The Bcl-2 family member Bfl-1/A1 is strongly repressed in normal and malignant plasma cells but is a potent anti-apoptotic factor for myeloma cells.

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Karin Tarte[1], Michel Jourdan[1], Jean Luc Veyrune[2], Ingolf Berberich[3], Geneviève Fiol[2], Nicole Redal[2], John Shaughnessy Jr[4], and Bernard Klein[1,2]

1 INSERM U475, Rue Puech Villa, 34197 Montpellier, France

2 Unité de Thérapie Cellulaire, CHU Montpellier, Hôpital St Eloi, 34295 Montpellier, France.

3 University of Würzburg, Institute for Virology and Immunology, Versbacherstraße 7, D-97078 Würzburg, Germany.

4 Donna and Donald Lambert Laboratory of Myeloma Genetics, Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR

Running title Bfl-1/A1 in normal and tumor plasma cells

Correspondance: Bernard KLEIN
INSERM U475
99, rue Puech Villa
34197 MONTPELLIER Cedex 5
FRANCE
Phone: +33 4 67 63 61 25
Fax: + 33 4 67 04 18 63
e-mail: klein@montp.inserm.fr

Summary

Terminal B-cell differentiation is a multi-step process, from short-lived plasmablasts to mature long-lived plasma cells (PC). The anti-apoptotic Bcl-2 family member Bfl-1/A1 plays a critical role in the survival of mature B cells. However, its potential involvement at later stages of B-cell development remains highly controversial. Our aim was thus to clarify the place of Bfl-1/A1 in the biology of normal human PC and in the pathogenesis of multiple myeloma (MM), the major PC dyscrasia. Using gene expression profiling and quantitative RT-PCR experiments, we found a similar down-regulation of Bfl-1/A1 in both normal immature plasmablasts and mature PC, when compared to B cells. In myeloma cells, the level of Bfl-1/A1 was low and Bfl-1/A1 was not an NF-κB-inducible gene. Collectively, these data demonstrate that Bfl-1/A1 is not involved in the prolonged survival of normal mature PC, and that Bfl-1/A1 deregulation is not a common oncogenic event in MM. However, overexpression of Bfl-1/A1 by retroviral transduction promoted autonomous survival of an IL-6 dependent myeloma cell line and rendered it less sensitive to dexamethasone. Thus, Bfl-1/A1 transduction could be an interesting tool to obtain myeloma cell lines from primary samples and to favour the *in vitro* generation of antibody-secreting long-lived normal PC.

Keywords Plasma cells, Bfl-1/A1, apoptosis, myeloma, B-cell differentiation, gene expression profile

Introduction

Plasma cells (PC) are the final effectors of humoral immunity and as such are fully specialized in the secretion of immunoglobulin (Ig). Three subsets of PC have been recently identified in humans: early PC in inductive lymphoid organs such as tonsils and spleen, transitional PC in peripheral blood, and fully mature PC in bone marrow and intestinal lamina propria (Medina*, et al* 2002, Medina*, et al* 2003). PC precursors are short-lived cells whereas mature PC can survive for periods longer than 1 year *in vivo* in close contact to stromal cells (Manz*, et al* 2002, Slifka*, et al* 1998). Recently, gene expression profiling of purified tonsil PC (TPC) and bone marrow PC (BMPC) have shed new lights into the understanding of PC heterogeneity (Zhan*, et al* 2003). In addition, we have described an *in-vitro* model of B lymphocyte differentiation that makes it possible to obtain highly proliferative and short-lived polyclonal plasmablastic cells (PPC) (Tarte*, et al* 2002). We recently performed a detailed transcriptional analysis of late B-cell development including PPC, TPC, BMPC, peripheral blood B cells (PBB), and tonsil B cells (TBC) (Tarte*, et al* 2003).

The late stages of normal B-cell differentiation are tightly regulated by the coordinate expression of several transcription factors (Calame*, et al* 2003). Two of them, X box-binding protein I (XBP-1) and positive regulatory domain I-binding factor I (PRDI-BF1)/Blimp-1, are specifically involved in the terminal differentiation of B cells into PC. PRDI-BF1/Blimp-1, which is expressed in all PC subsets (Angelin-Duclos*, et al* 2000) is a transcriptional repressor whose expression is necessary and sufficient to drive mature B cells to become Ig-secreting cells (Piskurich*, et al* 2000, Schliephake and Schimpl 1996, Shapiro-Shelef*, et al* 2003). Five genes have been formerly shown to be direct targets of PRDI-BF1/Blimp-1-dependent repression: *c-myc, CIITA,*

BSAP/Pax-5, ID3, and *SPI-B* (Lin*, et al* 2002, Lin*, et al* 2000, Piskurich*, et al* 2000, Shaffer*, et al* 2002). In addition, PRDI-BF1/Blimp-1 initiates a complex cascade of gene expression changes since 228 genes were found to be downregulated following its introduction into human B cell lines (Shaffer*, et al* 2002). The anti-apoptotic Bcl-2 family member Bfl-1/A1 is one of these PRDI-BF1/Blimp-1 target genes. Accumulating evidences suggest that this extinction of Bfl-1/A1 may play a central role in the short life span of early PC. First, whereas the transduction of PRDI-BF1/Blimp-1 into murine B lymphoma cells leads to their differentiation into short-lived PC, the concomitant ectopic expression of Bfl-1/A1 rescues them from cell death and maintains Ig secretion (Knödel*, et al* 1999). Second, transgenic mice expressing an interfering truncated form of Blimp-1 show an increased number of $A1^+$ $Blimp1^+$ IgM-secreting early PC resulting from prolonged survival (Angelin-Duclos*, et al* 2002). Even if several studies have suggested that an upregulation of Bfl-1/A1 expression at the final stages of PC maturation could be involved in the long-term survival of normal bone-marrow PC, there is currently no experimental evidence to support this hypothesis. Bfl-1/A1 is an immediate-early gene which is inducible in myeloid, lymphoid, and endothelial cells by a variety of stimuli sharing the capacity to activate NF-κB (Lin*, et al* 1993). Moreover Bfl-1/A1 is a direct transcriptional target of NF-κB (Edelstein*, et al* 2003, Grumont*, et al* 1999, Zong*, et al* 1999). The exact mechanism of Bfl-1/A1 function is unknown, but probably involves binding and inactivation of pro-apoptototic proteins such as the BH3 domain-only protein Bid (Werner*, et al* 2002), and the multidomain proteins Bok (Hsu*, et al* 1997). Unlike Bcl-2, Bfl-1/A1 is a growth-permissive anti-apoptotic factor (Gonzalez*, et al* 2003) (D'Sa-Eipper and Chinnadurai 1998) with a short half life (Karsan*, et al* 1996, Moulding*, et al* 2001). In normal B cells, Bfl-1/A1 is upregulated as immature B cells are recruited

into the long-lived mature B cell pool (Tomayko and Cancro 1998) and Bfl-1/A1 induction through a CD40/NF-κB pathway is critical for preventing antigen receptor ligation-induced cell death in B-cell lines and mature B cells (Craxton*, et al* 2000, Grumont*, et al* 1999, Kuss*, et al* 1999, Lee*, et al* 1999, Wen*, et al* 2003). Several studies suggest that Bfl-1/A1 expression is also a key factor in B-cell neoplasias. Engagement of surface IgM elicits a survival program in chronic lymphocytic leukemia B cells that is associated with an upregulation of Bcl-2, Mcl-1 and Bfl-1/A1 anti-apoptotic proteins (Bernal*, et al* 2001). In mantle cell lymphomas (Pham*, et al* 2003) and Hodgkin's disease (Hinz*, et al* 2001), inhibition of the constitutively activated NF-κB pathway leads to tumor cell apoptosis in association with a downregulation of Bfl-1/A1 expression. In addition, the simultaneous induction of both Bfl-1/A1 and pim-1 expression was recently found to be essential for BCR/ABL-dependant leukemogenesis (Nieborowska-Skorska*, et al* 2002).

Multiple myeloma (MM) is a presently incurable B-cell neoplasia characterized by the accumulation of malignant PC in the bone marrow. Myeloma cells express several anti-apoptotic proteins, namely Bcl-2, Bcl-xL, and Mcl-1 (Jourdan*, et al* 2000). Among ten anti- and pro-apoptotic proteins, only Mcl-1 was regulated by interleukin (IL)-6, the major myeloma growth factor. Recently, we found that the tumor necrosis factor (TNF) family members BAFF (B cell activating factor, also known as BLys) and APRIL (a proliferation-inducing ligand) are also able to enhance Mcl-1 expression in myeloma cells (Moreaux*, et al* 2003). Furthermore, oligonucleotide antisenses to Mcl-1, unlike to Bcl-2 or Bcl-xL, can promote apoptosis of myeloma cell lines (Derenne*, et al* 2002) and constitutive Mcl-1 expression strongly reduces apoptosis induced by IL-6 withdrawal (Jourdan*, et al* 2003). Collectively, these data demonstrate that Mcl-1 is one of the major anti-apoptotic mediators involved in growth factor-mediated survival

in MM. Mcl-1 shares some properties with Bfl-1/A1, in particular a short half-life, and a specific heterodimerization with the pro-apoptotic protein Bok (Hsu*, et al* 1997). The expression of Bfl-1/A1 in myeloma cells is controversial. Whereas we cannot detect Bfl-1/A1 mRNA by a sensitive RNase protection assay in 14 human myeloma cell lines (HMCL) (Jourdan*, et al* 2003), Anderson's group reported a constitutive expression of Bfl-1/A1 in MM-1S HMCL, that is further increased in response to NF-κB activation by insulin-like growth factor-1 (IGF-1) (Mitsiades*, et al* 2002a, Mitsiades*, et al* 2002b). In addition, *Bfl-1/A1* was detected using RT-PCR in primary myeloma cells from two patients at a lower level than in normal plasma cells (Borson*, et al* 2002).

