine whether Blimp-1 must be continuously expressed to maintain survival in human multiple myeloma cells or mouse plasmacytoma cells. Using a lentiviral vector delivering short hairpin RNA (shRNA) specific to human and mouse Blimp-1, we found that Blimp-1 knockdown causes apoptosis but not cell cycle arrest. RNA interference (RNAi) for Blimp-1 resulted in induction of the proapoptotic protein Bim and reduction of the antiapoptotic protein Mcl-1. Additionally, caspase-9 and caspase-3 were activated on reduction of Blimp-1 expression in multiple myeloma lines. Interestingly, we found that Blimp-1 knockdown in multiple myeloma lines could not reprogram the plasma cell phenotype. Accordingly, our results indicate that proteasome inhibitors, the effective therapeutic strategy for multiple myeloma, cause a reduction of Blimp-1 expression that is required for proteasome inhibitor–mediated apoptosis. Lastly, our data imply that IFN regulatory factor 4 (IRF4) might function upstream of Blimp-1 in supporting survival. Our results reveal a novel role for Blimp-1 in maintaining malignant plasma cell survival.

Materials and Methods

Cell lines and reagents. Cells were grown and maintained as follows: human U266 multiple myeloma cells in RPMI 1640 (Life Technologies) containing 20% fetal bovine serum (FBS; Life Technologies), NCI-H929 (H929) multiple myeloma cells in RPMI 1640 containing 10% FBS and 50 μmol/L β-mercaptoethanol (Life Technologies), and RPMI8226 and IM9 in RPMI 1640 containing 10% FBS. All multiple myeloma cells were grown in medium containing penicillin/streptomycin (100 units/mL; Life Technologies). Mouse plasmacytoma P3X63Ag8 (P3X) cells were grown in RPMI 1640 plus 10% FBS, penicillin/streptomycin, and β-mercaptoethanol (50 μmol/L). 293T and 3T3 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS and penicillin/streptomycin (100 units/mL). Caspase-3 inhibitor (Z-DEVD-FMK) was purchased from R&D Systems, Inc.

Plasmids. The shRNA cassette was first cloned into a pBS/U6 vector (kindly provided by Dr. Yang Shi, Harvard University, Cambridge, MA). The

Requests for reprints: Kuo-I Lin, Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang District, Taipei 115, Taiwan. Phone: 886-2-2789-9930, ext. 336; Fax: 886-2-2789-9931; E-mail: kuoilin@gate.sinica.edu.tw.

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protocol for generating shRNA for Blimp-1, IRF4, or the negative control was essentially as described (20). The human Blimp-1 shRNA contains the sense targeting sequences 5′-GGGACTCCTACGCTTACTTGAA-3′ and 5′-GGGTGCAGCCTTTATGAGTCATC-3′, corresponding to nucleotides 1004 to 1025 and 2254 to 2276, respectively, of the human Blimp-1 coding sequence (Genbank accession no. NM_001198). These sequences were named U6Blimp1-716i and U6Blimp1-1966i, respectively. The mouse Blimp-1 shRNA U6Blimp1-3952i contains a sense targeting sequence located in the 3′-untranslated region, 5′-GGGTGGTTCGGTCCTGTATTCT-3′, nucleotides 3952 to 3973 downstream of the translational start site (Genbank accession no. NM_007548). The human U6IRF4-776i and U6IRF4-1278i contain the sense targeting sequences 5′-GGGAAATCCTCGTGAAGGAGCT-3′ and 5′-GGGCTACGATTTACCAGAACAC-3′, respectively, of the human IRF4 coding sequence (Genbank accession no. NM_002460). The mouse U6IRF4-536i and U6IRF4-1278i contain the sequence 5′-GGGATTAT-GCCCCTGACCAGTC-3′ and 5′-GGGCTACGAGTTACCTGAACAC-3′, respectively, which targets to mouse IRF4 coding sequence (Genbank accession no. NM_013674). The control shRNA (U6controli) contains scrambled nucleotides, 5′-GGGATATCGGCATATTGCGGCTA-3′, which has no homology to other known mRNAs. The U6-shRNA cassette was removed from the pBS/U6 vector by *Bam*HI digestion and the overhanging sequences from *Bam*HI digestion were filled in and then blunt-end ligation was performed at the *Pac*I site on the FUGW lentiviral vector (21), a generous gift from Dr. Jeremy Luban (Columbia University, New York, NY).

Retroviral and lentiviral transduction. The procedure for preparing retroviral vectors was performed as described (22). For pseudotyped virus, retroviral vector (15 μg), pSV-φ-E-MLV (15 μg), and VSV-G (pMD.G; 15 μg) were transfected into 293T cells. For generation of pseudotyped lentivirus, lentivirus vector (FUGW; 15 μg), Δ8.9 (15 μg), and VSV-G (15 μg) were used for transfection into 293T cells. Target multiple myeloma cells were transduced at a multiplicity of infection (m.o.i.) of 5 to 10 in the presence of 5 μg/mL polybrene (Sigma) and P3X cells were transduced at a m.o.i. of 10 to 15, and the percentage of transduced cells was analyzed by green fluorescent protein (GFP) or yellow fluorescent protein (YFP) expression with fluorescence-activated cell sorting (FACS) analysis 1 day after infection.

RNA isolation and reverse transcription-quantitative PCR analysis. Total RNA was isolated on an RNeasy spin column (Qiagen). Total RNA (1 μg) was used for cDNA synthesis by the Omniscript Reverse Transcriptase kit (Qiagen) performed according to the manufacturer's instructions. cDNA was used for reverse transcription-quantitative PCR (RT-QPCR) analysis with the Taqman Universal PCR Master Mix in an ABI/Prism 7000 sequence detection system (Applied Biosystems). The Taqman primer sets used in this study are the following: *PRKG1* (assay ID: Hs00183512_ml) *PRDM1* (assay ID: Hs00153357_ml), *PPIA* (assay ID: Hs99999904_ml), *PU.1* (assay ID: Hs00231368_m1), *BCL-6* (assay ID: Hs00153368_m1), *CIITA* (assay ID: Hs00172106_m1), *XBP-1* (assay ID: Hs00231936_ml), and *PAX-5* (assay ID: Hs00172003_m1). The primer sets used for RT-QPCR with the SYBR green method are as follows: *c-MYC*, 5′-GCCACGTCTCCACACATCAG-3′ and 5′-TCTTGGCAGCAGGA-TAGTCCTT-3′; *SPI-B*, 5′-GGCCACACTTCAGCTGTCTGTA-3′ and 5′-AGG-AGCCCCCTCTGAATCAG-3′; *ID3*, 5′-CCCTGGACCCCCTGATG-3′ and 5′-TTTGTCGTTGGAGATGACAAGTTC-3′; and *MTA3*, 5′-GGAGAGCCTGT-GAGAGCTGCTA-3′ and 5′-CATATTAGGTGGGCCCCAAGA-3′. The primer sets used for semiquantitative reverse transcription-PCR (RT-PCR) are as follows: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5′-GGGC-GCCTGGTCACCAGGGCTG-3′ and 5′-GGGGCCATCCACAGTCTTCTG-3′; *SPI-B*, 5′-CACGGAGAACTTCGCT-3′ and 5′-CTTGGCGTAGTTTCGG-3′; *XBP-1*, 5′-CGCTGAGGAGGAAACTGAA-3′ and 5′-GGGAGGCTGGTAAG-GAACT-3′; and *ID3*, 5′-TGTGCTGCCTGTCGGAACG-3′ and 5′-ACAAG-TTCCGGAGTGAGCT-3′.

