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IL-6-induced Bcl6 variant 2 supports IL-6-dependent myeloma cell proliferation and survival through STAT3

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Abstract

IL-6 is a growth and survival factor for myeloma cells, although the mechanism by which it induces myeloma cell proliferation through gene expression is largely unknown. Microarray analysis showed that some B-cell lymphoma-associated oncogenes such as *Bcl6*, which is absent in normal plasma cells, were upregulated by IL-6 in IL-6-dependent myeloma cell lines. We found that *Bcl6* variant 2 was upregulated by STAT3. ChIP assay and EMSA showed that STAT3 bound to the upstream region of variant 2 DNA. Expression of p53, a direct target gene of Bcl6, was downregulated in the IL-6-stimulated cells, and this process was impaired by an HDAC inhibitor. Bcl6 was knocked down by introducing small hairpin RNA, resulting in decreased proliferation and increased sensitivity to a DNA damaging agent. Thus, STAT3-inducible Bcl6 variant 2 appears to generate an important IL-6 signal that supports proliferation and survival of IL-6-dependent myeloma cells.

Keywords: Myeloma; IL-6; Microarray; Bcl6; STAT3; p53; PDCD4; Trichostatin A

Multiple myeloma is a malignant proliferation of human plasma cells in the bone marrow. Our group and others have reported that interleukin-6 (IL-6) is a growth factor for myeloma cells [1,2]. IL-6 also plays a crucial role in the onset of plasma cell tumors in mice [3]. IL-6 is required not only for proliferation but for survival of immature myeloma cells from myeloma patients in vitro; immature cells constitute a predominant fraction of proliferating cells in myeloma cells [4]. IL-6 signals originate from the IL-6 receptor complex, which consists of the IL-6 receptor α (IL-6Rα) and gp130. This complex activates Janus kinases (JAK) [5], which phosphorylate gp130 molecules that recruit the signal transducer and activator of transcription 3 (STAT3) as well as the src homology 2-containing phosphatase-2 (SHP2). Tyrosine-phosphorylated STAT3 translocates to nuclei where it activates target genes. SHP2 activates ras-mitogen activated protein kinase and/or phosphatidylinositol-3′ kinase pathways. The integration of these various signals causes myeloma cell proliferation and survival [6,7]. However, it is less well understood how these pathways regulate cell proliferation through gene expression. Earlier reports analyzing IL-6-inducible genes showed that Mcl-1 upregulation resulted in survival of IL-6-dependent myeloma cells, but the enforced expression of Mcl-1 protein was not sufficient for the survival of INA-6 cells in the absence of exogenously added IL-6 [8].

Transcription repressors play significant roles in many cell functions, including cell transformation and proliferation. The *Bcl6* gene was initially cloned from the breakpoint of a B-cell lymphoma DNA that encoded a transcription repressor. This factor encoded a BTB/POZ domain and a *Krüppel*-type zinc finger motif that were located in the NH_2-terminal and the COOH-terminal

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region, respectively [9–11]. The Bcl6 protein recruits co-repressor molecules, such as SMRT, NCoR, BCoR, and MTA3, as well as histone deacetylases (HDACs) [12–15]. Previous studies examining Bcl6 gene expression profiles in many tissues and cell types showed it to be highly regulated during B-cell differentiation. It was also found to be restricted to B cells in the germinal center (GC), but was not found in pre-B cells, mature progenitor cells, or plasma cells [16]. The functions of Bcl6 are significant for the processes of germinal center formation and B-cell development, as Bcl6 knockout mice lost GC formation and T-cell-dependent activation of B cells [17]. Moreover, the enforced expression of Bcl6 in a murine B-cell line, BCL1, resulted in a blockade of the cytokine-induced plasma cell differentiation and plasma cell specific gene expression [18]. These results indicated that loss of Bcl6 expression during the differentiation process of plasma cells was a prerequisite for terminal differentiation and growth arrest for these cells [19,20]. A recent report showed that among the Bcl6-regulated genes, Bcl6-mediated suppression of p53, the key tumor suppressor for oncogenesis, occurred in GC B cells. This implied that Bcl6 tolerized the GC B cells to DNA damage-induced apoptosis originating from somatic hypermutation and class switching in immunoglobulin genes [21]. Although inactivation of p53 was observed in more than 50% of all tumors, the frequency of p53 mutation was relatively low in multiple myeloma [22–24], indicating the existence of a different p53 suppression mechanism besides mutations in myeloma cells.

To further elucidate the role of IL-6-induced gene expression in myeloma cell proliferation and survival, we assessed IL-6-induced gene expression changes in the IL-6-dependent myeloma cell lines ILKM2 and ILKM8 using a microarray technique. Among the genes that showed heightened expression in response to IL-6, we noted a novel role for STAT3-induced Bcl6 in the survival and proliferation of myeloma cells.

Materials and methods

Cell culture and reagents. Human myeloma cell lines ILKM2, ILKM3, ILKM8, KM11, KMS11, and KMS18 were cultured as described previously [25]. Analysis of cell numbers and cell viability by flow cytometry was carried out as described in our previous report [25]. U0126, parthenolide, 4-hydroxytamoxyfen (4-HT), and trichostatin A (TSA) were purchased from Sigma–Aldrich.