Given the potential but not fully demonstrated role of Bfl-1/A1 in normal PC survival and the contentious issue of its presence in myeloma cells, we would like to clarify the implication of Bfl-1/A1 in normal and malignant PC biology. In this study, we show that Bfl-1/A1 is expressed at very low level in normal PC and myeloma cells. Bfl-1/A1 is not inducible, even following a strong and prolonged activation of NF-κB. In addition, we demonstrate that a constitutive Bfl-1/A1 expression rescues myeloma cell from apoptosis induced by IL-6 withdrawal and dexamethasone.

Materials and Methods

Cell samples

XG-1, XG-2, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-16, and XG-20 HMCL were previously obtained and characterized by our laboratory (Zhang*, et al* 1994). Their growth is dependent upon addition of exogenous IL-6 and they were maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS) and 2 ng/ml of IL-6 (Peprotech, Rocky Hill, NJ). The IL-6-independent HMCL U266, NCI-H929, EJM, and L363 were obtained from DMSZ (Braunschweig, Germany). They were cultured in RPMI-10% FCS. All cell lines were free of *Mycoplasma*, as assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany).

Primary samples were obtained after informed consent either from healthy volunteers for PBB, TBC, and BMPC, or from patients with MM for malignant myeloma cells. Cell populations were isolated as previously described (Tarte*, et al* 2002, Zhan*, et al* 2003). PBB and TBC were purified using anti-CD19, BMPC and myeloma cells using anti-CD138 MACS microbeads (Miltenyi-Biotec, Paris, France). PPC were generated *in vitro* from purified $CD19^{+}$ PBB. Briefly, PBB were cultured in RPMI 1640-10% FCS in the presence of CD40L transfectant, IL-2, IL-4, IL-10, and IL-12 (R&D Systems, Abington, UK). After 4 days of culture, B cells were harvested and seeded without CD40 signal but with IL-2, IL-10, IL-12, and IL-6. On day 6 of culture, $CD20^{-}CD38^{++}$ PPC were sorted with a FACSvantage (Becton Dickinson).

RT-PCR and quantitative RT-PCR

Total RNA was converted to cDNA using the Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). PCR were performed with AmpliTaq DNA polymerase (Applied Biosystems, Courtaboeuf, France) and analyzed on a 1.5% agarose gel containing ethidium bromide. The following primers were used: β2-microglobulin: 5'-CTCGCGCTACTCTCTCTTTCTGG-3' and 5'-GCTTACATGTCTCGCCCACTTAA-3'; PRDI-BF1/Blimp-1: 5'-AGCTGACAATGATGAACTCA-3' and 5'-CTTGGGGTAGTGAGCGTTGTA-3', Bfl-1/A1: 5'-CGGCATCATTAACTGGGGAAG-3' and 5'-CTCTCTGGTCCGTAGTGTTACTTG-3'. For quantitative RT-PCR, we used the Assays-on-demand primers and probes and the Taqman Universal Master Mix from Applied Biosystems according to the manufacturer's instructions. Measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System. Serial dilutions of a single standard cDNA were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve. Normalization of samples was performed by dividing the value of the gene of interest by the value of the endogenous reference gene β2-microglobulin. PCR reactions for each sample were run in duplicate, except for the study of Bfl-1/A1 induction by NF-κB activators where each sample was tested in quadruplicate.

Microarray data

RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA) from 7 BMPC, 7 PPC, 7 PBB, 7 TBC, and the tumor PC purified from 16 patients with MM. cRNA preparation and hybridization to the HuGeneFL GeneChip microarray (Affymetrix, Santa Clara, CA) were done as described (Tarte*, et al* 2003, Zhan*, et al* 2002).

Arrays were scaled to an average intensity of 1500 and a threshold of 200 was assigned to small values. The visualization was obtained using Gene Cluster and Treeview softwares (Eisen*, et al* 1998).

Induction of Bfl-1/A1 expression

XG-13 HMCL was starved of IL-6 for 3 hours and seeded at 5 x 10^5 cells/ml with IL-6 (2 ng/ml), BAFF (200 ng/ml), APRIL (200 ng/ml), or no cytokine. NF-κB activation was determined after 1 hour using a Trans-AM NF-κB p50 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions. Cell lysates were diluted at 1:10 and added to the ELISA plate. Each condition was run in triplicate. In addition, cells were harvested after 1, 3, and 12 hours and RNA was extracted for quantitative RT-PCR evaluation of *Bfl-1/A1* expression.

Bfl-1/A1 gene transfer

The bicistronic vector pEYZ/MCS containing a Flag-tagged Bfl-1/A1 cDNA (pEYZ/FA1) was previously described (Kuss*, et al* 1999). This vector contained a chimeric marker gene (EYFP/Zeo) and Bfl-1/A1 expression is achieved via an internal ribosomal entry site derived from encephalo-myocarditis virus. pEYZ/MCS and pEYZ/FA1 were stably transfected into the HEK293E17 packaging cell line (Transgene, Strasbourg, France). Viral supernatants were added to XG-6 cells in the presence of 8 μg/ml of polybrene (Sigma, St Louis, MO). After 24 h, the cells were washed and cultured with 100 ng/ml of zeocin (Invitrogen) to select empty vector (XG-6MCS) or Bfl-1/A1 (XG-6A1) transduced cells that were further enriched for high EYFP expression using a Vantage cell sorter (Becton Dickinson, San Jose, CA).

Western Blot analysis

XG-6, XG-6MCS, and XG-6A1 were lysed in 10 mM tris-HCl (pH7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPPi), 1% Triton X-100, 5µM $ZnCl_2$, 100 µM Na_3VO_4, 1 mM DTT, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenolphosphate (PNPP), 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM benzamidine, 5 µg/ml pepstatin and 50 nM okadaik acid. Lysates were cleared by centrifugation at 10000 *g* for 10 min and resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were blocked for 1hour at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM tris-HCl (pH 7.4), 0.1% Tween 20 (TBS-T), 5% BSA, then incubated for 1 hour at room temperature with rabbit polyclonal anti-Mcl-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). This primary antibody was visualized using a goat anti-rabbit peroxidase-conjugated antibody (Sigma, St Louis, MO) and an enhanced chemiluminescence detection system. As control of protein loading, we used a monoclonal anti-β actin antibody (Sigma).

Study of apoptosis

After 3 hours of IL-6 starvation, XG-6, XG-6MCS, and XG-6A1 HMCL were washed twice and cultured at 10^5 cells/ml in RPMI 1640-10% FCS culture medium with either no cytokine, IL-6 (2 ng/ml), dexamethasone (DEX, 10^{-6}M), or IL-6 and DEX. After 3 days of culture, cells were washed twice in PBS and stained with Annexin-V-biotin (Boehringer Mannheim, Mannheim, Germany) followed by incubation with PE-conjugated streptavidin (Coulter-Immunotech, Marseilles, France). When indicated the anti-IL-6 antibody B-E8 (kindly provided by John Widjenes, Diaclone, Besançon,

France) was added at 10 μg/ml at the beginning of the culture. Fluorescence was analysed on a FACScan flow cytometer.

Statistical analysis

Statistical significance was tested using a non-parametric Wilcoxon or Mann-Whitney test.

Results

Expression of Bfl-1/A1 in normal and malignant PC

We first evaluated, using Affymetrix microarray, the level of *Bfl-1/A1* in primary myeloma cells purified from 16 patients, in comparison with 4 cell populations representing all stages of normal late B-cell differentiation, from resting B cells (PBB) to fully mature plasma cells (BMPC), including activated tonsil B cells (TBC) and polyclonal plasmablastic cells (PPC). As seen in Figure 1A and in agreement with previous studies on B-cell activation (Grumont*, et al* 1999), TBC expressed significantly more Bfl-1/A1 mRNA than PBB (*P* = .002). *Bfl-1/A1* was statistically downregulated in the two normal PC-subsets, *i.e.* PPC and BMPC, and in MM cells compared with PBB and TBC (*P* < .0001). Interestingly, we have previously pointed out using Affymetrix microarray a NF-κB signature in mature PC when compared to PPC (Tarte*, et al* 2003). In particular, we identified *NF-κB2*, *RelB*, *IKKε*, *IKKα*, and several NF-κB target genes such as *CD40* and the anti-apoptotic factors *A20*, and *IAP-2* as significantly overexpressed in BMPC versus PPC. However, despite these hallmarks of NF-κB activation in mature PC and even if Bfl-1/A1 is a direct NF-κB target (Edelstein*, et al* 2003, Grumont*, et al* 1999, Zong*, et al* 1999), we showed here that *Bfl-1/A1* was poorly expressed in normal mature PC, not significantly different from expression in plasmablasts (*P* = .073). Finally, we found no difference in *Bfl-1/A1* level between normal and tumor PC (*P* = .377).