FACS analysis. Apoptotic and dead cells were determined by Annexin V-phycoerythrin (PE) or Annexin V-allophycocyanin (APC) plus 7-amino-actinomycin D staining according to the protocol provided by the manufacturer (BD PharMingen). Cell cycle phase was determined by propidium iodide (BD PharMingen) staining according to the manufacturer's protocol. The flow cytometry protocol determining surface marker staining was essentially as described (6). The following antibodies used in this study were from BD PharMingen: PE-conjugated anti-human CD138/syndecan-1 (clone DL-101), PE-conjugated anti-human CD38 (clone HB7), PE-conjugated anti-human CD19 (HIB19), APC-conjugated anti-human CD20 (clone 2H7), PE-conjugated anti-human MHCII (HLADR, clone TU36), and PE-conjugated anti-human CD54 (clone HA58). Experimental results were analyzed by a FACSCalibur or FACSCanto machine (Becton Dickinson) with CellQuest or FCS Express 3.0 software.

Immunoblotting and ELISA. Nuclear extracts and total cell lysates were performed as described (3). Nuclear extracts (10–20 μg) or total cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotting as described using antibodies specific to Blimp-1 (6): IRF4 (M17, Santa Cruz Biotechnology), Bcl-2 (Ab-1, Calbiochem), Bcl-xL (2H12, Santa Cruz Biotechnology), Mcl-1 (S-19, Santa Cruz Biotechnology), Bim (AB17003, Chemicon International, Inc.), procaspase-4 (4B9, MBL), α-tubulin (Sigma), or actin (Sigma). Blots were then hybridized with one of the following horseradish peroxidase–conjugated secondary antibodies: rabbit anti-goat IgG (Sigma), goat anti-mouse IgG (Promega), or goat anti-rabbit IgG (Sigma). The immunoreactive proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions, and the chemiluminescent immunocomplexes were scanned and detected by the Fujifilm LAS-3000 system. The ELISA was essentially following the previous protocol (23).

Generation of ABL-MYC(Ψ2) virus and plasmacytoma in BALB/c mice. Helper virus-free ABL-MYC(Ψ2) stocks were obtained from the cultured supernatant of a packaging line as described (24). The Ψ2 packaging cell line (kindly provided by Dr. J. Frederic Mushinski, National Cancer Institute, Bethesda, MD) was cultured in DMEM with 10% FCS and 1 mmol/L sodium pyruvate (Invitrogen). ABL-MYC(Ψ2) virus titers were determined by NIH3T3 cell transformation assays by counting the number of foci formation after 2 weeks as previously reported (24). Eight-week-old BALB/c mice (purchased from BioLASCO Taiwan Co.) were injected with pristane (0.5 mL; Sigma) 2 days before i.p. injection with ABL-MYC(Ψ2) titer (2×10^5 foci/mL). Ascites and tumors from mice were collected ~14 days after ABL-MYC(Ψ2) injection and then subjected to immunoblot analysis of Blimp-1 and actin expression. In parallel, tumor cells were cultured in RPMI 1640 containing 10% FBS, 50 μmol/L β-mercaptoethanol, 1 mmol/L sodium pyruvate, 100 μmol/L nonessential amino acids, and murine interleukin-6 (IL-6; 5 ng/mL; PeproTech) at a density of 10^6/mL. After ~2 weeks, the surviving, nonadhering cells were isolated and subcultured to develop into an IL-6–independent line. Lentiviral vectors carrying shRNA for Blimp-1 or control sequences were used for transduction in established plasmacytoma lines at a m.o.i. of 10 to 15 in the presence of 5 μg/mL polybrene.

Caspase activity assay. Cells were harvested 3 and 4 days after lentiviral infection. Activity of caspase-3, caspase-8, and caspase-9 was assayed according to the manufacturer's instructions (Calbiochem). We used the following colorimetric peptide substrates in this assay: caspase-3–specific Ac-DEVD-*p*NA, caspase-8–specific Ac-IETD-*p*NA, and caspase-9–specific LEHD-*p*NA. The free chromophore *p*NA light emission was quantified using a microtiter plate reader (SpectraMax M2) at 405 nm.

Results

Blimp-1 shRNA causes apoptosis in plasma cell lines. We first used lentiviral vector to deliver the expression of shRNA for Blimp-1 to determine the importance of continuous Blimp-1 expression. Two designed shRNAs against human Blimp-1 (1004i and 2254i) and one shRNA against mouse Blimp-1 (3952i) were generated. The efficiency of endogenous Blimp-1 expression knockdown after lentiviral transduction in human or mouse plasma cell lines was monitored by immunoblot (Fig. 1*A*). Human U6Blimp1-1004i seemed to be more effective than U6Blimp1-2254i (Fig. 1*A*). A lentiviral vector carrying either the U6 promoter alone or the U6 promoter plus shRNA with scrambled sequences (U6controli) was used as controls. Notably, we found enhanced cell death in multiple myeloma lines, including H929 and U266, on knockdown

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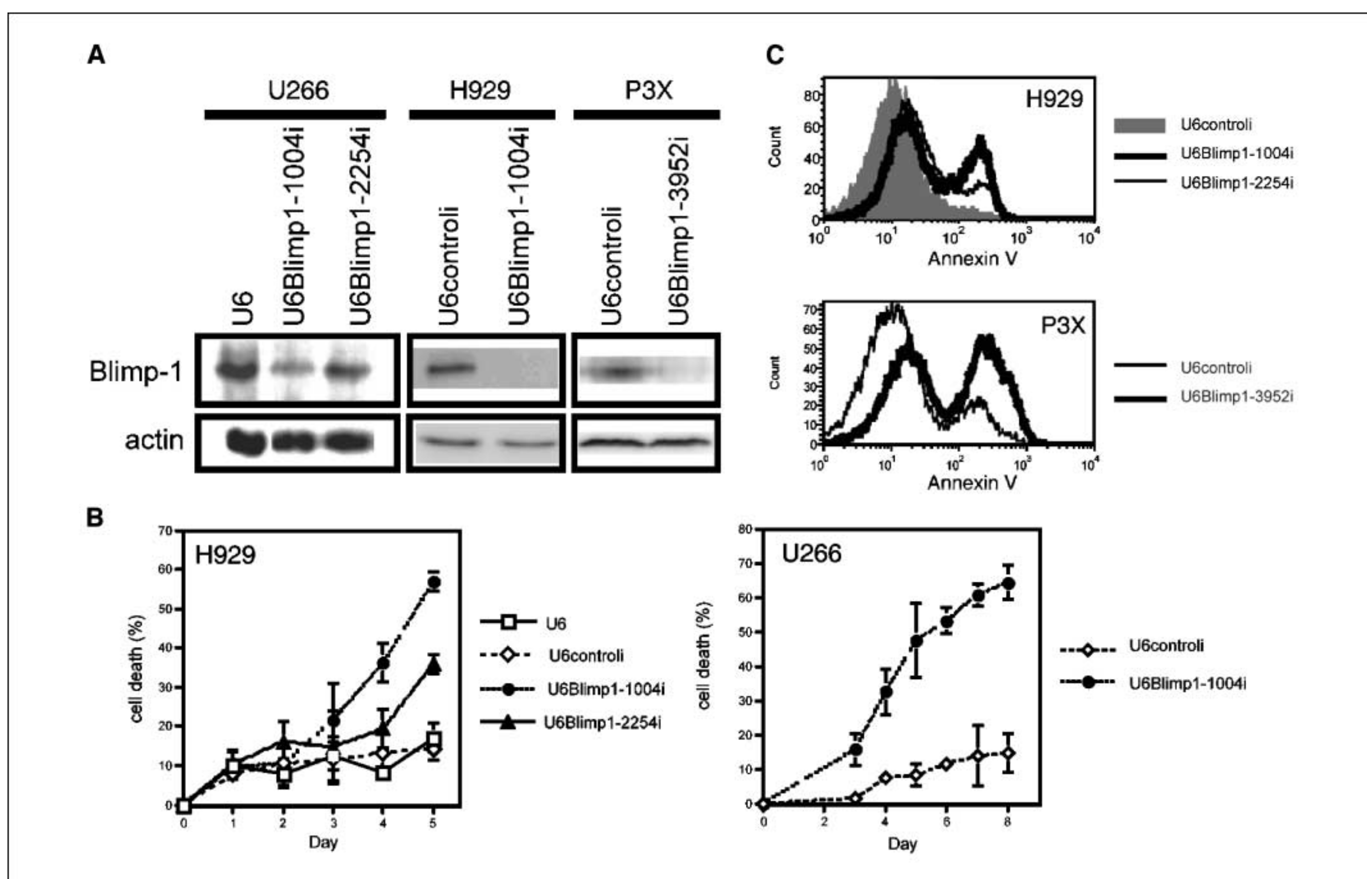