Primary myeloma cell analysis. Bone marrow mononuclear cells (BMMNCs) were obtained from a myeloma patient with informed consent according to the Helsinki protocol. These were cultured with or without IL-6 for 24 h and then stained with FITC-anti-CD38 and PE-anti-MPC1 as described previously [26]. Next, $CD38^{++}$ $MPC1^{-}$ cells were sorted using an Epics Elite cell sorter (Coulter).

Microarray analysis. Logarithmically proliferating ILKM2 and ILKM8 cells were starved for IL-6 for 12 h (ILKM2) or for 4 days (ILKM8) and then re-stimulated with IL-6 (10 ng/ml). Total RNA was prepared from 10^7 cells using Trizol reagent (Invitrogen). A detailed protocol for sample preparation and hybridization with an oligonucleotide microarray, GeneChip HG-U133A, was provided by Affymetrix. Briefly, double-stranded cDNA was synthesized using the T7 promoter-$(dT)_{24}$ as a primer. The product was then transcribed with T7 RNA polymerase to generate biotinylated complementary RNA (cRNA). Biotinylated cRNA was fragmented to generate shorter cRNA fragments. Hybridization of fragmented cRNA to GeneChips was carried out at 45 °C for 16 h with constant rotation at 60 rpm. The Chips were then washed and stained on a fluidics station, and scanned. The GeneChip Operating System (GCOS) was used for data manipulation (Affymetrix). The fluorescence intensity was measured for each microarray, and we normalized the raw data of each microarray by setting the mean of the signal intensities of all probe sets to 1000 to compare mRNA expression levels among samples. The data obtained at time 0 were used as a control for comparison analysis.

Real-time RT-PCR. Gene expression was quantified using real-time reverse transcription polymerase chain reaction (RT-PCR) detection with a LightCycler (Roche Diagnostics), RevTraAce reverse transcriptase (Toyobo), and SYBR Premix Ex Taq (Takara-Bio). The primer pairs used were 5′-TGATGGCCACGGCTATGTAC-3′ and 5′-TTCTCCACC ACCTCACGACC-3′ for Bcl6 (common to both variants), 5′-TTCCGAG AATTGAGCTCTGTTG-3′ and 5′-GCCTTGCTTCACAG TCCAAAA T-3′ for Bcl6 variant 1, 5′-TGGGATTGAGTGACT GGCACTT-3′ and 5′-AATGCCTTGCTTCACAGTCCA-3′ for variant 2, 5′- TGGATGT CCCACATTCATACTCTG-3′ and 5′-TCTGGTTTAAGACGACC TCCATCT-3′ for PDCD4, 5′-GCAGTCAGA TCCTAGC GTCGA-3′ and 5′-CATCAAATCATCCATTGCTTGG-3′ for p53, and 5′-GGTGA AGGTCGGAGTCAACG-3′ and 5′-AATTT GCCATGGGTGGAATC-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Signals for the genes of interest from each RNA sample were normalized to that sample's signal for GAPDH. The fold change was calculated relative to the signal of samples from IL-6-starved cells and the results were shown as means ± SD ($n = 3$).

Construction of a STAT3ER expression vector and a small hairpin RNA (shRNA) expression vector, and retrovirus-mediated gene transfer. STAT3ER was constructed by replacing mouse STAT3 with human STAT3 from pCAG musstat3ER [27] (a kind gift of Dr. Yokota, Kanazawa Univ. Sch. Med.). The STAT3ER fusion gene was then cloned into the pQCXIP vector (Clonetech). To generate shRNA expression viral vectors, double-stranded oligonucleotides coding for shRNA that specifically target the Bcl6 transcript (Bcl6 sh1: 5′-CCCGTCGAGACATCTTGACTGAGGAA TTCGTCAGTCAAGATGTCTCGACTTTTT-3′ and Bcl6 sh2: 5′-CCC GACACGGATCTGAGAATCTGGAATTCGAGATTCTCAGATCCG TGTCTTTTT-3′) [21], as well as control shRNA 1 consisting of scrambled nucleotides from the Bcl6 sh1 target sequence (Bcl6 shC1: 5′-CCCT GAACTGCTATACAGCGGTGGAATTCGACCGCTGTATAGCAGT TCATTTTT-3′), were cloned into the pSUPER.retro.puro vector (OligoEngine) according to the manufacturer's protocol. Another control shRNA expression vector was constructed by recloning of the p*Silencer* 3.0-H1 control (Ambion) into pQCXIP. The DNA sequences of plasmids containing the inserts were verified. Retrovirus-mediated gene transfer was performed as described in our previous report [26].