To further confirm a lack of Bfl-1/A1 deregulation in myeloma cells, and since commercially available anti-Bfl-1/A1 antibodies gave inconstant results (Moreb and Zucali 2001) and data not shown, we next tested 12 IL-6-dependent HMCL, 4 autonomously growing HMCL, and purified $CD138^+$ myeloma cells from 10 patients

at diagnosis using quantitative RT-PCR. In agreement with our previous results obtained by ribonuclease protection assay (RPA) (Jourdan*, et al* 2003), *Bfl-1/A1* expression in all HMCL and primary samples was dramatically decreased compared with PBB and TBC and was similar to that found in non-malignant BMPC and PPC. There is no statistical difference in the level of *Bfl-1/A1* between HMCL and primary myeloma cells (*P* = .737). Interestingly, *Bfl-1/A1* expression was highly heterogeneous in myeloma cells, from 3- to more than 300-fold less than in PBB. Altogether these data demonstrate that Bfl-1/A1 is strongly repressed in immature and mature PC and indicate that Bfl-1/A1 deregulation is not a common event associate with PC transformation into MM.

Since Bfl-1/A1 is inducible in several cellular models, we tested whether a strong and sustained NF-κB activation was able to promote Bfl-1/A1 upregulation in MM cells. In agreement with our recent data (Moreaux*, et al* 2003), BAFF and APRIL were more potent than TNF-α, a known stimulator of NF-κB in MM cells, in enhancing NF-κB DNA binding activity in XG-13 HMCL (Figure 2A). XG-13 cells were thus starved of IL-6 for 3 hours and IL-6, BAFF, or APRIL was added for 1, 3, and 12 hours before RNA extraction and determination of *Bfl-1/A1* level by quantitative RT-PCR. When compared with its level in XG-13 harvested during the exponential growth phase, *Bfl-1/A1* was never found to be upregulated whatever the time point or the cytokine considered (Figure 2B).

Constitutive expression of Bfl-1/A1 rescued HMCL from apoptosis induced by IL-6 deprivation and dexamethasone

Recently, we have demonstrated that a constitutive expression of Mcl-1 in XG-6 is sufficient to render this IL-6-dependent HMCL autonomous, *i.e.* able to grow in the

absence of exogenous IL-6 (Jourdan*, et al* 2003). To look for a biological role of Bfl-1/A1 in myeloma cell survival, we transduced XG-6 with a Bfl-1/A1–containing retroviral vector (XG-6A1) or with the empty control vector (XG-6MCS). Each vector contained EYFP as a marker gene. After selection with zeocin and cell-sorting for high EYFP expression, XG-6A1 and XG-6MCS highly and similarly expressed EYFP (Figure 3A). In order to show the reproducibility of Bfl-1/A1 effect and to avoid the selection of an autonomous subclone, five independent transduction/selection experiments were performed with each vector. The five XG-6A1 and the five XG-6MCS HMCL expressed EYFP at the same level (data not shown). Whereas only XG-6A1 expressed detectable *Bfl-1/A1* mRNA using RT-PCR, XG-6, XG-6MCS, and XG-6A1, expressed a high and comparable level of PRDI-BF1/Blimp-1 mRNA, showing that the induction of Bfl-1/A1 in XG-6A1 was not associated with an extinction of its transcriptional repressor (Figure 3B). Importantly, XG-6, XG-6MCS, and XG-6A1 expressed a similar level of the anti-apoptotic protein Mcl-1 (Figure 3C).

We next investigated the potential effect of Bfl-1/A1 expression on HMCL survival using two apoptotic stimuli: IL-6 deprivation and dexamethasone (DEX). XG-6, XG-6MCS, and XG-6A1 were IL-6 starved, cultured for 3 days with or without IL-6 and DEX, and apoptosis was then quantified using Annexin-V staining. As shown in figure 4A, Bfl-1/A1 constitutive expression had a strong although not complete protective effect on IL-6-deprivation-induced cell death. In particular, addition of IL-6 increased survival of XG-6, XG-6MCS, but also of XG-6A1. Results obtained for the 5 XG-6A1 and the 5 XG-6MCS HMCL were detailed in Figure 4B. Transduction of Bfl-1/A1 reduced apoptosis induced by IL-6 withdrawal by 49% (n=5, P = .008). Similarly, Bfl-1/A1 partially protected XG-6 from DEX-induced apoptosis in the absence of IL-6

(Figure 4B, *P* = .008) whereas only IL-6 addition was able to completely restored XG-6, XG-6MCS and XG-6A1 viability in the presence of DEX (Figure 4A).

Finally, we tested if XG-6A1 was able to grow independently of the addition of exogenous IL-6 (Figure 5). As expected, XG-6 and XG-6MCS died within 7 to 11 days upon IL-6 removal. IL-6 promoted exponential growth with a doubling time of 27 to 28 hours for XG-6, XG-6MCS, and XG-6A1. In the absence of IL-6, XG-6A1 was able to grow with a doubling time of 59 hours, *i.e*. at a 2.5 fold slower rate than in the presence of IL-6.

The survival effect of Bfl-1/A1 was independent of IL-6

To determine if the autonomous growth of XG-6A1 was due to an autocrine loop involving IL-6, we cultured XG-6, XG-6MCS, and XG-6A1 in the presence of the B-E8 anti-IL-6 monoclonal antibody (Figure 6). Whereas B-E8 completely abrogated the IL-6 mediated cell-survival of XG-6 and XG-6MCS, the survival of XG-6A1 in the absence of exogenous IL-6 was not affected by B-E8. As described above, addition of IL-6 further enhanced XG-6A1 viability. This later effect was blocked by B-E8 anti-IL-6 antibody.

Discussion

Several studies have recently suggested that the anti-apoptotic Bcl-2 family member Bfl-1/A1 could be involved in the long-term survival of normal mature bone marrow PC but there is no evidence that Bfl-1/A1 is actually highly expressed at the late stage of PC-differentiation. In addition, the expression of Bfl-1/A1 in malignant PC is still a matter of controversy (Jourdan*, et al* 2003, Mitsiades*, et al* 2002a, Mitsiades*, et al* 2002b). The aim of this study was thus to definitively clarify the role of Bfl-1/A1 in the biology of normal PC and in the pathogenesis of MM.

We demonstrated here using a combination of microarray experiments and quantitative RT-PCR that Bfl-1/A1 mRNA level was very low in normal PC compared to normal B cells and was not statistically different between short-lived plasmablasts and long-lived BMPC. The downregulation of *Bfl-1/A1* during B-cell differentiation into PC is in good agreement with previous studies showing that PRDI-BF1/Blimp-1 is expressed in all PC subsets (Angelin-Duclos*, et al* 2000, Tarte*, et al* 2003) and strongly represses Bfl-1/A1 expression (Knödel*, et al* 1999, Shaffer*, et al* 2002). Moreover, our current work suggests that the prolonged survival of BMPC within specialized microenvironment niches is not due to an up-regulation of Bfl-1/A1, but probably to other anti-apoptotic proteins, such as A20, c-IAP2, and Bcl-2, that were all recently described as overexpressed in BMPC compared to PPC (Tarte*, et al* 2003). Concerning malignant PC, we found a similarly low expression of *Bfl-1/A1* in 16/16 HMCL and 26/26 purified primary myeloma cell samples. This confirms our previous results obtained using ribonuclease protection assay (Jourdan*, et al* 2003) and Affymetrix microarrays (Tarte*, et al* 2002, Tarte*, et al* 2003, Zhan*, et al* 2002). In particular, in our gene expression profiling of 74 newly diagnosed MM patients *Bfl-1/A1* was not identified as a “spiked gene” *i.e.* a gene overexpressed in a small

subset of patients (Zhan*, et al* 2002). Interestingly, *Bfl-1/A1* levels evaluated using quantitative RT-PCR were very heterogeneous in MM samples. This could reflect the heterogeneity of myeloma disease where the tumor cell compartment comprised different subpopulations based on growth and maturation criteria (Bataille*, et al* 2003). Of note, there was no difference in Bfl-1/A1 levels between primary myeloma samples collected at diagnosis and HMCL obtained from patients at the terminal stage of the disease. Collectively, these data suggest that Bfl-1/A1 is not a major anti-apoptotic protein in the survival of myeloma cells and plays no crucial role in the emergence of myeloma disease.

