A proto-oncogene Bcl-6 is upregulated in the bone marrow microenvironment in multiple myeloma cells

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Abbreviations: MM, multiple myeloma; BM, bone marrow; BMSC, BM stromal cell; SCCS, stromal cell culture supernatant; IKK, IκB kinase
Abstract

Constitutive Bcl-6 expression was undetectable in MM cell lines, except U266 cells. However, it was upregulated by co-culture with bone marrow (BM) stromal cell culture supernatant (SCCS). Bcl-6 expression in patient MM cells in the BM was positive. Anti-IL-6 neutralizing Ab significantly blocked SCCS-induced Bcl-6 in MM cells. Indeed, IL-6 strongly triggered Bcl-6 expression in MM cells, whereas JAK inhibitor and STAT3 siRNA downregulated Bcl-6. TNFα also triggered Bcl-6, but independently of STAT3, whereas and IKKβ inhibitor downregulated TNFα-induced Bcl-6, indicating that the canonical NF-κB pathway mediates TNFα-induced Bcl-6 expression. Importantly, downregulation of Bcl-6 by shRNA significantly inhibited MM cell growth in the presence of SCCS. Our results therefore suggest that Bcl-6 expression in MM cells is modulated, at least in part, via JAK/STAT3 and canonical NF-κB pathways; and that targeting Bcl-6, either directly or via these cascades, inhibits MM cell growth in the BM milieu.
**Introduction**

BCL6 (B- cell lymphoma 6) is a 95-kD nuclear protein belonging to the Pox virus zinc finger transcription factor family. It is a proto-oncogene encoding a transcriptional repressor which regulates germinal center B cell differentiation. BCL6 is constitutively expressed in a significant fraction of B-cell lymphomas. Importantly, Bcl-6 is deregulated either by chromosomal translocations (3q27) or aberrant somatic hypermutation in a subset (35-40%) of diffuse large B cell lymphomas (DLBCLs) \(^1,2\), and its biologic significance has been extensively studied in this setting \(^3\).

Bcl-6 function is regulated by acetylation; specifically, histone deacetylase (HDAC)-2 binds to Bcl-6 and modulates its function \(^4\). Conversely, inhibition of HDAC2 induces hyper-acetylation of Bcl-6, resulting in loss of its function. Therefore HDAC inhibitors (especially class I inhibitors) have been used for functional inhibition of Bcl-6 \(^5\). Most importantly, a small peptide inhibitor of BCL-6 induces cytotoxicity in primary human DLBCL cells both in vitro and in vivo, without effecting normal lymphoid tissue \(^6,7\), suggesting that Bcl-6 is a promising novel therapeutic target in DLBCL. However, the biologic significance of Bcl-6 in MM has not yet been elucidated.
Materials and Methods
Detailed information pertinent to tumor cell lines and primary tumor specimens; growth of long-term bone marrow stromal cells (BMSCs); reagents; immunoblotting; cell growth assays; real-time polymerase chain reaction (RT-PCR); and shRNA infection are included as Supplementary Methods on the Blood website. Primary CD138+ tumor cells from MM patients were obtained using negative selection, as previously described after IRB-approved (Dana-Farber Cancer Institute) informed consent and in accordance with the Declaration of Helsinki protocol.

Results and Discussion
To examine whether patient MM cells in the BM express Bcl-6, we first performed immunohistochemical analysis on BM tissue microarrays from both healthy donors (NBM) and MM patients. Importantly, Bcl-6 was strongly expressed within the nucleus in MM cells of all cases (Figure 1A), suggesting that Bcl-6 might play a role in MM pathogenesis. To examine whether soluble factors modulated Bcl-6 expression in MM cells in the context of the BM microenvironment, MM.1S and RPMI8226 cells were cultured with stromal cell culture supernatants (SCCS) or BMSCs for 12h. Induction of Bcl-6 was similarly upregulated by SCCS and BMSC co-culture (Figure 2B), suggesting that induction of Bcl-6 by BMSC was predominantly by soluble factors and we carried out further experiments using SCCS. Although MM cell lines express weak or undetectable constitutive Bcl-6 expression, it is markedly upregulated by SCCS (Figure 2C). Since the pattern of Bcl-6 induction by SCCS was similar to phospho-STAT3 and phospho-ERK, we hypothesized that IL-6 in SCCS may be triggering Bcl-6 in MM cells. We therefore cultured MM cell lines with recombinant IL-6 and confirmed that Bcl-6 was markedly upregulated, as was p-STAT3 (Figure 2D). Interestingly, U266 has high baseline Bcl-6 and p-STAT3 expression, which is associated with constitutive phosphorylation of gp130 (Figure S1). Dose-dependent (Figure 2E) and time-dependent (Figure 2F) effects of IL-6 on Bcl-6 expression showed maximum induction by 4h with 3 and 10 ng/ml IL-6. Importantly, IL-6 also triggered Bcl-6 expression in patient MM cells (Figure 2G). Real-time RT-PCR showed that IL-6, in a time-dependent fashion, significantly increased Bcl-6 mRNA levels in INA6 cells (Figure 2H), indicating that IL-6-induced transcriptional upregulation of Bcl-6. We also examined the kinetics of Bcl-6 downregulation after IL-6 withdrawal. As shown in Figure 2I, Bcl-6 expression rapidly decreased to baseline levels at 5h to 10h after IL-6 withdrawal.
To define the extent to which IL-6 accounts for SCCS-induced Bcl-6 expression, we next cultured MM cells with SCCS, in the presence or absence of neutralizing anti-IL-6 Ab. Neutralizing anti-IL-6 Ab only partially inhibited SCCS-induced Bcl-6 and p-STAT3 expression (Figure 2A). Since other gp130 family cytokines also trigger phosphorylation of STAT3 and ERK; and BMSCs also secrete OSM (Figure S2); we examined whether exogenous oncostatin M (OSM) also triggered Bcl-6 in MM cell lines. As expected, OSM markedly upregulated Bcl-6 expression in MM.1S and INA6 cell lines, associated with induction of p-STAT3 (Figure S3). In contrast, other cytokines including IGF-1, VEGF, SDF-1α, or IL-3 did not induce Bcl-6 expression (data not shown). These results suggest that gp130 family cytokines trigger Bcl-6 expression in MM cells.