Figure 1. Inhibition of Blimp-1 expression causes apoptosis in plasma cell lines. *A,* introduction of shRNAs for Blimp-1 reduces the expression of Blimp-1 in human U266, H929, and P3X mouse plasmacytoma after 3 d of lentiviral vector transduction. Nuclear extracts from U266 cells (transduction rate >95%), total extracts from H929 (transduction rate >95%), or sorted shRNA-expressing, GFP-positive P3X cells were subjected to immunoblot analysis using a Blimp-1–specific antibody. Actin antibody was used for normalization. *B,* transduction of shRNAs for Blimp-1 (U6Blimp1-1004i or U6Blimp1-2254i), but not a shRNA with a scrambled sequence (U6controli), causes cell death. H929 or U266 cells were transduced with Blimp-1 shRNA-expressing lentiviral vectors. The percentage of cell death was assayed by trypan blue exclusion following various days of infection. *Points,* data from at least three independent experiments; *bars,* SD. *C,* transduction of shRNA for Blimp-1 induces apoptotic cell death. H929 cells transduced with U6Blimp1-1004i (*bold line*) or U6Blimp1-2254i (*fine line*) showed increased proportion of Annexin V–positive cells at day 4 compared with cells transduced with U6controli. U6Blimp1-3952i (*bold line*) causes increased apoptosis in mouse P3X cells determined by Annexin V staining after 3 d of lentiviral transduction.

of Blimp-1 expression (Fig. 1*B*). This is a specific effect because U6controli caused almost identical cell survival to U6 (Fig. 1*B*). The effect of mouse Blimp-1 shRNA, U6Blimp1-3952i, on reducing cell survival was also observed in the P3X plasmacytoma cell line (data not shown). Annexin V staining of GFP-positive cells showed that Blimp-1 RNAi-mediated cell death is apoptotic (Fig. 1*C*). Similar results were also observed in human RPMI8226 cells (data not shown). Taken together, we found that reduction of Blimp-1 expression via shRNA-mediated knockdown results in apoptotic cell death in human multiple myeloma lines and in a mouse plasmacytoma cell line.

Inhibition of Blimp-1 expression causes apoptosis in plasmacytomas. We next tested whether the above-mentioned Blimp-1 knockdown-mediated apoptosis in cell lines could be recapitulated in a newly established plasmacytoma line derived from a freshly isolated plasmacytoma. For this purpose, we generated plasmacytomas induced by inoculation of a murine retrovirus that encodes the *v-abl* and *c-myc* oncogenes, ABL-MYC(Ψ2), into BALB/c mice (24, 25). As previously reported, superficial, mesenteric connective tissue tumors and ascites developed in nearly all mice injected with ABL-MYC(Ψ2) i.p. (25). Immunoblot analysis showed expression of Blimp-1 protein in freshly isolated tumor and ascites but not in spleen from mice injected with ABL-MYC(Ψ2) (Fig. 2*A*). The isolated tumor cells were further established as a stromal cell–independent, IL-6–independent CD138, the plasma cell surface marker, positive and IgM-secreting line after ~2 months of culture *in vitro* (data not shown). We then verified the expression of Blimp-1 and showed the efficiency of decreasing Blimp-1 expression by U6Blimp1-3952i in the newly established plasmacytoma line (Fig. 2*B*). Consistently, we also found an increased proportion of Annexin V–positive apoptotic cells in the newly established plasmacytoma line after various days of U6Blimp1-3952i transduction (analyzed from gated CD138-positive and GFP-positive cells; Fig. 2*C*). After 4 days, the production of IgM from U6Blimp1-3952i–transduced cells was also largely reduced compared with that from U6controli-infected cells (Fig. 2*D*). Thus, knockdown of Blimp-1 causes apoptosis in a newly established plasmacytoma line.

Knockdown of Blimp-1 expression cannot reprogram the plasma cell phenotype. Previous results using a conditional knockout strategy to delete *prdm1*, the gene encoding Blimp-1, after the formation of plasma cells in mice showed reduced numbers of antigen-specific plasma cells from bone marrow (14). This observation suggests two possible models for the role of

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Blimp-1 in maintaining plasma cells in the bone marrow. Blimp-1 may regulate cell survival or, alternatively, may maintain the plasma cell phenotype. We therefore tested whether Blimp-1 knockdown could reprogram the multiple myeloma cell surface phenotype and gene expression, in addition to triggering apoptosis. We found that expression of plasma cell surface markers, such as CD138 and CD38, remained similar after 4 days of U6Blimp1-1004i or U6 transduction (Fig. 3*A*). The levels of other surface markers, CD54, CD19, CD20, and MHC class II, also remained unchanged (Fig. 3*A*). We also examined the mRNA levels of known Blimp-1 target genes, such as *PAX-5, XBP-1, SPI-B, ID3, PU.1, BCL-6, c-MYC*, and *CIITA*, in this setting. The levels of *PAX-5* were too low to be accurately estimated by RT-QPCR; however, the mRNA levels of the majority of target genes tested remained unchanged, including *SPI-B, ID3, PU.1, XBP-1*, and *c-MYC*, suggesting that Blimp-1 knockdown could not globally alter the Blimp-1–dependent transcription program (Fig. 3*B* and *C*). However, we observed a dramatic increase in *CIITA* and *BCL-6* mRNA expression by U6Blimp1-1004i in H929 (Fig. 3*C*), indicating that a small subset of Blimp-1 target genes could be derepressed in this cell context.