Western blotting. In order to detect Bcl6, cells were starved for IL-6 for 12 h (for ILKM2 cells) or for four days (for ILKM8 cells). Cells that were unstimulated or stimulated with IL-6 (10 ng/ml) for 8 h were recovered, lysed, and analyzed by Western blotting as previously described [26]. For detection of STAT3ER, ILKM8 mock infected or STAT3ER infected cells cultured with IL-6 were recovered and analyzed by Western blotting. Antibodies used were anti-Bcl6 (N-3, Santa Cruz), and anti-STAT3 (#9132, Cell Signalling).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay (ChIP) analysis was performed according to the previous report [28]. Antibodies used for immunoprecipitation of protein–DNA complexes were anti-STAT3 (F-2) or anti-Bcl6 (N-3) or control rabbit IgG (Santa Cruz). Primers used were 5′-GGCAGCTTCCTGGAAAGTTACTT-3′ and 5′-T TTTTCCTGTTACGCCGTCAA-3′ for Bcl6 variant 2 upstream (indicated as b in Fig. 3A), 5′-GAAGACTGTGCTGCGTGTAGTGC-3′ and 5′-TCT GTGTTTAAGAGATCAGCCGG-3′ for Bcl6 variant 1 upstream (indicated as a in Fig. 3A), 5′-ATGAACGTTTTCCTCCCTAATGG-3′ and 5′-GCATAATCAGGCTGTGTTGGGT-3′ for the PDCD4 promoter region (negative control), and 5′-ATGACTTAAACCCCATAAAACTTGGT-3′ and 5′-AACTAGAAAATATGCTTCTGTGTGCTG-3′ for the PDCD4 3′-untranslated region (UTR). The resulting data were compared to the

signal of the input (total chromatin-bound DNA before immunoprecipitation) from IL-6-starved cells.

Electrophoretic-mobility shift assay. Nuclear extracts were isolated from ILKM2 cells starved for IL-6 for 12 h and from cells restimulated with IL-6 (10 ng/ml) as described [29]. Electrophoretic-mobility shift assay (EMSAs) were performed using double-stranded ^{32}P-labeled oligonucleotide probes. Five micrograms of nuclear extracts was incubated with 1 pmol of labeled probes and 2 μg poly(dI–dC) in 10 mM Hepes (pH 7.9), 10% glycerol, 1 mM DTT, and 1 mM EDTA (final NaCl concentration was 80 mM) at room temperature for 30 min. The protein–DNA complexes were separated on a 4% polyacrylamide gel in Tris–glycine buffer (25 mM Trishydroxyaminomethane, 250 mM glycine). Gels were then dried and autoradiographed. For the supershift assay, nuclear extracts were preincubated with 1 μl each of anti-STAT3 or anti-STAT1 rabbit polyclonal antibody (Cell Signalling), followed by binding to the labeled probes. For the competition assays, a 100-fold molar excess of unlabeled competitor oligonucleotides was added to the binding reaction ten minutes the reactions were initiated. The probe used for the EMSAs was TG, 5′-TACGTTCCGGGAATGAGAGGGCTGTGTCAT-3′. For the competition assays, TG sequences containing a mutated STAT3 recognition site were constructed: TGm, 5′-TACGTGCCGGGACTGAGAGGGCTGTGTCAT-3′ (mutated sequences are underlined), and m67SIE: 5′-GATCCGGGAGGGATTTACGGGAAATGCTG-3′ [30].

Statistical analysis. Student's *t* test was performed to analyze the statistical significance of differences between sample data using the Microsoft Excel statistical analysis tool. *P* values less than 0.05 were considered significant.

Results

Bcl6 upregulation by IL-6

The IL-6-dependent myeloma cell lines ILKM2 and ILKM8 proliferated in the presence of IL-6 (Fig. 1A). To identify the genes regulated by IL-6 in myeloma cells, these IL-6-dependent myeloma cell lines were starved for IL-6 and then were restimulated with IL-6 (10 ng/ml) for 0.5, 3, and 8 h. The IL-6-induced gene expression was analyzed using an Affymetrix U133A DNA chip comprised of approximately 22,000 probe sets specific for 18,400 human genes. The relative expression changes were compared between IL-6 starved and IL-6 stimulated samples. A total of 48 different genes showed more than a 2-fold increase and 10 different genes showed more than a 2-fold decrease consistently at one of four time points in ILKM2 and ILKM8 cells (supplementary table). Our data demonstrate differential expression of both known (e.g., SOCS3, junB, and Mcl-1) genes and novel IL-6 target genes. Several of these genes, such as *Bcl6* and *Bcl3*, are transcriptionally activated by chromosomal translocation and are deeply involved in the pathogenesis of B cell malignancies. Brocke-Heidrich et al. [8] also reported that both *Bcl3* and *Bcl6* were upregulated by IL-6 in IL-6-dependent INA-6 and XG-1 myeloma cells, but they did not describe this upregulation mechanistically. As IL-6 withdrawal resulted in cell death in ILKM2 cells and in cell cycle arrest in ILKM8 cells (Fig. 1A), we hypothesized that IL-6-induced Bcl6 could suppress proapoptotic genes and/or growth suppressor genes. Therefore, further analysis was performed focusing on Bcl6. We confirmed the IL-6-induced upregulation of Bcl6 mRNA and protein by quantitative RT-PCR and Western blotting in ILKM2 and ILKM8 cells (Fig. 1B and C).

