Cells

MM.1S, RPMI8226, and U266 cell lines were obtained from the ATCC (Manassas, VA) or kindly provided by sources and maintained as previously described.[8] INA6 cells were kindly provided from Dr Renate Burger (University of Kiel, Kiel, Germany). BM specimens were obtained from patients with MM, and mononuclear cells (MNCs) separated by Ficoll-Hipaque density sedimentation. Primary $CD138^+$ tumor cells from MM patients were obtained using negative selection, as previously described [8] after IRB-approved (Dana-Farber Cancer Institute) informed consent and in accordance with the Declaration of Helsinki protocol. BMMNCs were used to establish long-term BMSC cultures, as previously described.[8]

Reagents

IKKβ inhibitor MLN120B [9] was provided by Millennium Pharmaceuticals (Cambridge, MA), dissolved in DMSO, stored at −20°C, and diluted in culture medium immediately before use; control media contained < 0.1% DMSO. Human interleukin (IL)-6, neutralizing anti–IL-6 Ab, oncostatin M (OSM), and tumor necrosis factor (TNF)α were purchased from R&D Systems (Minneapolis, MN). AG490 and U0126 were obtained from Calbiochem (San Diego, CA) and Cell Signaling Technology (Danvers, MA), respectively.

Cell growth assay

MM cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye absorbance, as previously described.[10] All experiments were performed 3 times in quadruplicate. Data represent mean ± SD.

Immunoblotting and immunoprecipitation

Protein lysates were obtained by cell lysis in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholat, 0.1% SDS, 5 mM EDTA, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 5 µg/ml leupeptin, and 5 µg/ml aprotinin). Whole cell lysates were immunoblotted with anti-Bcl6, -ERK1/2, -p53, –β-actin, –α-tubulin, -GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); as well as anti-phospho (p)-STAT3 (Tyr705), -STAT3, –p-Akt (Ser473), –p-ERK (Thr202/Tyr204), –p-JNK (Thr183/Tyr185), –p-p38MAPK (Thr180/Tyr182), and -p38MAPK (Cell Signaling Technology) Abs. Anti-gp130 and –p-tyrosine Abs were purchased from Upstate Biotechnology (Lake Placid, NY), and immunoprecipitation was carried out as described previously.[11]

Immunohistochemistry

Immunohistochemistry was performed on bone marrow from healthy volunteers and patients with MM, as described previously.[12] Anti-human Bcl6 Ab (catalog number 9479) was obtained from Abcam (Cambridge, MA). Anti-CD138 Ab was obtained from BD Biosciences (San Jose, CA). The primary Abs were visualized with the corresponding biotinylated Ab coupled to streptavidin-peroxidase complex (Vector Laboratories, Burlingame, CA). All Abs, conditions, and reactivity were tested with positive control slides. Histological micrographs were taken using a Leica DM200 microscope (aperture HC PLANs 10X/22, objective lenses: N PLAN 100X/1.25 oil) and a SPOT Insight QE Model camera with SPOT Advanced acquisition software (Diagnostic Instruments, Sterling Heights, MI).

Real-time quantitative PCR

BCL6 gene expression was examined by real-time PCR and normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as previously described.[13] Briefly, total RNA was extracted from untreated or IL-6 (5 ng/ml)-treated INA6 cells using RNeasy Kit and RNase-Free DNase Set (Qiagen, Valencia, CA). cDNA was synthesized using first strand cDNA synthesis Kit (SuperArray Bioscience, Frederick, MD). Quantitative real-time polymerase chain reaction (PCR) was carried out in a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). cDNAs were amplified in 25 µl reactions containing SYBR® Green PCR Master Mix (SuperArray Bioscience). Amplification was done at 95°C for 10 min, followed by 95 °C for 15 sec and 60°C for 1 min, for a total of 40 cycles. Gene-specific oligonucleotide primers including BCL6 (forward 5′-CCGCTCTTGCCAAATGCTTTG-3′ and reverse 5′-CACGATACTTCAT-CTCATCTGG-3′) and GAPDH (forward 5′-AATCCCATCACCATCTTCCA-3′ and reverse 5′-TGGACTCCACGACGTACTCA-3′) were purchased from SuperArray Bioscience Corp. (Frederick, MD). Data represent mean ± SD of triplicate wells.

Detection of cytokines in BMSC culture supernatants (SCCS)

Seventy-nine cytokines produced by BMSCs were detected by "RayBio® Human Cytokine Antibody Array V" (RayBiotech, Inc. Norcross, GA) according to manufacturer's instructions.

Bcl-6 knockdown

Lentiviral shRNA were used to knockdown Bcl6 expression in MM cells. Scrambled and bcl6 pLKO shRNA vectors were provided by Dr. William Hahn (Dana-Farber Cancer Institute). Recombinant lentivirus was produced by transient infection of 293T cells following a standard protocol, as described previously.[8] After 24h, MM.1S and U266 cells were incubated with culture supernatants from 293T cells containing crude virus for 6h, and then washed with media. A second infection was repeated on the next day. After 48h culture of MM.1S cells with SCCS, cell proliferation and immunoblotting analyses were performed. U266 cells were not cultured with SCCS, since they express constitutive Bcl-6. STAT3 siRNA (Thermo Scientific Dharmacon, Lafayette, CO) was used for STAT3 knockdown in U266 cells.

Statistical analysis

Statistical significance of differences observed in drug-treated versus control cultures was determined using the Wilcoxon signed-ranks test. The minimal level of significance was $p < 0.05$.