Changes of antiapoptotic and apoptotic protein expression on inhibition of Blimp-1 expression. The above results suggested that Blimp-1 is not involved in maintaining the plasma cell phenotype. We therefore determined the role of Blimp-1 in regulating cell survival by examining the mechanism of Blimp-1 RNAi-mediated apoptosis. We first examined the cell cycle status.

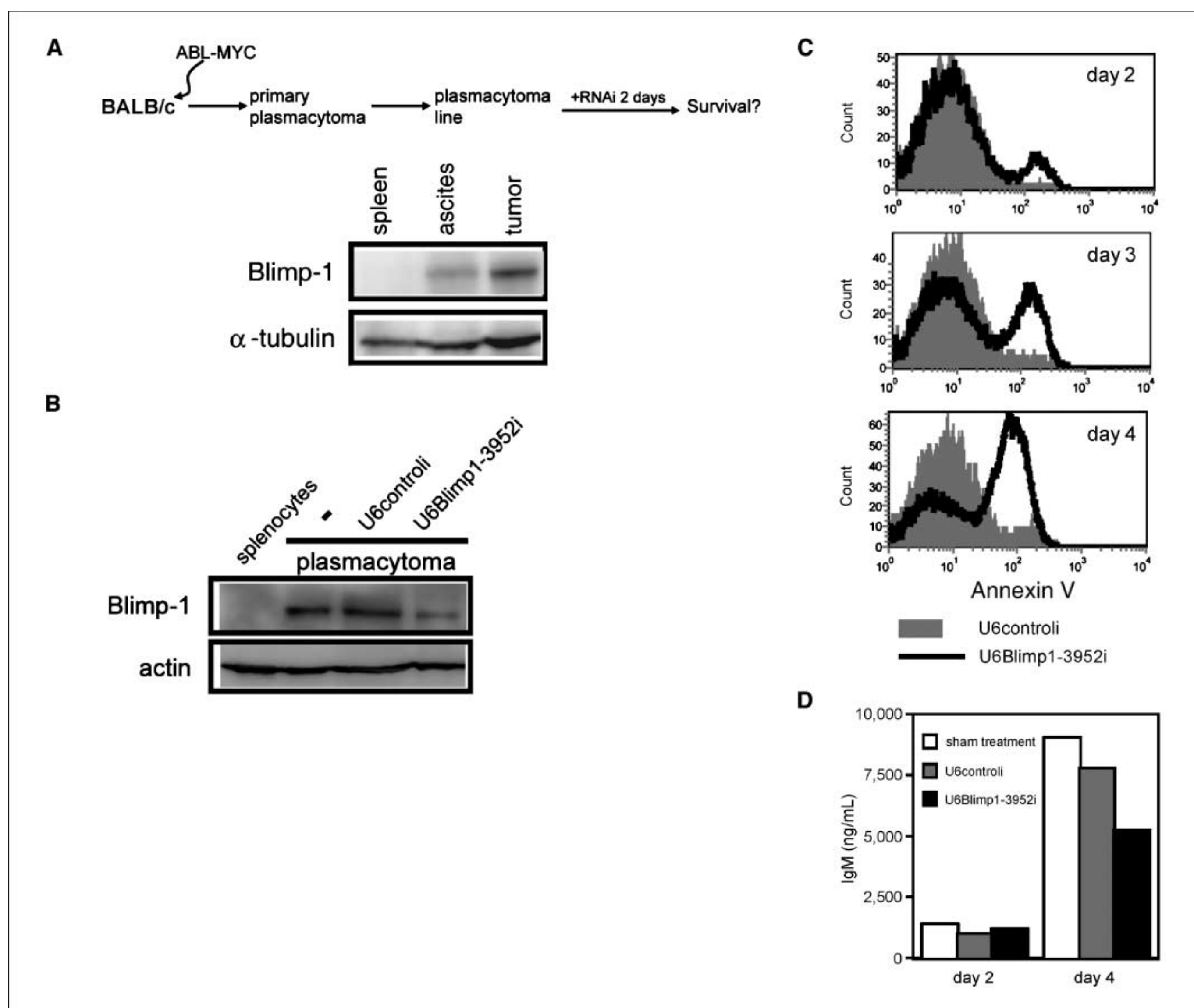

Figure 2. Blimp-1 knockdown causes apoptotic cell death in a newly established plasmacytoma line. *A,* ABL-MYC(Ψ2)-induced plasmacytoma or ascites express Blimp-1 as shown by immunoblotting. Total extracts isolated from 10^6 splenocytes, ascites, or plasmacytoma from ABL-MYC(Ψ2)-injected mouse were subjected to immunoblot analysis. One representative result from at least three independent isolated samples is shown. α-Tubulin expression was analyzed as a control. *B* and *C,* U6Blimp1-3952i causes apoptosis in a newly established plasmacytoma line. The established plasmacytoma line was infected with either U6controli or U6Blimp1-3952i, and 2 d after infection (transduction rate >95%), the cells were harvested to determine the efficiency of shRNA-mediated Blimp-1 knockdown by immunoblot analysis (*B*) and, 2, 3, and 4 d after infection, to determine the apoptotic status of gated shRNA-expressing, GFP-positive cells by Annexin V staining (*C*). *D,* reduction of IgM production in the newly established plasmacytoma line by Blimp-1 knockdown. Cell culture supernatants harvested from *B* and *C* at indicated numbers of days were subjected to ELISA determining IgM production. Results from *B* to *D* were representative data from three independent experiments.

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We found that Blimp-1 knockdown in H929 cells did not dramatically affect cell cycle progression; the proportion of cells in G_1, S, and G_2-M phases were practically identical between U6Blimp1-1004i–transduced and U6controli-transduced cells at day 2 (Fig. 4*A, top*). After 4 days of transduction, the proportion of cells in each phase were similar between the two groups, although more cells accumulated in sub-G_1 phase in the U6Blimp1-1004i–transduced cells (Fig. 4*A, bottom*). Similar results were found in U266 cells (data not shown).

We then looked at the expression of antiapoptotic or proapoptotic proteins involved in regulating apoptosis. Caspase activity assays showed that U6Blimp1-1004i caused activation of caspase-9 at day 4 of transduction as well as the activation of the downstream effector caspase-3 (Fig. 4*B*). Notably, we did not observe the activation of caspase-8 on reduction of Blimp-1 (Fig. 4*B*), suggesting that Blimp-1 knockdown-mediated apoptosis in plasma cells results from intrinsic apoptotic pathways (26). Accordantly, we observed decreased expression of procaspase-4, due to protein cleavage (27), by U6Blimp1-1004i (Fig. 4*D*). Moreover, Blimp-1 RNAi-induced apoptosis could be blocked by caspase-3 inhibitor, Z-DEVD-FMK (Fig. 4*C, bottom*). Expression of various antiapoptotic and proapoptotic Bcl-2 protein family on inhibition of Blimp-1 expression was monitored by immunoblot analysis. In multiple myeloma cells, the levels of Bcl-2, Bcl-xL, and Mcl-1 are elevated (19, 28). We therefore examined the expression of these proteins at day 4 of U6Blimp1-1004i transduction in H929 cells and showed that Mcl-1 was reduced and Bim was increased, whereas Bcl-2 and Bcl-xL remained unchanged (Fig. 4*D*). Thus, our collective data suggested that Blimp-1 knockdown-mediated apoptosis works through activation of an intrinsic apoptotic pathway as well as through the down-regulation of the anti-apoptotic protein Mcl-1 and the induction of the proapoptotic protein Bim.