Bcl6 variant 2 expression was strongly augmented in IL-6 treated myeloma cells

Two transcriptional variants of *Bcl6* that differed in 5′-UTR but encoded the same protein were registered in the RefSeq database of GenBank (variant 1: NM_001706 and variant 2: NM_138931, Fig. 3A). Variant specific real-time RT-PCR showed that variant 2 was upregulated by IL-6 stimulation, while variant 1 was downregulated only in the IL-6-dependent myeloma cell lines ILKM2, ILKM3, and ILKM8 (Fig. 2A). In contrast, the IL-6-independent cell lines KM11, KMS11, and KMS18 showed quite low expression of both variants compared to the IL-6-dependent cell lines. To determine whether this variant 2 upregulation was observed in patients' cells, we

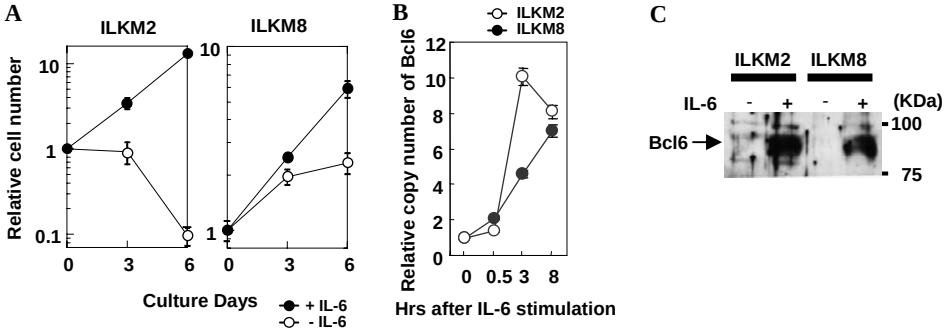

Fig. 1. Bcl6 mRNA and protein were upregulated by IL-6. (A) Growth curve of IL-6-dependent cells. Logarithmically proliferating cells responsive to IL-6 were inoculated in the presence (10 ng/ml) or absence of IL-6, cultured for the indicated times, and counted by flow cytometry. Relative cell number (day 0 = 1) is indicated as means ± SE ($n = 3$). (B) Real-time RT-PCR analysis of Bcl6. ILKM2 and ILKM8 cells were starved for IL-6 and restimulated with IL-6 (10 ng/ml) for 0.5, 3, and 8 h, and then analyzed. Relative copy number was calculated compared to time 0. The data are representative of three similar experiments and are shown as means ± SE ($n = 3$). (C) Western blotting of Bcl6. IL-6 starved ILKM2 and ILKM8 cells were restimulated with IL-6 for 8 h, and analyzed by Western blotting using a Bcl6 antibody. Arrow indicates Bcl6 protein.