It was previously reported that the MM1-S myeloma cell line constitutively expressed Bfl-1/A1, cIAP-2, FLIP, survivin, and XIAP and that further activation of NF-κB pathway by insulin-like growth factor-1 (IGF-1) is associated with an up-regulation of these four anti-apoptotic proteins in this cell line (Mitsiades*, et al* 2002a). The NF-κB pathway plays probably a key role in the survival of normal and malignant PC, as it is the case for normal and malignant B cells. Interestingly, we have previously detected a NF-κB signature, *i.e*. an up-regulation of several NF-κB family members and target genes, in fully mature long-lived BMPC unlike in PPC that are characterized by their short survival (Tarte*, et al* 2003). In addition, NF-κB blockade, using the proteasome inhibitors PS-341/Bortezomib, the IκB kinase inhibitor PS-1145, or SN50, a synthetic peptide that specifically inhibits NF-κB nuclear translocation, induces apoptosis in HMCL and primary myeloma cells (Hideshima*, et al* 2002, Mitsiades*, et al* 2002b). Recently, a phase II clinical trial has demonstrated that Bortezomib is active *in vivo* in patients with relapsed refractory MM (Richardson*, et al* 2003). BAFF and APRIL, two members of the TNF superfamily, strongly induce NF-κB activation in MM cells, in association with an induction of Bcl-2 and Mcl-1, and protect them against apoptosis

induced by IL-6 deprivation and dexamethasone (Moreaux*, et al* 2003). We thus tested them for *Bfl-1/A1* induction. However, we show here that neither BAFF nor APRIL is able to upregulate Bfl-1/A1 in XG-13 HMCL.

The low level of *Bfl-1/A1* found in BMPC despite hallmarks of NF-κB activation, in association with data showing that *Bfl-1/A1* was poorly expressed and not inducible in MM cells in response to potent NF-κB activators, suggests that the active repression of Bfl-1/A1 by PRDI-BF1/Blimp-1 in normal and malignant PC is fairly strong to overcome NF-κB signaling. In addition, the apoptotic effect of Bortezomib and SN50 on MM cells is probably not essentially due to a downregulation of Bfl-1/A1, but rather to the reported inhibition of expression of Bcl-2, XIAP, cIAP-2, or survivin.

Overexpression of Bfl-1/A1 through retroviral transduction protects B cells from antigen receptor-mediated (Craxton*, et al* 2000, Kuss*, et al* 1999) and PRDI-BF1/Blimp-1-mediated (Knödel*, et al* 1999) apoptosis. We thus evaluated if Bfl-1/A1 could also protect MM cells from apoptotic stimuli. We showed here that transduction with Bfl-1/A1 resulted in the survival of an IL-6-dependent HMCL in the absence of IL-6, making it possible its long-term autonomous growth. It also conferred resistance to dexamethasone-induced apoptosis. Until now, only Mcl-1 and Bcl-2 overexpression were reported to protect MM cells from apoptosis induced respectively by IL-6 deprivation (Jourdan*, et al* 2000, Jourdan*, et al* 2003) and dexamethasone (Feinman*, et al* 1999). The survival of Bfl-1/A1-transduced HMCL was not due to the induction of an IL-6 autocrine loop since it was not abrogated by B-E8 anti-IL-6 antibody whereas the IL-6-dependent survival of parental and mock-transduced HMCL was completely blocked by B-E8. Interestingly, Bfl-1/A1

constitutive expression protected only partially MM cells from IL-6 deprivation, at a level similar to Mcl-1 constitutive expression (Jourdan*, et al* 2003).

In conclusion, Bfl-1/A1 expression is strongly repressed at all stages of normal PC differentiation as well as in malignant PC. Ectopic expression of Bfl-1/A1 promotes myeloma cell survival, as it was previously reported for normal B cells committed *in vitro* to a PC fate (Knödel*, et al* 1999). A protection of malignant PC from apoptosis by Bfl-1/A1 overexpression might be of potential interest, especially to promote the development of immunotherapy strategies in MM. Indeed, myeloma cell lines can only be obtained from patients with secondary extramedullary proliferation and fulminant disease (Zhang*, et al* 1994). At the early stages of myeloma disease, malignant PC can be harvested only in small amount, do not proliferate and rapidly dye *in vitro*, even in the presence of exogenous cytokines such as IL-6. In this context, Mcl-1 transduction would not be sufficient to promote prolonged primary myeloma cell growth since a high level of Mcl-1 is already achieved in these cells through addition of exogenous cytokines, without promoting their survival. On the contrary, Bfl-1/A1 ectopic expression could be a first step in the generation of myeloma cell lines at diagnostic. In addition, generation of long-lived, antibody-secreting, normal PC is an essential goal for the development of protective humoral vaccination strategies. We recently demonstrated that peripheral blood memory B cells can be reproducibly induced to differentiate into short-lived plasmablastic cells producing somatically mutated immunoglobulin (Tarte*, et al* 2002). Transduction of Bfl-1/A1 in these human *in vitro* derived plasmablastic cells might prevent them from apoptosis, as previously described in the context of murine *in vitro* generated plasmablasts, and help obtaining PC lines producing human antibodies.

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Legends to Figures

Figure 1. Expression of *Bfl-1/A1* in normal and malignant plasma cells

A. *Bfl-1/A1* expression was determined using Affymetrix HuGeneFL microarrays on 7 purified $CD138^+$ bone marrow plasma cells (BMPC), 16 purified $CD138^+$ multiple myeloma cells (MM), 7 purified $CD20^-CD38^{++}$ polyclonal plasmablastic cells (PPC), 7 purified $CD19^+$ tonsil B cells (TBC), and 7 purified $CD19^+$ peripheral blood B cells (PBB). Arrays were scaled to an average intensity of 1500 and a threshold of 200 was assigned to small values. The colored image is a graphic representation generated using Cluster and Treeview softwares. Red represents an expression greater than the median, green represents an expression less than the median, and the deeper color intensity represents a greater magnitude of deviation from the median.

B. *Bfl-1/A1* expression was studied by quantitative RT-PCR on 12 IL-6 dependent human myeloma cell lines (HMCL), 4 IL-6 independent HMCL, 10 purified primary myeloma cells, 3 PPC, 3 BMPC, 3 TBC, and 3 PBB. Data represent the expression ratio between the samples and the mean value obtained for PBB.

Figure 2. *Bfl-1/A1* is not inducible in myeloma cells

XG-13 HMCL was starved of IL-6 for 3 hours and cultured with no cytokine, IL-6 (2 ng/ml), BAFF (200 ng/ml), or APRIL (200 ng/ml). (**A**) NF-κB activity was determined after 1 hour. Results are the mean +/- SD of data obtained on triplicate ELISA wells. (**B**) *Bfl-1/A1* RNA was quantified using quantitative RT-PCR after 1, 3, and 12 hours. Results are expressed as the expression ratio between the samples and the value obtained for XG-13 in exponential growth phase.

Figure 3. Transduction of HMCL with Bfl-1/A1-containing retrovirus

A. XG-6 was transduced with Bfl-1/A1-containing retroviral vector (XG-6A1) or with empty retroviral vector (XG-6MCS), cultured with zeocin and IL-6, and cell-sorted for high EYFP expression. After this selection process, all XG-6A1 and XG-6MCS cells expressed EYFP. Results are representative of 5 independent transduction experiments.

B. *Bfl-1/A1* and *PRDI-BF1/Blimp-1* expression was determined using RT-PCR on XG-6, XG-6MCS, and XG-6A1.

C. XG-6, XG-6MCS, and XG-6A1 were cultured with IL-6 and harvested for Western Blot analysis. Cell lysates were analyzed using anti-Mcl-1 antibody. Immunoblotting for β-actin confirmed equal protein loading. Western Blot is of one representative experiment out of two.

Figure 4. Bfl-1/A1 reproducibly protects HMCL from apoptosis induced by IL-6 deprivation and dexamethasone.

A. XG-6, XG-6MCS, and XG-6A1 were starved of IL-6 for 3 hours and cultured with no cytokine, IL-6 (2 ng/ml), dexamethasone (DEX, 10^{-6}M), or IL-6 and DEX. Cells were recovered after 3 days of culture and apoptosis was evaluated using annexin-V staining. Results are those of one experiment out of three.

B. Five XG-6MCS and five XG-6A1 clones were generated in five separate transduction experiments. They were starved of IL-6 and cultured for 3 days without cytokine, in the presence or in the absence of DEX (10^{-6}M). Percentage of apoptotic cells was evaluated by annexin-V staining.

Figure 5. Bfl-1/A1 supports long-term growth of XG-6 in the absence of IL-6

XG-6, XG-6MCS, and XG-6A1 were cultured at 10^5/ml with or without IL-6 (2 ng/ml). Every 3-4 days, cells were counted and replated in the same culture conditions. Results are the cumulative cell numbers obtained from 25 days of culture and are from one experiment representative of three.