Proteasome inhibitor–induced apoptosis involves reduced expression of Blimp-1. To examine whether the current effective agents for multiple myeloma therapy affect Blimp-1 expression, we treated various cell lines with the proteasome inhibitors MG132 and bortezomib. Bortezomib (Velcade, formerly known as PS-341)

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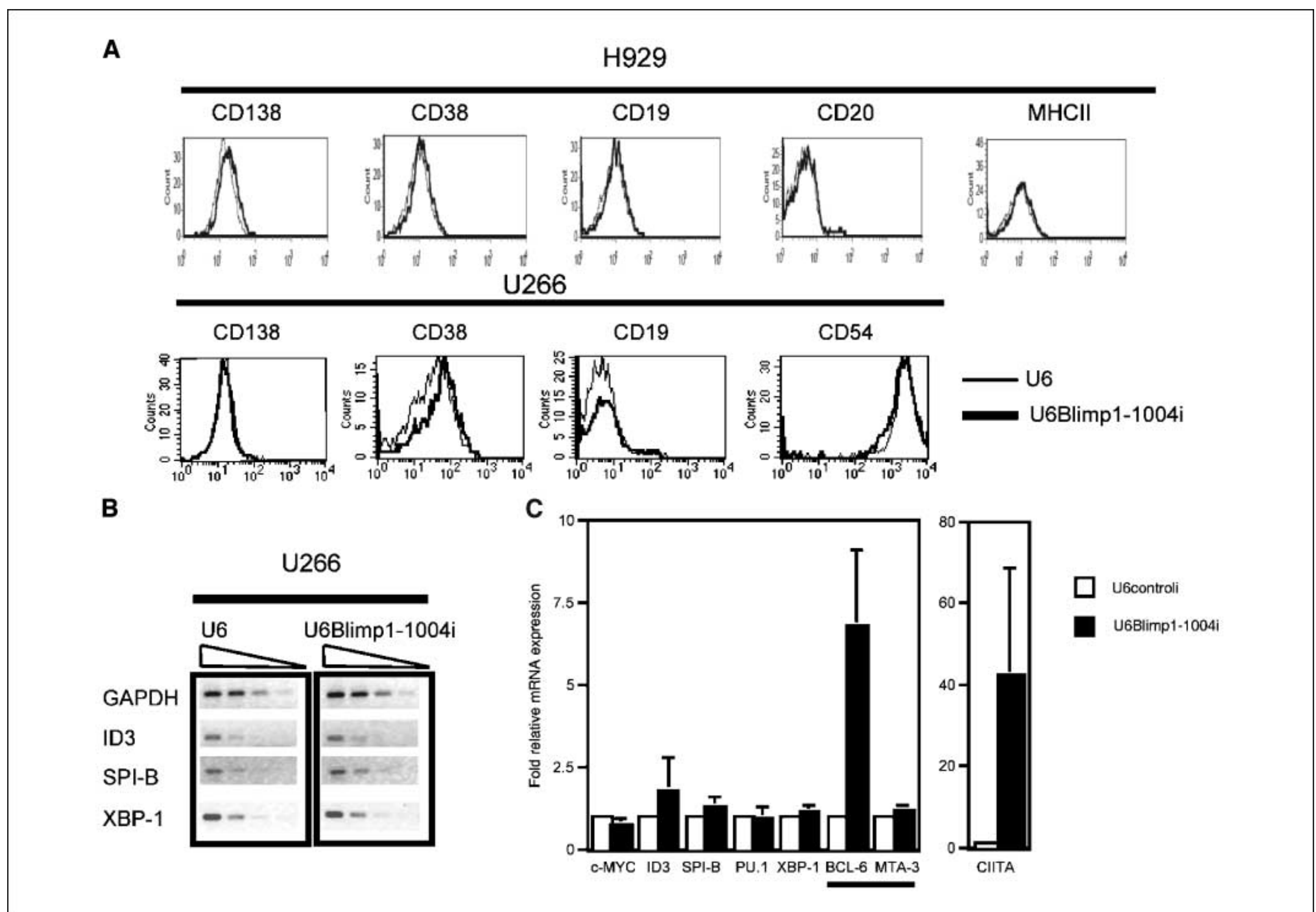

Figure 3. Blimp-1 knockdown cannot alter general plasma cell fate. *A,* RNAi for Blimp-1 does not change the expression of markers on the plasma cell surface. The expression of CD138, CD38, CD19, CD20, MHC class II, and CD54 was determined by FACS analysis from GFP-positive U266 or H929 cells transduced with either U6 or U6Blimp1-1004i for 4 d. *B,* RNAi for Blimp-1 does not alter the expression of *ID3, SPI-B*, and *XBP-1* in U266 cells. Semiquantitative RT-PCR analysis was performed using a 3-fold serial dilution of cDNA isolated from U266 cells transduced with either U6 or U6Blimp1-1004i for 4 d. GAPDH cDNA was used as a control. *C,* RNAi for Blimp-1 does not alter the expression of *c-MYC, ID3, SPI-B, XBP-1, PU.1*, and *MTA-3* but leads to increased expression of *CIITA* and *BCL-6* in H929 cells. RT-QPCR was used to analyze the mRNA levels of the above-mentioned genes from H929 cells transduced with either U6controli or U6Blimp1-1004i for 4 d. *Columns,* data from three independent experiments; *bars,* SD. Results were normalized to internal control PRKG1 mRNA and compared with the expression of each gene in U6controli-transduced cells.