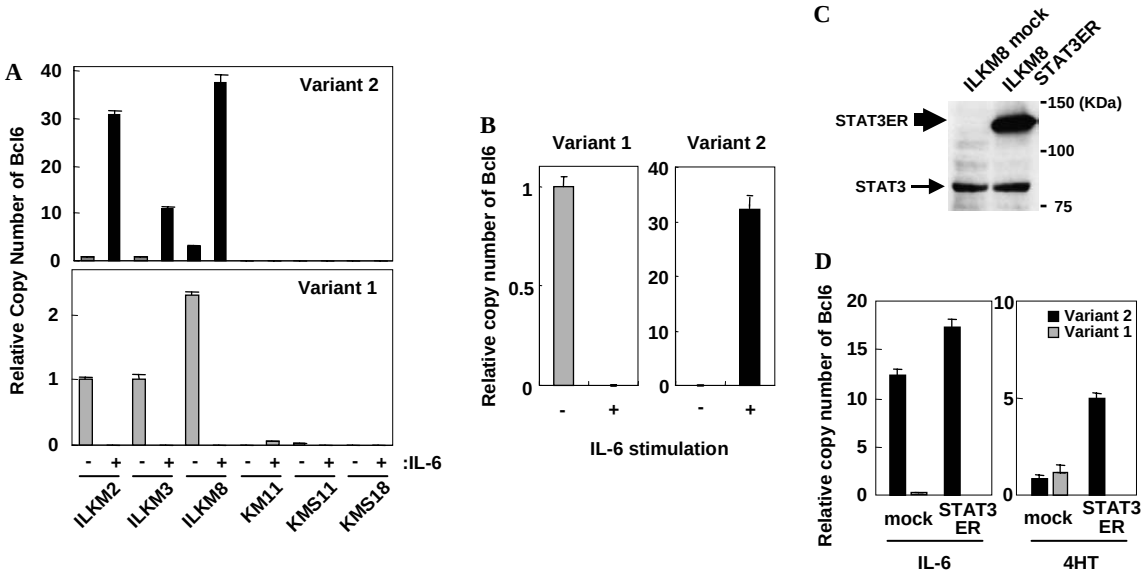

Fig. 2. Bcl6 variant 2 was upregulated by activation of STAT3ER. (A) Real-time RT-PCR analysis of Bcl6 variants 1 and 2 in myeloma cell lines. IL-6 starved ILKM2, ILKM3, and ILKM8 cells (IL-6-dependent cell lines), and KM11, KMS11, and KMS18 (IL-6-independent cell lines) (−) were stimulated with IL-6 for 8 h (+), and were then analyzed. Data are presented compared to time 0 of IL-6-starved ILKM2 cells. Representative data of three similar experiments are shown as means ± SE ($n = 3$). $P < 0.01$ versus time 0. (B) Real-time PCR analysis of Bcl6 variants 1 and 2 in immature myeloma cells obtained from a myeloma patient. $CD38^{++}$ $MPC1^{-}$ immature myeloma cells were separated from IL-6 treated (+, 10 ng/ml, 30 h) or untreated (−) BMMNCs by a cell sorter. Relative copy number of Bcl6 variants 1 and 2 is indicated (untreated = 1). (C) Western blotting of STAT3ER. Cell lysates of ILKM8 cells infected with empty vector or STAT3ER expression vector were analyzed by Western blotting using an anti-STAT3 antibody. Thick and thin arrows indicate STAT3ER and endogenous STAT3, respectively. (D) Real-time RT-PCR of Bcl6 variants 1 and 2 in STAT3ER expressing ILKM8 cells. Control vector-infected or STAT3ER-infected ILKM8 cells were starved with IL-6 and stimulated with IL-6 (10 ng/ml), or 4-HT (10 μM) for 4 h, and then analyzed. Data are presented compared to time 0. $P < 0.01$ versus time 0 (IL-6-treated ILKM8 mock and STAT3ER, and 4HT-treated ILKM8 STAT3ER).

cultured BMMNCs isolated from a multiple myeloma patient in the presence or absence of IL-6 for 30 h. $CD38^{++}$ $MPC1^{-}$ immature myeloma cells that contained cells proliferating in response to IL-6 were isolated by a cell sorter [4]. Real-time RT-PCR of patient-derived immature cells that were treated with or without IL-6 also showed that variant 2 was upregulated, whereas variant 1 was downregulated by IL-6 (Fig. 2B). These data indicated that Bcl6 variant 2 upregulation really was induced in IL-6-dependent myeloma cells.

Bcl6 variant 2 was upregulated by STAT3ER

We next analyzed how variant 2 was upregulated by IL-6 stimulation. Treatment with the MEK inhibitor U0126, or with an NF-κB inhibitor, parthenolide, did not inhibit the IL-6 induced upregulation of variant 2 (data not shown). We thus deduced that STAT3 might regulate variant 2 induction, because there was a candidate STAT binding site in the upstream region of putative exon 1 of variant 2 (−1369 to −1377 bases from the 5′ end of NM_001706, Fig. 3A). To prove that Bcl6 variant 2 was upregulated by STAT3, a construct with STAT3 cDNA fused to the tamoxifen-responsive estrogen receptor fragment (STAT3ER) was introduced into the ILKM8 cell line. Western blotting showed that ILKM8 cells containing STAT3ER expressed fusion protein having the expected size (123 kDa) in addition to the endogenous STAT3 protein (Fig. 2C). Stimulation of ILKM8 STAT3ER cells with 10 μM 4-HT resulted in upregulation of variant 2 and downregulation of variant 1, whereas both variants did not show any significant change by 4-HT treatment in ILKM8 mock cells (Fig. 2D). We also confirmed that SOCS3 expression, the well-established target gene of STAT3, was induced by 4-HT in ILKM8 STAT3ER cells (data not shown).

STAT3 binds to the upstream region of variant 2

We next checked to see if the STAT3 protein could bind to the 5′-upstream region of variant 2 using ChIP analysis and EMSAs. In ILKM2 cells cultured with IL-6, real-time PCR analysis of the candidate STAT3 binding site (amplimer b) showed a strong signal with anti-STAT3 antibody-immunoprecipitated chromatin DNA after IL-6 stimulation, whereas a control amplimer that locates in the upstream region of variant 1 did not show any significant difference among samples (Fig. 3B). EMSAs probed with the STAT recognition sequence (TG as shown in Fig. 3C) in the upstream region of variant 2 indicated that a specific DNA–protein complex was formed after IL-6 stimulation (the bands shown as B in Fig. 3D). Preincubation of the anti-STAT3 antibody resulted in disappearance of the bands B and the concomitant appearance of the band SS, which had a slower mobility, showing that the bands B included STAT3. In