Figure 6. The protective effect of Bfl-1/A1 is not abrogated by an anti-IL-6 antibody.

XG-6, XG-6MCS, and XG-6A1 were starved of IL-6 and cultured for three days with or without IL-6 (2 ng/ml), with or without 10 μg/ml of B-E8 anti-IL-6 antibody. The apoptosis was evaluated by annexin-V staining. Results are those of one experiment out of two.

References

Angelin-Duclos, C., Cattoretti, G., Lin, K.I. & Calame, K. (2000) Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *Journal of Immunology,* **165,** 5462-5471.

Angelin-Duclos, C., Johnson, K., Liao, J., Lin, K.I. & Calame, K. (2002) An interfering form of Blimp-1 increases IgM secreting plasma cells and blocks maturation of peripheral B cells. *Eur J Immunol,* **32,** 3765-3775.

Bataille, R., Robillard, N., Pellat Deceunynck, C. & Amiot, M. (2003) A cellular model for myeloma cell growth and maturation based on an intraclonal CD45 hierarchy. *Immunological Review,* **194,** 105-111.

Bernal, A., Pastore, R.D., Asgary, Z., Keller, S.A., Cesarman, E., Liou, H.C. & Schattner, E.J. (2001) Survival of leukemic B cells promoted by engagement of the antigen receptor. *Blood,* **98,** 3050-3057.

Borson, N.D., Lacy, M.Q. & Wettstein, P.J. (2002) Altered mRNA expression of Pax5 and Blimp-1 in B cells in multiple myeloma. *Blood,* **100,** 4629-4639.

Calame, K.L., Lin, K.I. & Tunyaplin, C. (2003) Regulatory mechanisms that determine the development and function of plasma cells. *Annu Rev Immunol,* **21,** 205-230.

Craxton, A., Chuang, P.I., Shu, G., Harlan, J.M. & Clark, E.A. (2000) The CD40-inducible Bcl-2 family member A1 protects B cells from antigen receptor-mediated apoptosis. *Cell Immunol,* **200,** 56-62.

Derenne, S., Monia, B., Dean, N.M., Taylor, J.K., Rapp, M.J., Harousseau, J.L., Bataille, R. & Amiot, M. (2002) Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood,* **100,** 194-199.

D'Sa-Eipper, C. & Chinnadurai, G. (1998) Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities. *Oncogene,* **16,** 3105-3114.

Edelstein, L.C., Lagos, L., Simmons, M., Tirumalai, H. & Gelinas, C. (2003) NF-kappa B-dependent assembly of an enhanceosome-like complex on the promoter region of apoptosis inhibitor Bfl-1/A1. *Mol Cell Biol,* **23,** 2749-2761.

Eisen, M.B., Spellman, P.T., Brown, P.O. & Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences of the United States of America,* **95,** 14863-14868.

Feinman, R., Koury, J., Thames, M., Barlogie, B., Epstein, J. & Siegel, D.S. (1999) Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. *Blood,* **93,** 3044-3052.

Gonzalez, J., Orlofsky, A. & Prystowsky, M.B. (2003) A1 is a growth-permissive antiapoptotic factor mediating postactivation survival in T cells. *Blood,* **101,** 2679-2685.

Grumont, R.J., Rourke, I.J. & Gerondakis, S. (1999) Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev.,* **13,** 400-411.

Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munshi, N., Dang, L., Castro, A., Palombella, V., Adams, J. & Anderson, K.C. (2002) NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem,* **277,** 16639-16647.

Hinz, M., Loser, P., Mathas, S., Krappmann, D., Dorken, B. & Scheidereit, C. (2001) Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. *Blood,* **97,** 2798-2807.

Hsu, S.Y., Kaipia, A., McGee, E., Lomeli, M. & Hsueh, A.J. (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc Natl Acad Sci U S A,* **94,** 12401-12406.

Jourdan, M., De Vos, J., Mechti, N. & Klein, B. (2000) Regulation of Bcl-2-family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-alpha and insulin-like growth factor 1. *Cell Death Differ,* **7,** 1244-1252.

Jourdan, M., Veyrune, J.L., Vos, J.D., Redal, N., Couderc, G. & Klein, B. (2003) A major role for Mcl-1 antiapoptotic protein in the IL-6-induced survival of human myeloma cells. *Oncogene,* **22,** 2950-2959.

Karsan, A., Yee, E. & Harlan, J.M. (1996) Endothelial cell death induced by tumor necrosis factor-α is inhibited by the Bcl-2 family member, A1. *J Biol Chem,* **271,** 27201-27204.

Knödel, M., Kuss, A.W., Lindemann, D., Berberich, I. & Schimpl, A. (1999) Reversal of Blimp-1-mediated apoptosis by A1, a member of the Bcl-2 family. *European Journal of Immunology,* **29,** 2988-2998.

Kuss, A.W., Knodel, M., Berberich-Siebelt, F., Lindemann, D., Schimpl, A. & Berberich, I. (1999) A1 expression is stimulated by CD40 in B cells and rescues WEHI 231 cells from anti-IgM-induced cell death. *Eur J Immunol,* **29,** 3077-3088.

Lee, H.H., Dadgostar, H., Cheng, Q., Shu, J. & Cheng, G. (1999) NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc Natl Acad Sci U S A,* **96,** 9136-9141.

Lin, E.Y., Orlofsky, A., Berger, M.S. & Prystowsky, M.B. (1993) Characterization of A1, a Novel Hemopoietic-Specific Early- Response Gene with Sequence Similarity to bcl-2. *Journal of Immunology,* **151,** 1979-1988.

Lin, K.I., Angelin-Duclos, C., Kuo, T.C. & Calame, K. (2002) Blimp-1-dependent repression of pax-5 is required for differentiation of B cells to immunoglobulin m-secreting plasma cells. *Mol Cell Biol,* **22,** 4771-4780.

Lin, K.I., Lin, Y. & Calame, K. (2000) Repression of c-myc is necessary but not sufficient for terminal differentiation of B lymphocytes in vitro. *Mol Cell Biol,* **20,** 8684-8695.

Manz, R.A., Arce, S., Cassese, G., Hauser, A.E., Hiepe, F. & Radbruch, A. (2002) Humoral immunity and long-lived plasma cells. *Curr Opin Immunol,* **14,** 517-521.

Medina, F., Segundo, C., Campos-Caro, A., Gonzalez-Garcia, I. & Brieva, J.A. (2002) The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. *Blood,* **99,** 2154-2161.

Medina, F., Segundo, C., Campos-Caro, A., Salcedo, I., Garcia-Poley, A. & Brieva, J.A. (2003) Isolation, maturational level, and functional capacity of human colon lamina propria plasma cells. *Gut,* **52,** 383-389.

Mitsiades, C.S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S.P., Munshi, N.C., Richardson, P.G. & Anderson, K.C. (2002a) Activation of NF-kappaB and upregulation of intracellular anti-

apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene,* **21,** 5673-5683.

Mitsiades, N., Mitsiades, C.S., Poulaki, V., Chauhan, D., Richardson, P.G., Hideshima, T., Munshi, N., Treon, S.P. & Anderson, K.C. (2002b) Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. *Blood,* **99,** 4079-4086.

Moreaux, J., Legouffe, E., Jourdan, E., Quittet, P., Reme, T., Lugagne, C., Moine, P., Rossi, J.F., Klein, B. & Tarte, K. (2003) BAFF and APRIL protect myeloma cells from apoptosis induced by IL-6 deprivation and dexamethasone. *Blood,* **in press**.

Moreb, J.S. & Zucali, J. (2001) Human A1 expression in acute myeloid leukemia and its relationship to Bcl-2 expression. *Blood,* **97,** 578-579.

Moulding, D.A., Akgul, C., Derouet, M., White, M.R. & Edwards, S.W. (2001) BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. *J Leukoc Biol,* **70,** 783-792.

Nieborowska-Skorska, M., Hoser, G., Kossev, P., Wasik, M.A. & Skorski, T. (2002) Complementary functions of the antiapoptotic protein A1 and serine/threonine kinase pim-1 in the BCR/ABL-mediated leukemogenesis. *Blood,* **99,** 4531-4539.

Pham, L.V., Tamayo, A.T., Yoshimura, L.C., Lo, P. & Ford, R.J. (2003) Inhibition of constitutive NF-kB activation in mantle cell lymphoma B cells leads to induction of cell cycle arresr and apoptosis. *Journal of Immunology,* **171,** 88-95.

Piskurich, J.F., Lin, K., Lin, Y., Wang, Y., Ting, J.P.Y. & Calame, K. (2000) BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nature Immunology,* **1,** 526-532.

Richardson, P.G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S.V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R.Z., Kuter, D., Limentani, S.A., Lee, S., Hideshima, T., Esseltine, D.-L., Kauffman, M., Adams, J., Schenkein, D.P. & Anderson, K.C. (2003) A phase 2 study of Bortezomib in relapsed, refractory myeloma. *N Engl J Med,* **348,** 2609-2617.