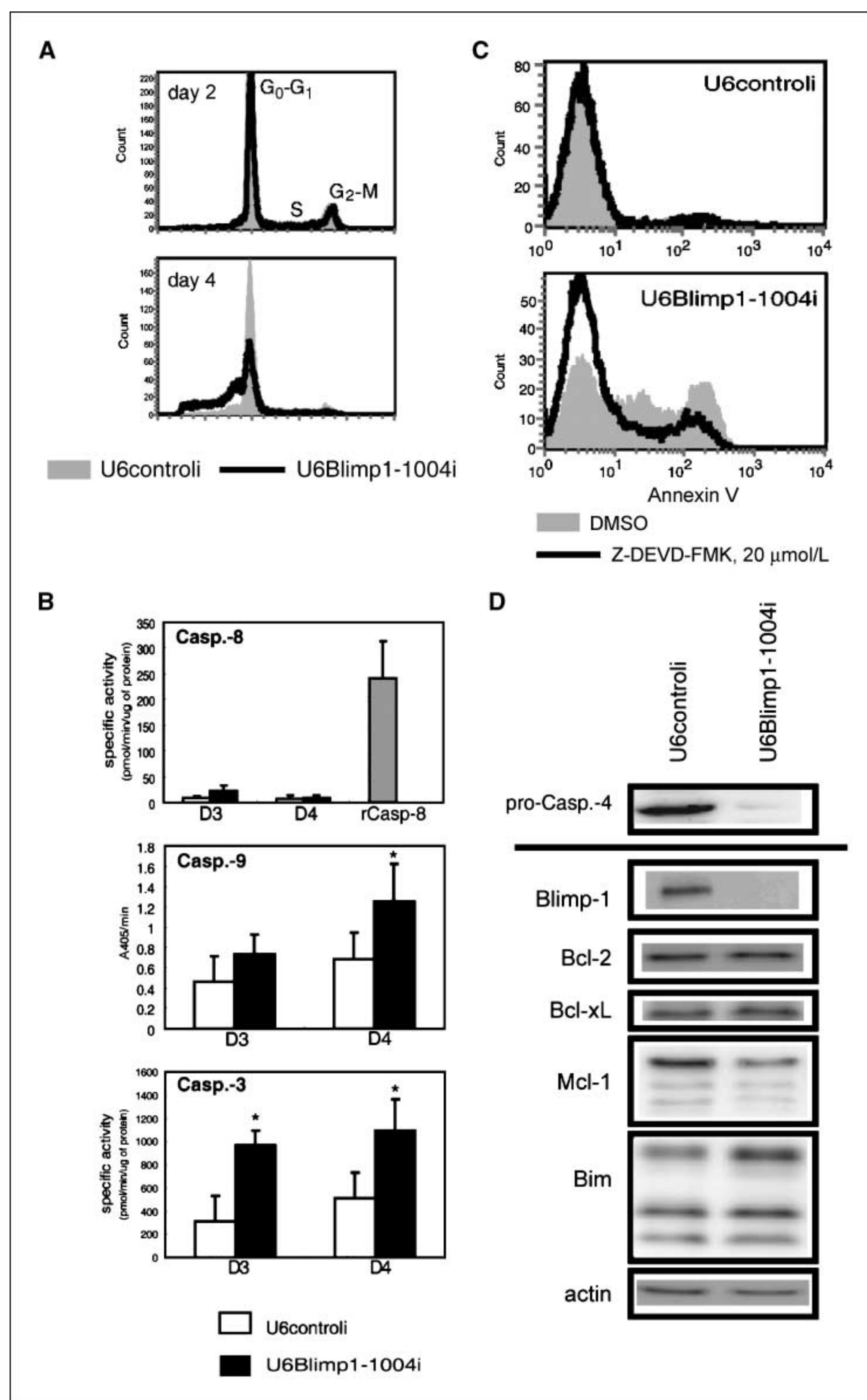

Figure 4. Activation of caspases, induction of Mcl-1, and reduction of Bim during Blimp-1 RNAi-mediated apoptosis. *A,* Blimp-1 knockdown in H929 cells does not significantly alter cell cycle status. H929 cells were transduced with U6Blimp1-1004i or U6controli. After 2 and 4 d of transduction (transduction rate >95%), cells were subjected to propidium iodide staining and FACS analysis. *B,* caspase-9 and caspase-3 were activated by Blimp-1 RNAi in H929 cells. The activities of caspase-8, caspase-9, and caspase-3 were determined from H929 cells after 3 and 4 d of transduction with U6Blimp1-1004i or U6controli. Recombinant caspase-8 was included as the positive control for caspase-8 activity assay. *Columns,* data from three independent experiments; *bars,* SD. *, $P < 0.05$. *C,* Blimp-1 knockdown-induced apoptosis was blocked by caspase-3 inhibitor. After 2 d of transduction with U6Blimp1-1004i or U6controli, H929 cells were treated with caspase-3 inhibitor, Z-DEVD-FMK, or DMSO solvent control. The apoptotic status of GFP-positive cells was determined by Annexin V staining at day 4. *D,* immunoblot analysis of procaspase-4 and antiapoptotic or proapoptotic Bcl-2 family protein expression from H929 cell lysates after 4 d of transduction with U6controli or U6Blimp1-1004i. Actin expression was analyzed for the loading control.

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is approved for treatment of multiple myeloma patients who have relapses from prior therapies (29). Treatment of MG132 at 1 μmol/L or of bortezomib at 10 nmol/L induced significant cell death in H929, U266, RPMI8226, and IM9 cells (data not shown), as previously reported (30). Both proteasome inhibitors caused ~50% cell death in H929 cells after 24 h of treatment (data not shown). Notably, we found that Blimp-1 expression was reduced in H929 cells after treatment with proteasome inhibitors (Fig. 5*A*). Likewise, proteasome inhibitors caused reduction of Blimp-1 mRNA expression in multiple myeloma cells (Fig. 5*B*).

To test the causal relationship of down-regulation of Blimp-1 expression in proteasome inhibitor–mediated apoptosis, we ectopically expressed Blimp-1 using a bicistronic retrovirus expressing Blimp-1 and YFP (pGC-HABlimp-YFP; ref. 5). We observed a

reduced proportion of apoptotic cells on MG132 treatment in pGC-HABlimp-YFP–transduced U266 cells compared with the control pGC-YFP–transduced cells (Fig. 5*C*), supporting the notion that Blimp-1 may be required for maintaining multiple myeloma cell survival. Because Blimp-1 has a survival role in multiple myeloma cells, we next examined whether Blimp-1 RNAi could sensitize multiple myeloma cells to proteasome inhibitors. We found that H929 cells transduced with U6Blimp1-1004i for 24 h showed increased apoptosis at early phase, ∼16 h, of 10 nmol/L bortezomib treatment (Fig. 5*D*). However, likely due to the strong cytotoxicity of bortezomib, we did not observe the enhanced cell death in U6Blimp1-1004i–infected H929 cells at later phase of drug treatment (data not shown).

Knockdown of IRF4 expression leads to apoptosis and reduced Blimp-1 expression. Interestingly, when examining the levels of Blimp-1 expression on proteasome inhibitor treatment, we also observed that the mRNA (data not shown) and protein levels (Fig. 5*A*) of another plasma cell transcription factor, IRF4, were significantly reduced. Because Blimp-1 is regulated by IRF4 (31), we examined whether IRF4 also regulates multiple myeloma cell survival by expressing shRNA against human and mouse IRF4 using a lentiviral vector in plasma cell lines. We found human U6IRF4-1278i to be more effective than U6IRF4-776i, and mouse U6IRF4-1278i to be more effective than U6IRF4-536i (Fig. 6*A*), for repressing IRF4 expression. Indeed, either human or mouse U6IRF4-1278i–transduced cells showed reduced levels of Blimp-1 (Fig. 6*A*). However, we did not observe reduced IRF4 expression in the reciprocal experiment using RNAi for Blimp-1 (data not shown). These results confirm that IRF4 may act upstream of Blimp-1 expression in plasma cells (31). Notably, we found that knockdown of IRF4 expression also caused cell apoptosis in H929, U266 multiple myeloma cells, and mouse P3X plasmacytoma cells (Fig. 6*B* and *C*) and that, for human or mouse U6IRF4-1278i, a high percentage of cell death correlated with a high efficiency of IRF4 and Blimp-1 knockdown (Fig. 6*B*). Together, these data suggested that IRF4 may regulate plasma cell survival through the regulation of Blimp-1.

Discussion

Blimp-1 functions as a master regulator controlling a cascade of gene expression during plasma cell differentiation (6). The role of continuous Blimp-1 expression in plasma cells or in their neoplastic counterparts is not clear. Conditional deletion of the Blimp-1 gene, *prdm1*, after the formation of plasma cells in mice showed a reduced number of antigen-specific plasma cells from bone marrow (14). This observation implies that Blimp-1 may either regulate plasma cell survival or maintain plasma cell phenotype. Our data favor the former possibility. Failure to reprogram plasma cell fate by depletion of Blimp-1 expression is probably due to continuous suppression of *PAX-5* because *PAX-5* has been shown to repress plasma cell differentiation (32, 33).