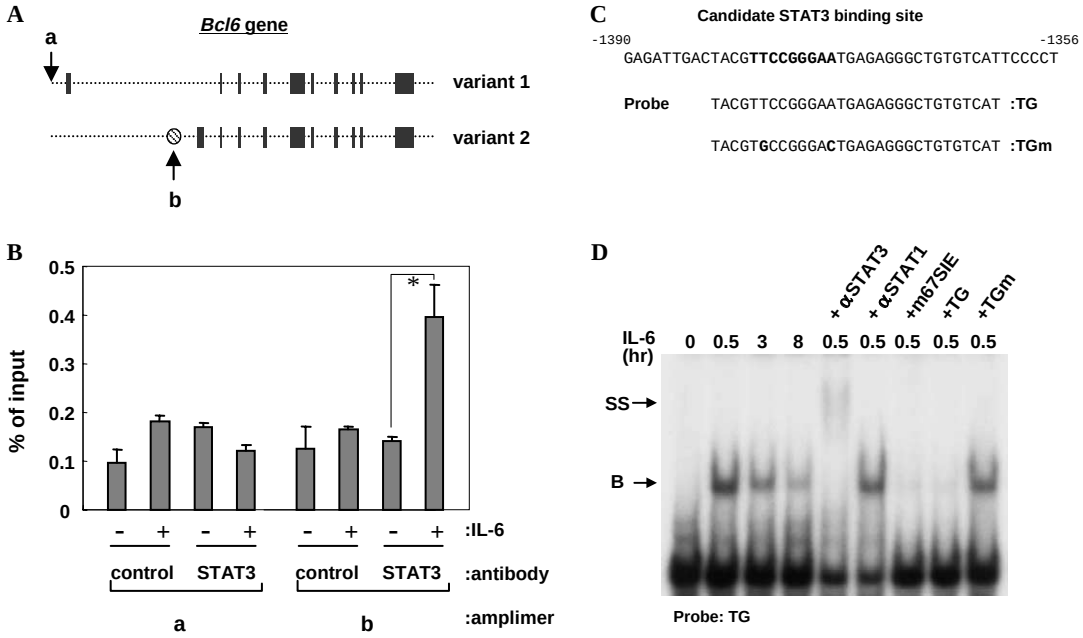

Fig. 3. STAT3 bound to the Bcl6 variant 2 upstream region. (A) Genome structure of Bcl6 variants 1 and 2. Bold lines shows exons and thin dotted lines show other regions including introns, promoter and enhancer regions. Arrows (a and b) indicate the positions of PCR amplimers for ChIP. Hatched circle in variant 2 shows STAT binding site. (B) ChIP analysis quantified by real-time PCR. Chromatin DNA immunoprecipitated from IL-6-treated (10 ng/ml, 3 h) or -starved ILKM2 cells by control or anti-STAT3 antibody was analyzed by real-time PCR. Data are shown as percent of input DNAs without immunoprecipitation. The results are representative of three independent experiments and are shown as means ± SE ($n = 3$). *$P < 0.05$ versus IL-6 starved sample. (C) Sequence of STAT3 binding site and probes used in EMSA. Numbers written over the sequence show positions from the 5′ end of variant 2 mRNA. Nucleotides shown in bold character are the consensus STAT binding sequence. TG is the probe for the EMSA. TGm is a mutant of TG used for the competition assay. Nucleotides in TGm that differ from TG are shown in bold characters. (D) EMSA of STAT3 binding sequence in variant 2. ILKM2 cells were IL-6-starved and restimulated for the indicated times. Specific binding of STAT3 to the probe was indicated by incubation of anti-STAT3 or anti-STAT1 antibody with nuclear extract (designated as αSTAT3 and αSTAT1, respectively) before adding probe that resulted in supershift of the signal. +m67SIE, +TG, +TGm indicates addition of cold probes at 100 times molar excess of the labeled probe. B, IL-6-induced DNA-binding complex; SS, supershifted DNA-binding complex. Lower bands were nonspecific signals.

contrast, preincubation with an anti-STAT1 antibody did not influence the mobility or the intensity of the complexes, showing that STAT1 was not included in the complexes. The bands B disappeared when excess amounts of cold m67SIE, a well-established specific STAT3 binding sequence, or TG itself were added, but not when mutated TG (TGm) was added. These results showed that the STAT3 activated by IL-6 stimulation might target this recognition sequence, and that Bcl6 variant 2 was transcriptionally upregulated through STAT3.

Decreased expression of Bcl6 target genes by IL-6

Among the genes reported to be targets for Bcl6-mediated suppression, several genes, such as syndecan1, XBP1, p53, CD69, and CXCR4 [19–21], were listed as downregulated genes in the microarray data, although the decrease in their expression was less than twofold, or the decrease was in one but not both of the ILKM2 and ILKM8 cell lines (data not shown). Among these genes, we confirmed that the amount of p53 mRNA was decreased by IL-6 stimulation by real-time RT-PCR (Fig. 4A). As transcriptional repression mediated by Bcl6 was carried by HDACs, and HDAC inhibitors can reverse the effect of Bcl6-mediated transcriptional repression [31], we treated IL-6 starved cells with IL-6 and an HDAC inhibitor, TSA, simultaneously. As shown in Fig. 5B, TSA treatment partially revived the expression of p53 in cells stimulated with IL-6.

Furthermore, we checked the expression of the PDCD4 gene, a candidate tumor suppressor gene, which was commonly decreased in ILKM2 and ILKM8 cells by IL-6. Quantitive RT-PCR analysis confirmed the decrease in PDCD4 mRNA by IL-6 both in ILKM2 and ILKM8 cells (Fig. 5A). The decrease in PDCD4 mRNA was also observed in tamoxifen-treated ILKM8 STAT3ER cells (data not shown). TSA treatment diminished the decreases in both PDCD4 and p53 mRNA that were induced by IL-6 (Fig. 5B). Although these data could not support the idea that PDCD4 was the direct target of Bcl6, in silico analysis of a consensus Bcl6 binding sequence, 5′-TTCYWNGAA-3′ [19], identified a candidate binding sequence just after the 3′ end of the PDCD4 transcript (+232 to +240 from NM_014456). A ChIP assay using an anti-Bcl6 antibody showed that the anti-Bcl6 antibody immunoprecipitated this putative binding sequence in ILKM2 after IL-6 stimulation showing that PDCD4 is a direct target of Bcl6 (data not shown).