Our studies indicated that phosphorylation of both ERK and STAT3 are correlated with Bcl-6 expression (Figure 1C). MEK inhibitor U0126 completely blocked both constitutive and IL-6-induced phospho-ERK; however, it did not inhibit phospho-STAT3 or Bcl-6 expression (Figure S4A). In contrast, pan-JAK inhibitor AG490 markedly downregulated phospho-STAT3 and Bcl-6 expression (Figure S4B). We further confirmed the significance of STAT3 downregulation using STAT3 siRNA: STAT3 siRNA transfectants have lower levels of Bcl-6 than scrambled siRNA transfectants (Figure S4C). Taken together, these results suggest that IL-6-induced Bcl-6 expression is modulated via JAK/STAT3 pathway.

We next examined the impact of IL-6 on Bcl-6 expression in MM cells in the context of the BM microenvironment by culturing three IL-6 responsive MM cell lines (MM.1S, U266) with SCCS or IL-6. IL-6 was a more potent inducer of p-STAT3 than SCCS; however, SCCS more potently induced Bcl-6 than IL-6 in MM.1S (Figure 2A). We further examined three distinct SCCS and observed that induction of Bcl-6 by SCCS was correlated with phosphorylation of JNK and p38MAPK (Figure 2B). Since we have previously shown that transcription and secretion of IL-6 by BMSCs is regulated by NF-κB; and TNFα is a potent activator of NF-κB, JNK, and p38MAPK, we hypothesized that TNFα in SCCS may also trigger Bcl-6 expression. As expected, TNFα markedly upregulated Bcl-6 in RPMI8226 cells (Figure 2C). This upregulation was completely independent of p-STAT3, indicating that IL-6 or other gp130 family cytokines were not induced by TNFα-NF-κB axis in this setting. Importantly, both SCCS and TNFα also triggered Bcl6 in patient MM cells even when IL-6 did not (Figure 2D). We have previously shown that IKKβ inhibitor MLN120B blocks TNFα-induced NF-κB activation. We next showed that MLN120B completely blocked TNFα-induced Bcl-6 expression in
patient MM cells (Figure 2E) and RPMI8226 cells (Figure 2F), suggesting that TNFα-induced Bcl-6 expression is mediated via canonical NF-κB pathway. Since secretion of cytokines from primary BMSCs varies, we further compared the induction of Bcl-6 in RPMI8226 cells by SCCSs from three different individuals, in the presence or absence of neutralizing anti-IL-6 Ab or MLN120B (Figure 2F). All SCCSs markedly induced Bcl-6 expression; however, the inhibitory effect of neutralizing anti-IL-6 Ab or MLN120B on Bcl-6 induction varied (Figure 2G). These results suggest that SCCS may have different cytokines, and that the signaling cascades triggering Bcl-6 may therefore also differ. These results indicate that upregulation of Bcl-6 by SCCS is not induced by a single cytokine, but the total effect of cytokines in the BM milieu.