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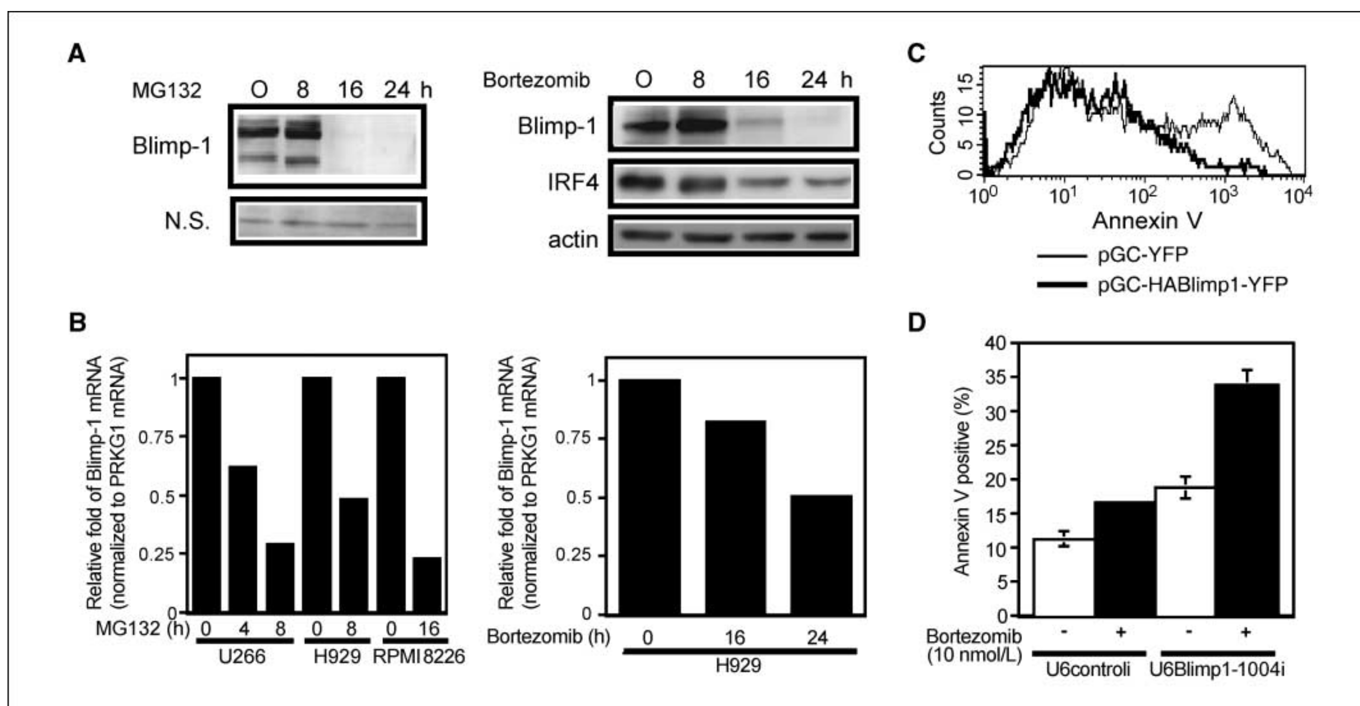

Figure 5. Down-regulation of Blimp-1 in proteasome inhibitor–mediated cell death. *A,* decreased expression of Blimp-1 and IRF4 proteins during proteasome inhibitor treatment. U266 cells were treated with MG132 (1 μmol/L) or bortezomib (10 nmol/L) for various periods, and cell lysates were subjected to immunoblot analysis. A nonspecific band (*top*) or actin expression (*bottom*) was analyzed for the loading control. *B,* down-regulation of Blimp-1 mRNA in multiple myeloma cells treated with proteasome inhibitors. Various multiple myeloma cells were treated with MG132 (1 μmol/L; *left*) or H929 cells were treated with bortezomib (1 nmol/L) for indicated periods; cells were then harvested for RNA isolation and subsequent RT-QPCR analysis. Results were normalized to internal control PRKG1 mRNA and compared with 0-h treatment. *C,* ectopic expression of Blimp-1 delays MG132-mediated apoptosis. U266 cells were transduced with retrovirus expressing Blimp-1 and YFP, pGC-HABlimp-YFP, or the control virus, pGC-YFP. Two days after transduction, YFP-positive cells were selected by sorting and then treated with MG132 (1 μmol/L) for 16 h. The apoptotic status from YFP-positive cells was monitored by Annexin V staining. One representative result from three independent experiments is shown. *D,* Blimp-1 knockdown cells are more sensitive to bortezomib treatment. H929 cells were transduced with either U6controli or U6Blimp1-1004i for 1 d and then treated with bortezomib (10 nmol/L). Annexin V–positive cells determined by flow cytometry were analyzed after overnight bortezomib treatment.