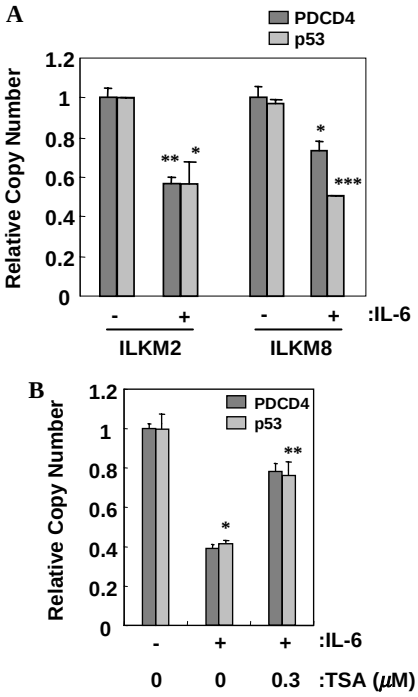

Fig. 4. Bcl6 target genes were downregulated by IL-6 stimulation. (A) Real-time RT-PCR of p53 and PDCD4. ILKM2 and ILKM8 cells starved for IL-6 (−) and then restimulated with IL-6 (10 ng/ml) for 8 h (+) were recovered and analyzed by real-time RT-PCR. Representative data of three similar experiments are shown as means ± SE ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control (-IL-6). (B) Effect of TSA on the IL-6-suppressed expression of p53 and PDCD4 genes. ILKM2 were starved for IL-6 and then restimulated with IL-6 for 8 h. TSA (300 nM) was added 30 min before IL-6 stimulation. *$P < 0.01$ versus control (-IL-6), and **$P < 0.05$ versus IL-6 treated sample.

Accordingly, these data showed that the IL-6-induced Bcl6 protein bound to the target sequences and recruited HDACs, resulting in suppression of these target genes.

Suppression of Bcl6 expression downregulated the proliferation and survival of IL-6-dependent cells

To determine the physiological relevance of IL-6-induced Bcl6 upregulation, we investigated whether Bcl6 downregulation by retroviral transduction of short hairpin RNAs (shRNAs) specifically targeting Bcl6 mRNA might affect proliferation and survival of myeloma cells. Bcl6 shRNA expression resulted in significant suppression of the Bcl6 transcript and protein, as quantified by real-time PCR and Western blotting carried out 4 days after infection (Fig. 5A, data not shown). The cell proliferation rate and the sensitivity to a DNA damaging agent, bleomycin, were next compared between control shRNA 1- (c1) infected and Bcl6 shRNA 1 (sh1) infected cells in the presence of IL-6 starting from four days after infection. The Bcl6 shRNA 1 infected cells showed slower proliferation and lower viability by bleomycin treatment than the control cells (Figs. 5B and C). Another Bcl6 shRNA, sh2, has similar effect on the Bcl6 mRNA expression, proliferation, and survival (data not shown). Thus, upregulation of Bcl6 positively participated in the IL-6-dependent myeloma cell proliferation and gave resistance to DNA damage.

Discussion

The overexpression of Bcl6 by chromosomal translocation has been reported in many studies, and the roles of Bcl6 in the oncogenesis of B-cell lymphomas are well established. In this study, we report that Bcl6 also functions through STAT3-induced variant 2, and this is important in IL-6-responsive myeloma cell proliferation and survival.

Previous reports have shown that the expression of Bcl6 protein and mRNA was high in B cells but not in pre-B cells nor in plasma cells, including myeloma cell lines, whereas we have detected IL-6-responsive expression of