To determine the biologic significance of Bcl-6, we next knocked down Bcl-6 expression by lentiviral Bcl-6 shRNA. MM.1S cells were cultured with SCCS to induce Bcl-6. Both sh #1 and sh #2 constructs sufficiently downregulated Bcl-6 expression (Figure S5A). Importantly, Bcl-6 knockdown infectants had decreased number of viable cells compared to non-infected or Sc shRNA infectants (Figure S5B). Analogous experiments were carried out in U266 cells with high constitutive Bcl-6 expression without SCCS and we observed that Bcl-6 shRNA showed cell growth inhibition associated with downregulation of Bcl-6 (Figure S6). In this study, our results therefore suggest that Bcl-6 expression is mediated via both Jak/STAT3 and NF-κB pathways in MM cells, and that targeting these cascades could both inhibit Bcl-6 expression and inhibit growth of MM cells in the BM microenvironment.
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Author's contributions
T. Hideshima performed experiments and prepared the manuscript; C. Mitsiades designed experiments and analyzed the data; H. Ikeda performed experiments; D. Chauhan designed experiments and analyzed the data; N. Raje designed experiments; G. Gorgun performed experiments; K. H. Hideshima performed experiments; N. C. Munshi analyzed the data; P. G. Richardson analyzed the data; D. R. Carrasco designed and performed the experiments; K. C. Anderson designed experiments and analyzed the data.

Conflict-of-interest disclosure: C.M. discloses having received in the past consultant honoraria from Millennium, Novartis, Bristol-Myers Squibb, Merck, Kosan, and Pharmion, as well as research funding from Amgen, AVEO Pharma, EMD Serono, and Sunesis. K.C.A., N.C.M., and N.R. are consultants and on advisory board for Millennium, Celgene, and Novartis. P.G.R. is a consultant and on advisory boards for Millennium and Celgene. The remaining authors declare no competing financial interests.
References


Figure Legends

**Figure 1. IL-6 in SCCS induces Bcl-6.** (A) Immunohistochemical analysis for Bcl-6 expression was performed on BM tissue microarrays from healthy donors (NBM) and MM patients. Representative results are shown. CD138 is stained in red; Bcl-6 is stained in brown. Control sample was stained without ant-Bcl-6 Ab or anti-CD138. (B) MM.1S and RPMI8226 cells were cultured with SCCS (S) or BMSCs (Sc) for 12h. (C) MM.1S, IL-6 starved INA6, RPMI8226, and U266 cells were cultured with SCCS for 12h. (D) MM.1S, IL-6 starved INA6, RPMI8226 and U266 cells were cultured with IL-6 (5 ng/ml) for 12h. (E) MM.1S cells were cultured with IL-6 (5 ng/ml) for the indicated time periods. (F) MM.1S and IL-6 starved INA6 cells were cultured with IL-6 (1, 3, 10 ng/ml) for 12h. (G) Patient MM cells were cultured with IL-6 (5 ng/ml) for 12h. (H) INA6 cells were cultured with IL-6 (5 ng/ml for 3h and 8h) or SCCS for 8h. Total RNA was extracted and Bcl-6 gene expression was examined by real-time PCR (grey bar) and normalized to expression of GAPDH (white bar), which served as an internal control. *; p < 0.01. (I) INA6 cells were cultured with or without IL-6 (5 ng/ml) for 12h. Cells were then washed and cultured for indicated time periods. Whole cell lysates were subjected to immunoblotting with indicated Abs. phospho-STAT3 served as positive control for IL-6-induced signal transduction.

**Figure 2. TNFα upregulates Bcl-6 expression.** (A) MM.1S, and U266 cells were cultured with IL-6 (5 ng/ml) or SCCS (SC) for 12h. (B) RPMI8226 cells were cultured with three different SCCS (#1, #2, #3) for 12h. (C) RPMI8226 cells were cultured with TNFα (2.5 ng/ml) or IL-6 (5 ng/ml) for the indicated time periods. (D) Patient MM cells were cultured with IL-6 (5 ng/ml), TNFα (2.5 ng/ml) or SCCS (SC) for 12h. (E) RPMI8226 cells were cultured with or without SCCS (S), in the presence or absence of MLN120B (M, 10 µM) for 12h. (F) Patient MM cells were cultured with TNFα (T) for 12h in the presence or absence of MLN120B (M, 10 µM). (G) RPMI8226 cells were cultured with three different SCCS (sc#1, sc#2, sc#3), in the presence or absence of neutralizing IL-6 Ab (5 µg/ml) or MLN120B (M, 10 µM) for 12h. Whole cell lysates were subjected to immunoblotting with indicated Abs.
Figure 1
Figure 2

A Bcl-6 p-STAT3 STAT3

B SCCS Bcl-6 p-STAT3 STAT3 p-JNK2/3 p-p38MAPK p38MAPK GAPDH

C SCCS Bcl-6 p-STAT3 STAT3 p-JNK2/3

D Bcl-6 IL6 TNF SC

E Bcl-6 p-STAT3 STAT3 p-p38MAPK p38MAPK GAPDH

F Bcl-6 p-STAT3 STAT3 p-p38MAPK p38MAPK GAPDH

G SCCS #1 SCCS #2 SCCS #3

cont cont cont

SC+IL-6Ab SC+IL-6Ab SC+IL-6Ab

SC+MLN SC+MLN SC+MLN

Bcl-6 p-STAT3 STAT3 GAPDH

cont cont cont
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