Schliephake, D.E. & Schimpl, A. (1996) Blimp-1 overcomes the block in IgM secretion in lipopolysaccharide/anti-mu F(ab')2-co-stimulated B lymphocytes. *Eur J Immunol,* **26,** 268-271.

Shaffer, A.L., Lin, K.I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltnane, J.M., Yang, L., Zhao, H., Calame, K. & Staudt, L.M. (2002) Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity,* **17,** 51-62.

Shapiro-Shelef, M., Lin, K.I., McHeyzer-Williams, L.J., Liao, J., McHeyzer-Williams, M.G. & Calame, K. (2003) Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity,* **19,** 607-620.

Slifka, M.K., Antia, R., Whitmire, J.K. & Ahmed, R. (1998) Humoral immunity due to long-lived plasma cells. *Immunity,* **8,** 363-372.

Tarte, K., de Vos, J., Thykjaer, T., Zhan, F., Fiol, G., Costes, V., Rème, T., Legouffe, E., Rossi, J.F., Shaughnessy, J., Orntoft, T.F. & Klein, B. (2002) Generation of polyclonal plasmablasts from peripheral blood B cells: a normal counterpart of malignant plasmablasts. *Blood,* **100,** 1113-1122.

Tarte, K., Zhan, F., De Vos, J., Klein, B. & Shaughnessy, J. (2003) Gene expression profiling of plasma cells and plasmablasts: toward a better understanding of the late stages of B-cell differentiation. *Blood,* **102,** 592-600.

Tomayko, M.M. & Cancro, M.P. (1998) Long-lived B cells are distinguished by elevated expression of A1. *Journal of Immunology,* **160,** 107-111.

Wen, R., Chen, Y., Xue, L., Schuman, J., Yang, S., Morris, S.W. & Wang, D. (2003) Phospholipase Cgamma2 provides survival signals via Bcl2 and A1 in different subpopulations of B cells. *J Biol Chem,* **278,** 43654-43662.

Werner, A.B., de Vries, E., Tait, S.W., Bontjer, I. & Borst, J. (2002) Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. *J Biol Chem,* **277,** 22781-22788.

Zhan, F., Hardin, J., Bumm, K., Zheng, M., Tian, E., Sanderson, R.D., Yang, Y., Wilson, C., Zangari, M., Anaissie, E., Morris, C., Muwalla, F., van Rhee, F., Fassas, A., Crowley, J., Tricot, G., Barlogie, B. & Shaughnessy, J. (2002) Molecular profiling of multiple myeloma. *Blood,* **99,** 1745-1757.

Zhan, F., Tian, E., Bumm, K., Smith, R., Barlogie, B. & Shaughnessy, J. (2003) Profiling of human plasma cell differentiation and classification of multiple myeloma based on similarities to distinct stages of late-stage B-cell development. *Blood,* **101,** 1128-1140.

Zhang, X.G., Gaillard, J.P., Robillard, N., Lu, Z.Y., Gu, Z.J., Jourdan, M., Boiron, J.M., Bataille, R. & Klein, B. (1994) Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. *Blood.,* **83,** 3654-3663.

Zong, W.X., Edelstein, L.C., Chen, C., Bash, J. & Gelinas, C. (1999) The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. *Genes Dev,* **13,** 382-387.

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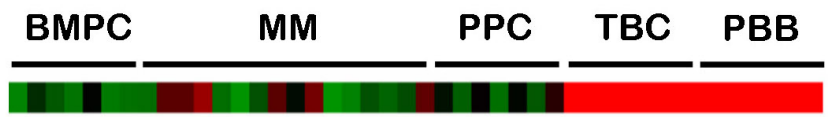

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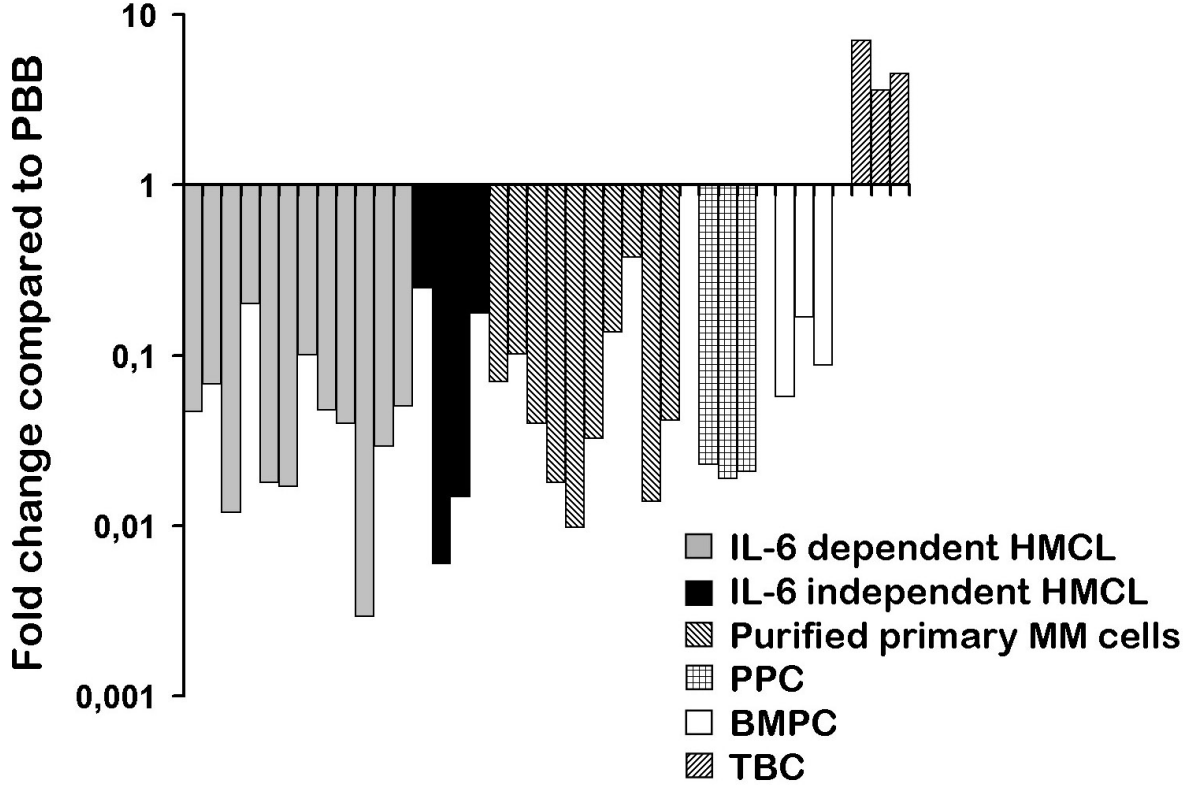
B.



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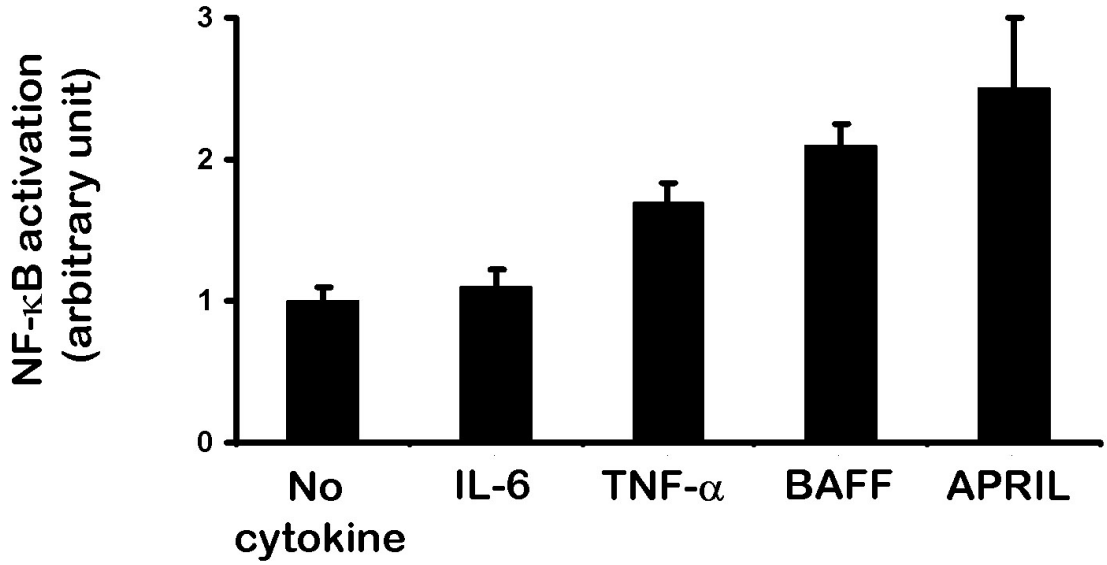
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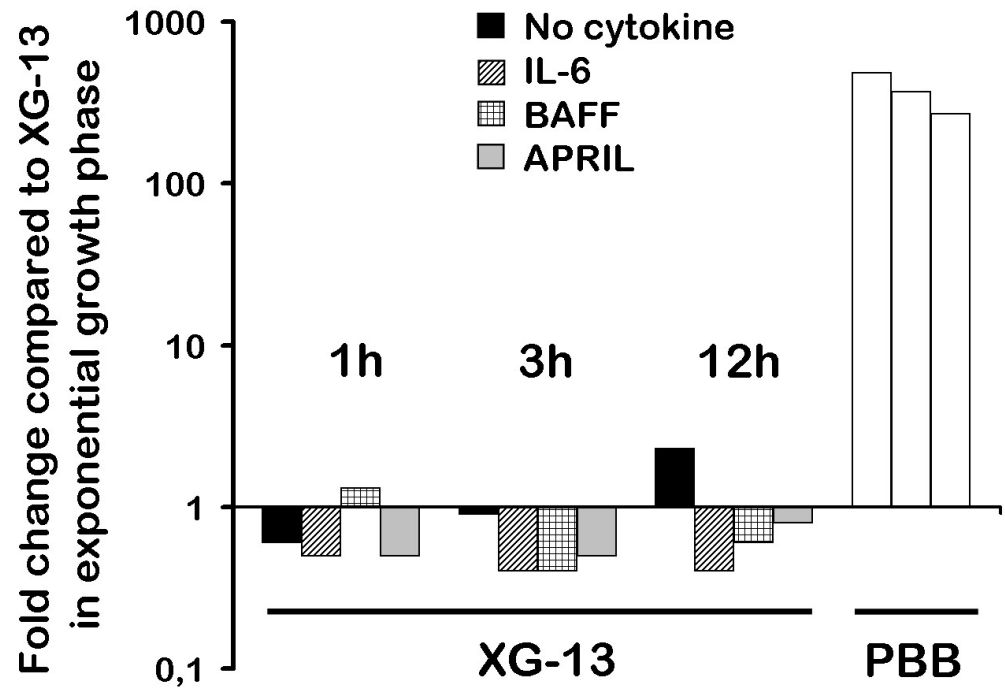
APRIL