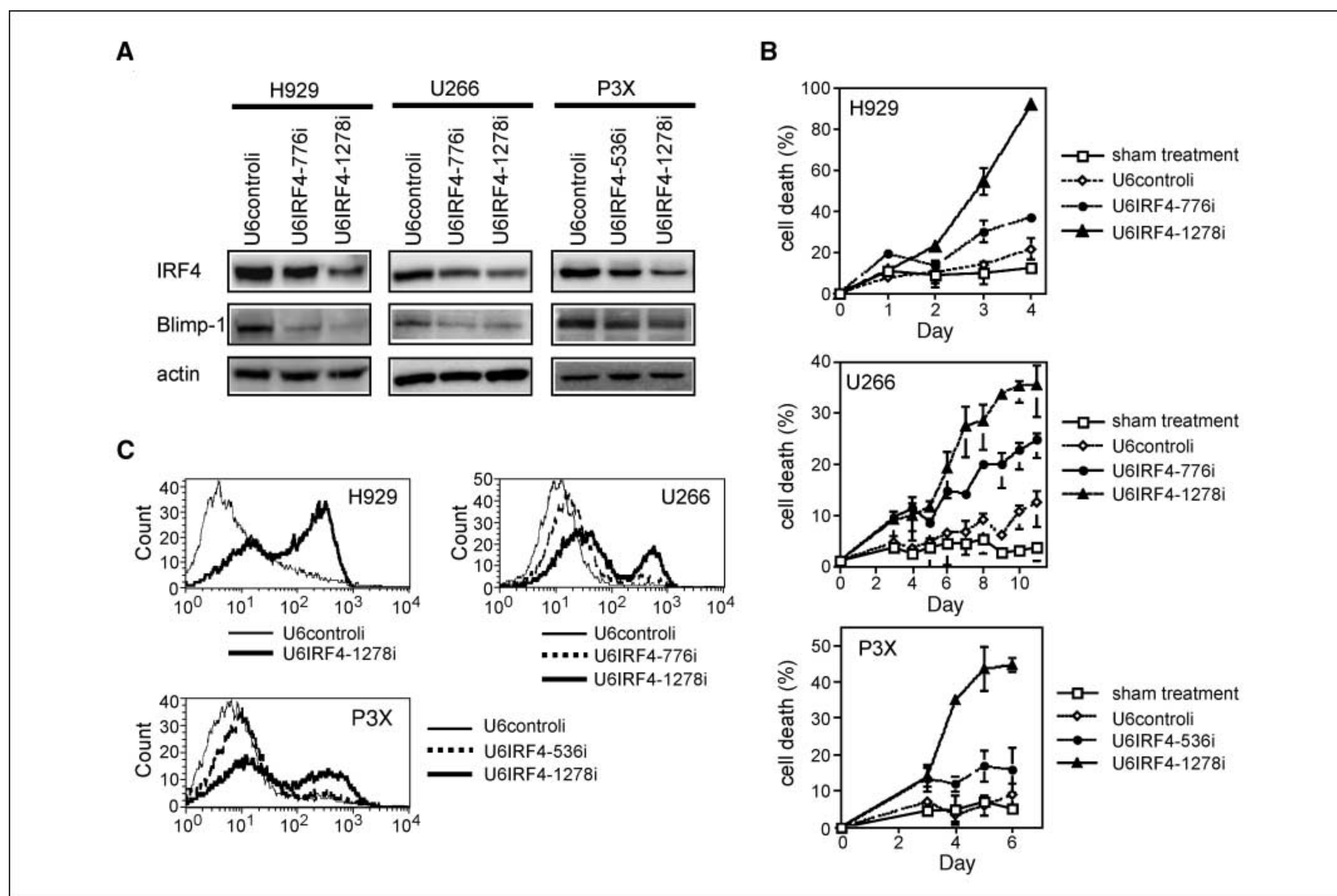

Figure 6. IRF4 knockdown leads to apoptosis in plasma cell lines. *A,* two lentiviral vectors carrying different shRNAs for human or mouse IRF4 show knockdown of IRF4 and Blimp-1. H929, U266, and P3X cells were transduced with the indicated lentiviral vector. After 3 d, immunoblot analysis was performed on cell extracts. *B,* RNAi for IRF4 causes cell death. H929, U266, and P3X cells were transduced with the indicated shRNA-expressing lentiviral vector (transduction rate >95%). The percentage of cell death was determined by trypan blue exclusion at the indicated number of days after infection. *Bars,* SD. *C,* IRF4 knockdown induces apoptotic cell death in H929, U266, and P3X cells. After 4 d of transduction, cells with IRF4 knockdown led to an increased proportion of Annexin V–positive cells compared with U6controli cells.

However, the mechanism of failure of reprogramming of plasma cell fate might also be due to a lack of the Blimp-1–independent coactivator/corepressor or an irreversible epigenetic modification. Although exogenous expression of BCL-6 has been shown to reprogram plasma cell fate in H929 and U266 cells (34), it is important to note that this was in an MTA3-dependent manner. It is therefore likely that our observed lack of plasma cell reprogramming in the presence of increased BCL-6 expression is linked to the fact that we also observed no concomitant change in MTA3 expression (Fig. 3*C*). The mode of action of Blimp-1 repression is through interaction with transcriptional repressors, including HDAC1/2 (8), Groucho (9), the histone methyltransferase G9a (10), and the arginine-specific histone methyltransferase Prmt5 (11). Our results showed that the majority of Blimp-1 target genes remained repressed whereas *CIITA* and *BCL-6* were significantly derepressed on Blimp-1 knockdown in H929 cells, suggesting differential mode of action by which Blimp-1 regulates expression of target genes in plasma cells.

The expression of the plasma cell–specific transcription factors IRF4, Blimp-1, and XBP-1 has been confirmed in multiple myeloma cells (1). The role of XBP-1 in maintaining myeloma cell survival has been shown, as evidenced by the fact that depletion or inactivation of XBP-1 in myeloma cells leads to increased apoptosis in response to endoplasmic reticulum stress (35). IRF4 and Blimp-1 have overlapping expression patterns in a subset of centrocytes in the germinal center and in plasma cells (12). Recent gene expression profiling data revealed that the molecular signature of stimulated *Irf4*$^{-/-}$ splenic B cells resembles that of *prdm1*$^{-/-}$ B cells (31). *Irf4*-deficient mice have severe defects in mature T-cell and B-cell function and lack memory B cells and plasma cells (36). A recent study using conditional deletion of *Irf4* in germinal center B cells indicated that IRF4 may be involved not only in plasmacytic differentiation but also in class switch recombination (37). In this study, we observed that IRF4 knockdown leads to the reduction of Blimp-1 (Fig. 6*A*), confirming the finding that IRF4 controls the expression of *prdm1* (31). We also observed that IRF4 knockdown causes apoptosis in transformed plasma cells, consistent with the notion that Blimp-1 works downstream of IRF4 in supporting plasma cell survival. The mechanism underlying IRF4 regulation of plasma cell survival and *prdm1* expression remains to be examined.

The mechanism that controls the continuous expression of Blimp-1 in plasma cells is unclear, but one possibility is through IL-6 signaling. IL-6 secreted from bone marrow plays an important role in promoting normal plasma cell and multiple myeloma cell growth/survival in the bone marrow milieu (38). In accord with these data, IL-6 and several IL-6 downstream signaling molecules,

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including signal transducers and activators of transcription 3 (STAT3) and activator protein-1, have been shown to induce Blimp-1 transcription (6, 39, 40). Our finding that Mcl-1 is reduced and Bim is induced on Blimp-1 knockdown seems to fit with the IL-6–mediated signaling scenario because blockade of IL-6 signaling down-regulates Mcl-1 and up-regulates Bim (41). In addition to regulating Mcl-1 and Bim, Blimp-1 suppresses another antiapoptotic Bcl-2 family protein, A1 (6, 42). However, A1 is an unlikely player in regulating plasma cell survival because it is strongly repressed in normal and malignant plasma cells (43). The mechanism that leads to up-regulation of Bim and down-regulation of Mcl-1 on Blimp-1 knockdown awaits further identification.

Proteasome inhibitors induce significant antitumor activity and apoptosis in multiple myeloma through multiple mechanisms, including inhibition of IL-6–triggered phosphorylation of the extracellular signal-regulated kinases 1/2, STAT3, and Akt (30), activation of mitochondrial cytochrome *c* release, activation of the caspase-9 and Fas/caspase-8–dependent apoptotic pathways (44), and inhibition of the activity of nuclear factor-κB (45). The molecular targets of proteasome inhibitor–mediated apoptosis in multiple myeloma, however, are not fully understood, although it has been shown that proteasome inhibitors, including MG132 and bortezomib, cause apoptosis by disrupting the unfolded protein response via suppression of the generation of the spliced form of XBP-1 in J558 myeloma cells (35). In this study, we found that IRF4 and Blimp-1 mRNA and protein expression is reduced on proteasome inhibitor treatment (Fig. 5), suggesting that factor(s) dependent on proteasome activity may regulate the expression of both genes in multiple myeloma. Additionally, Blimp-1 shRNA-transduced cells are more sensitive to bortezomib treatment (Fig. 5*D*), which may provide a clue for future therapeutic design.

Multiple myeloma is a largely incurable plasma cell malignancy that accounts for ∼20% of deaths from hematologic malignancy and ∼2% of deaths from cancer in the United States (46). The main obstacle for therapeutic elimination of multiple myeloma cells arises from the complex bone marrow microenvironment that provides growth factors and cell survival signals. Here, we have provided several lines of evidence showing that plasma cells rely on Blimp-1 for survival but not for maintaining plasma cell phenotype. Our results may help delineate future therapeutic regimens for plasma cell–related tumorigenesis, including multiple myeloma.

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