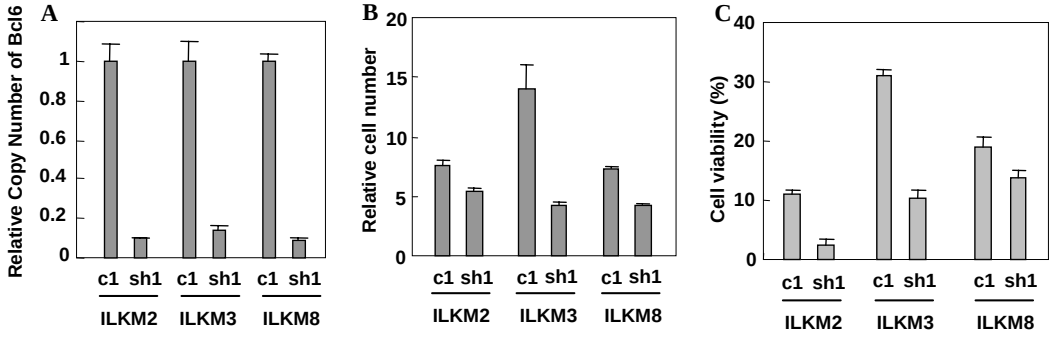

Fig. 5. Suppression of Bcl6 decreased the growth rate and increased the bleomycin sensitivity in IL-6-dependent myeloma cells. (A) Real-time RT-PCR of Bcl6 in shRNA introduced cells. ILKM2, 3, and 8 cells infected with shRNA expression vector coding control (c1) or Bcl6-targetting expression vector (sh1) were analyzed by real-time RT-PCR. Data are shown as means ± SE ($n = 3$). Suppression of Bcl6 mRNA was significant between c1 and sh1 in all cell lines ($P < 0.01$). (B) Growth of Bcl6-suppressed cells. Cells infected with shRNA expression vector were cultured with IL-6 for 4 days. Viable cell numbers were measured by flow cytometry at days 0 and 4. Data are shown relative to day 0. Presented data are representative of three similar experiments and are shown as means ± SE ($n = 3$). Growth differences were significant between c1 and sh1 in all cell lines ($P < 0.01$). (C) Bleomycin sensitivity of Bcl6-suppressed cells. Cells at four days after infection were cultured with 0, 20 μg/ml bleomycin for two days, and cell viability was measured by flow cytometry. Representative data of three similar experiments are shown as means ± SE ($n = 3$). Differences in bleomycin sensitivity were significant between c1 and sh1 in all cell lines ($P < 0.01$).

Bcl6 in malignant plasma cells from a myeloma patient as well as in IL-6-dependent myeloma cells. Such discrepancy is explainable from our previous data showing that the phenotypes of cells that proliferate in response to IL-6 were limited; specifically, only CD45 positive myeloma cells responded to IL-6 [25,26]. In addition, the level of IL-6Rα seems to be important, as plasmablast cells in the tonsil, which responded to IL-6 by proliferating, expressed IL-6Rα, whereas their precursor cells did not respond to IL-6 [32]. Actually, the IL-6-responsive myeloma cells used in this study expressed both CD45 and IL-6Rα at high levels, so they could express Bcl6 variant 2 in response to IL-6. The IL-6-inducible expression of Bcl6 was restricted to variant 2 in IL-6-dependent myeloma cells. Our data showed that some B cell lines stably expressed variant 1, whereas the variant 2 expression was relatively low (data not shown). The variant 2 expression was enhanced by IL-6 if the B cells were transduced with IL-6Rα. Thus, the mechanism of Bcl6 expression seemed to differ between B cells and myeloma cells; myeloma cells had obtained the ability to express Bcl6 when they also expressed IL-6Rα and CD45. Additionally it is less understood how Bcl6 is upregulated in the GC. Our data might shed light on the mechanism of Bcl6 upregulation in the GC B cells.

Most myeloma cells have genomic instability, such as chromosomal rearrangements, deletions, and amplifications that generate DNA damage signals, so that some mechanisms to escape from DNA damage checkpoint-induced growth arrest or apoptosis are required for proliferation. Cancer cells usually circumvent the checkpoint signals by inactivating pathways involving p53. Our data suggest that the IL-6-dependent myeloma cells might escape from DNA damage checkpoints by upregulation of Bcl6 through STAT3, resulting in downregulation of p53 mRNA. We have also reported that PDCD4 might be a candidate for Bcl6-mediated repression. In addition, our microarray data showed that $p27^{Kip1}$, a previously reported indirect Bcl6 target gene [20], was downregulated in ILKM2 cells, while in ILKM8 cells $p57^{Kip2}$ expression was downregulated instead. The downregulation of these pro-apoptotic genes and growth arrest genes might be also related to the anti-apoptic and growth promoting effect of IL-6-induced Bcl6.

The altered expression pattern of Bcl6 target genes caused by IL-6 in our cells was different from previous reports using B cell lines, and expression also differed between ILKM2 and ILKM8 cells. Shaffer et al. [20] showed that the expression of Bcl6 target genes varied among cell lines and in different experimental systems. It is possible that the target genes of Bcl6 might be determined by the amount of co-repressors present, some of which are expressed differentially in different cell types. For example, the combination of Bcl6 and MTA3 expression in a myeloma cell line, U266, induced plasma cell specific gene expression [15]. Indeed, our microarray data showed that ILKM2 and ILKM8 cells expressed both NCoR and BCoR but not MTA3, SMRT or ETO (data not shown).

In conclusion, our data indicate the possibility that IL-6-induced Bcl6 (STAT3-induced variant 2) protects IL-6-dependent myeloma cells against various pro-apoptotic signals and promotes cell proliferation, since both the IL-6-induced proliferation and the survival after DNA damage of myeloma cells were blocked by the Bcl6 shRNA. This is the first report revealing the physiological relevance of Bcl6 in myeloma cells. It is tempting to speculate that similar mechanisms to those involved in B-cell lymphoma support myeloma cell proliferation and survival. This would in turn imply that inhibition of Bcl6 function might be an effective therapy to eliminate the growing fraction of myeloma cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.09.036.

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