B.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APRIL
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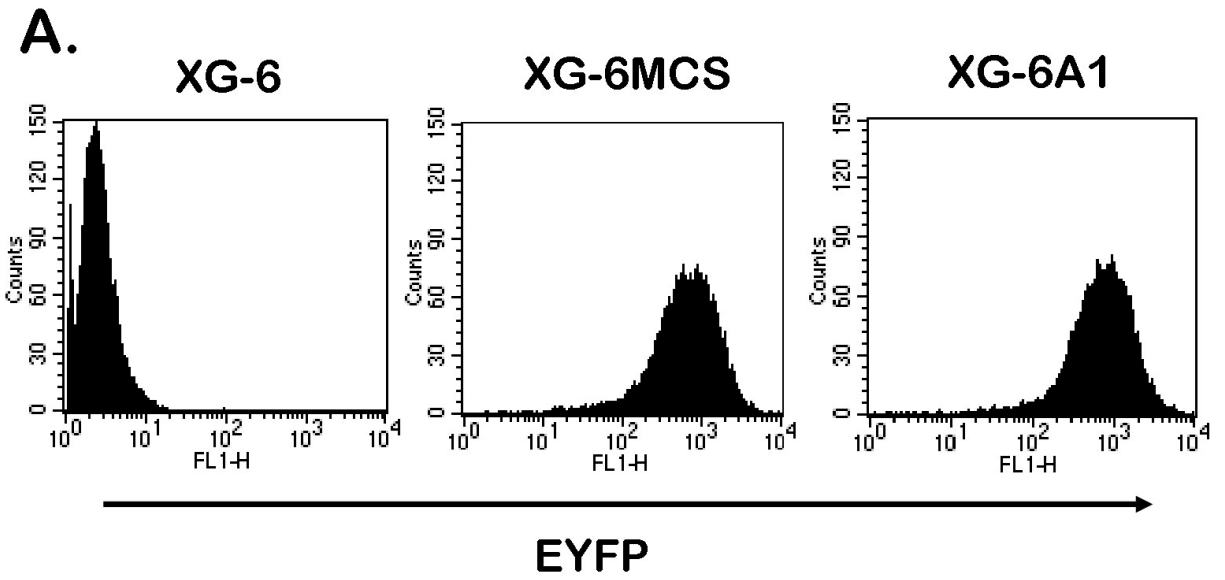
3h
12h
XG-13
PBB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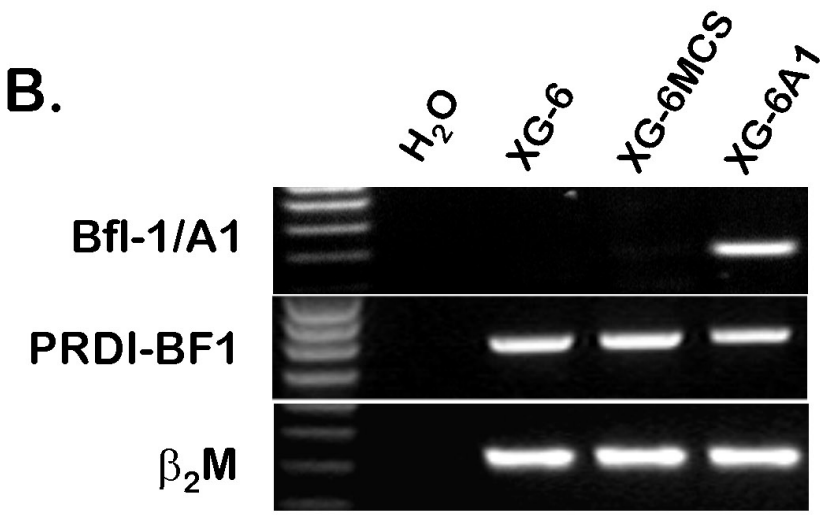
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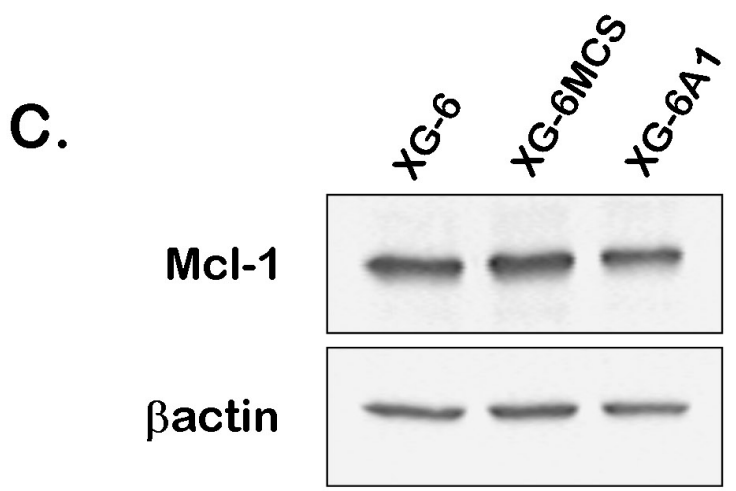
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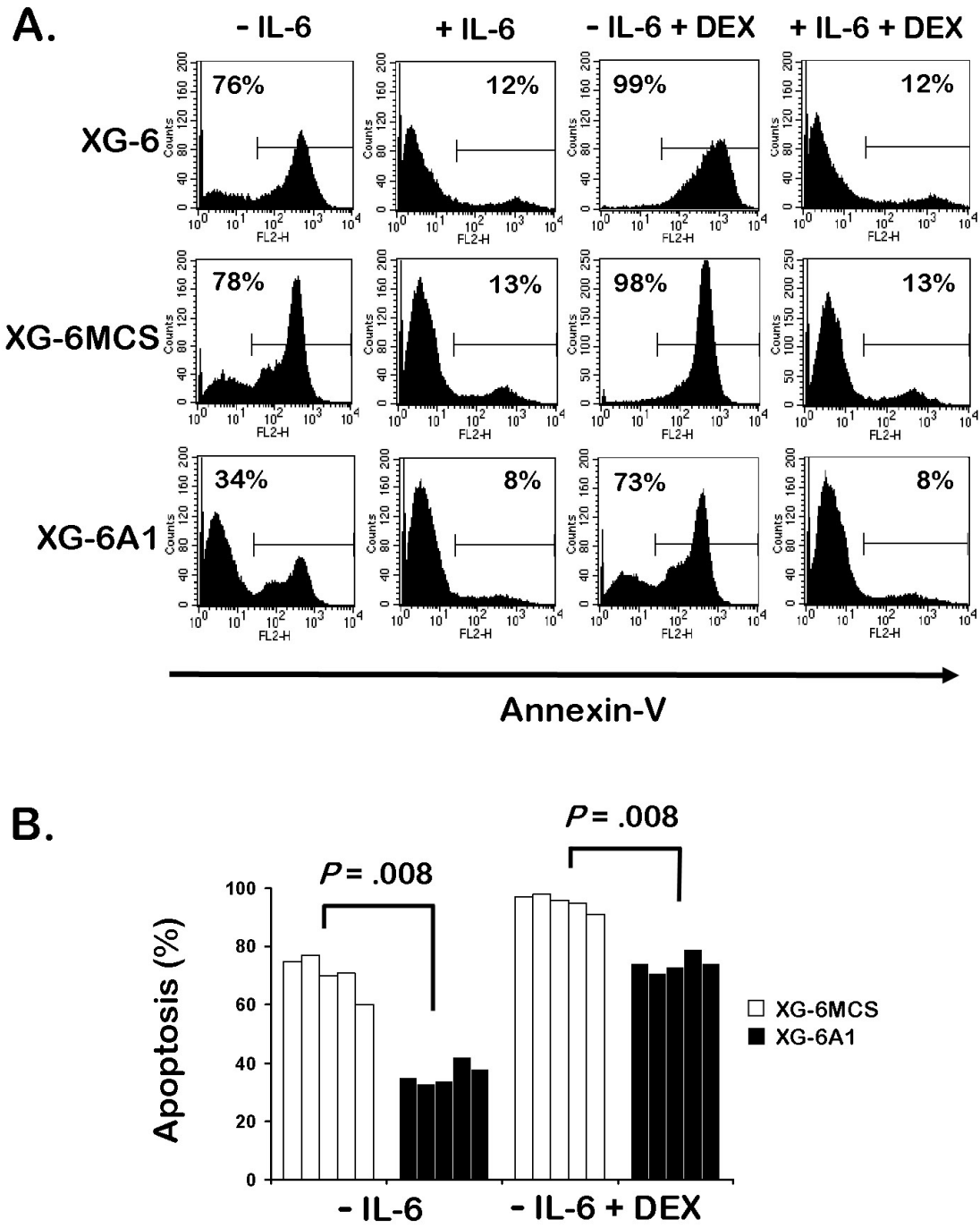
A.
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+ IL-6
- IL-6 + DEX
+ IL-6 + DEX
XG-6
XG-6MCS
XG-6A1
76%
12%
99%
12%
78%
13%
98%
13%
34%
8%
73%
8%
Counts
FL2-H
Annexin-V
B.
P = .008
P = .008
Apoptosis (%)
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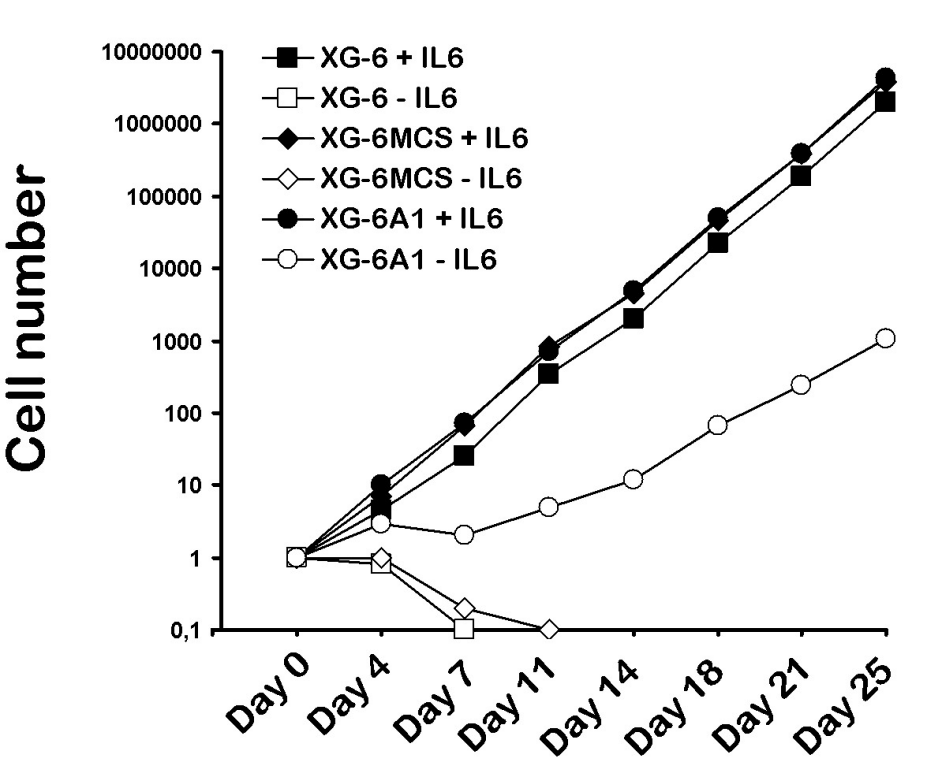
XG-6A1
- IL-6
- IL-6 + DEX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100000
10000
1000
100
10
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XG-6 - IL6
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XG-6MCS - IL6
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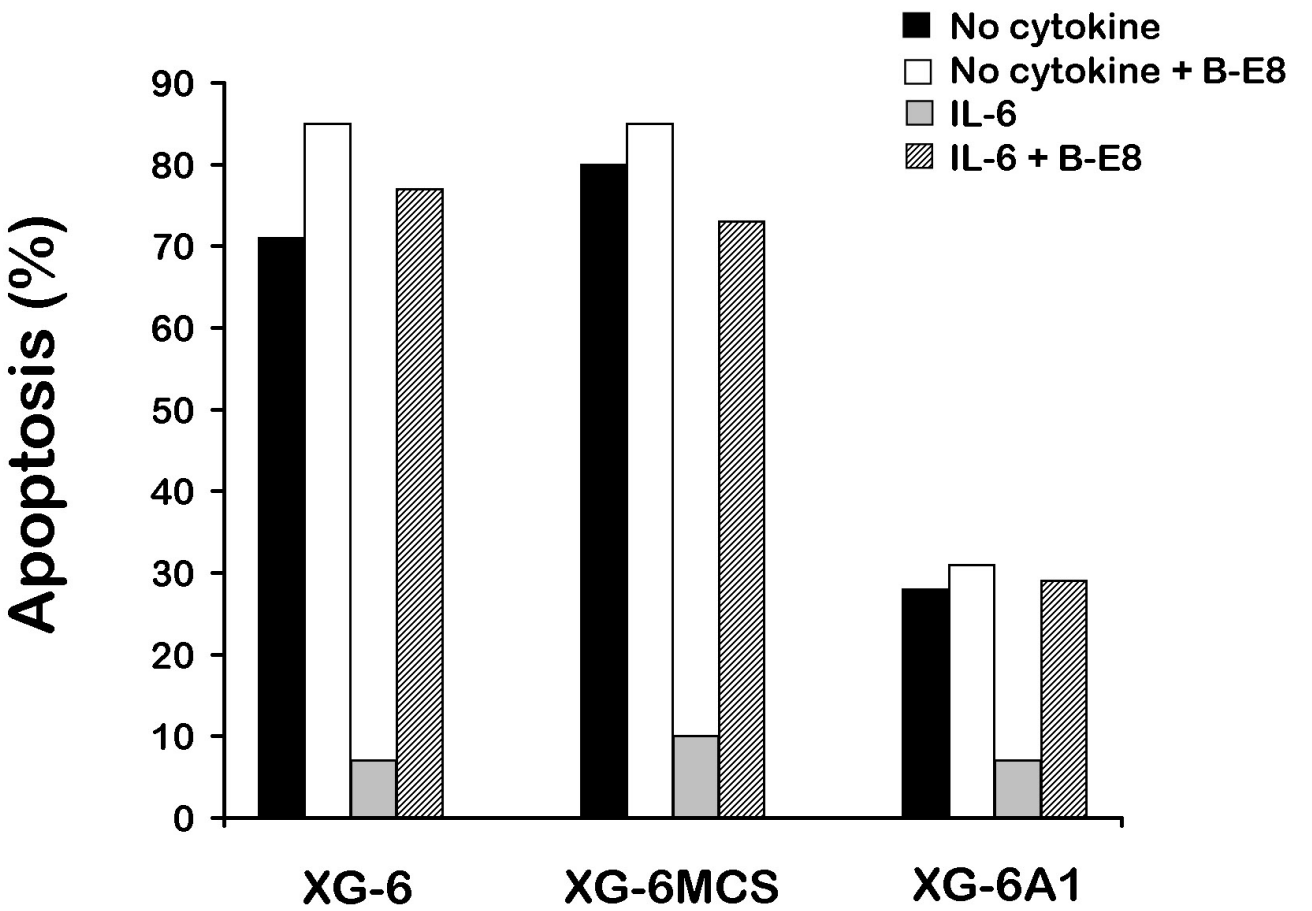
XG-6A1 - IL6
Day 0
Day 4
Day 7
Day 11
Day 14
Day 18
Day 21
Day 25


No cytokine
No cytokine + B-E8
IL-6
IL-6 + B-E8
Apoptosis (%)
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80
70
60
50
40
30
20
10
0
XG-6
XG-6MCS
XG-6A1