

IL-6 Triggers Cell Growth via the Ras-Dependent Mitogen-Activated Protein Kinase Cascade¹

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IL-6 mediates growth of some human multiple myeloma (MM) cells and IL-6-dependent cell lines. Although three IL-6 signaling pathways (STAT1, STAT3, and Ras-dependent MAPK cascade) have been reported, cascades mediating IL-6-triggered growth of MM cells and cell lines are not defined. In this study, we therefore characterized IL-6 signaling cascades in MM cell lines, MM patient cells, and IL-6-dependent B9 cells to determine which pathway mediates IL-6-dependent growth. IL-6 induced phosphorylation of JAK kinases and gp130, regardless of the proliferative response of MM cells to this growth factor. Accordingly, we next examined downstream IL-6 signaling via the STAT3, STAT1, and Ras-dependent mitogen-activated protein kinase (MAPK) cascades. IL-6 triggered phosphorylation of STAT1 and/or STAT3 in MM cells independent of their proliferative response to IL-6. In contrast, IL-6 induced phosphorylation of Shc and its association with Sos1, as well as phosphorylation of MAPK, only in MM cells and B9 cells that proliferated in response to IL-6. Moreover, MAPK antisense, but not sense, oligonucleotide inhibited IL-6-induced proliferation of these cells. These data suggest that STAT1 and/or STAT3 activation may occur independently of the proliferative response to IL-6, and that activation of the MAPK cascade is an important distal pathway for IL-6-mediated growth. *The Journal of Immunology*, 1997, 159: 2212–2221.

Interleukin-6 is a multifunctional cytokine that induces B cell differentiation, as well as proliferation of human multiple myeloma (MM)³ cells and IL-6-dependent cell lines. Specifically, it is an autocrine and paracrine growth factor for some human MM cells (1, 2). Kawano and colleagues initially postulated an autocrine growth mechanism, since MM cells secrete and specifically proliferate to IL-6 in vitro (1). More recently, triggering of MM cells via cell surface CD40 has induced IL-6-mediated autocrine MM cell growth (3, 4). On the other hand, IL-6-mediated paracrine MM cell growth is supported by the observations that bone marrow stromal cells (BMSC) are the major source of IL-6 in MM (2, 5, 6), that freshly isolated MM cells cultured without exogenous IL-6 rapidly stop proliferating (7), and that adhesion of MM cells to BMSC up-regulates IL-6 secretion by BMSC (8, 9). Most recently, IL-6 has also been demonstrated to inhibit MM cell apoptosis triggered by corticosteroids, serum starvation, and anti-Fas (10–12).

IL-6 specifically binds to a cell surface receptor consisting of two subunits, the ligand-binding gp80 IL-6R and the signal-transducing gp130 components (13). Binding of IL-6 to IL-6R induces

homodimerization of gp130 and activation of the JAK (Janus kinase) family of tyrosine kinases, JAK1, JAK2, and/or Tyk2 (14–18); activated JAK family kinases phosphorylate gp130 (17). Following activation of these tyrosine kinases, three downstream pathways have been reported (19, 20). First, the phosphorylated gp130 binds to STAT3, which is phosphorylated by JAK family kinases; homodimers of phosphorylated STAT3 rapidly migrate to the nucleus and bind to IL-6 response elements on the promoter of IL-6-induced genes (18, 21–26). Second, IL-6 phosphorylates STAT1, and heterodimer of tyrosine-phosphorylated STAT1 and STAT3 binds the nuclear DNA sequence termed GAS (IFN- γ -activated sequence) or SIE (*sis*-inducible element) (16, 18, 27–30). Finally, IL-6 can also activate the Ras-dependent mitogen-activated protein kinase (MAPK) cascade, with sequential activation of Shc (Src homology 2/ α -collagen related), Grb2, Son of sevenless 1 (Sos1), Ras, Raf, MEK, and MAPK; this cascade ultimately leads to activation of transcription factors NF-IL-6 or AP-1 complex (Jun/Fos) (31–36). Recent reports have demonstrated that STAT3 activation is a critical step in differentiation and growth arrest of the M1 murine myeloid leukemia cell line (37, 38), and that STAT3 also mediates inhibition of apoptosis (39). However, which of these cascades mediates IL-6-dependent MM or cell line growth has not been delineated.

In the present study, we examined activation of IL-6 signaling cascades in patient MM cells, MM-derived cell lines, and IL-6-dependent B9 cells. Activation of STAT1 and/or STAT3 in response to IL-6 was demonstrated in cells that do not proliferate in response to IL-6, as well as in IL-6-dependent B9 cells. In contrast, IL-6 triggered activation of the Ras-dependent MAPK cascade, evidenced by phosphorylation of Shc, coimmunoprecipitation of phosphorylated Shc with Sos1, and phosphorylation of Erk2 (MAPK) was observed only in B9 cells and MM2 patient cells, both of which proliferate in response to IL-6. Moreover, MAPK antisense (but not sense) oligonucleotide (ODN), which specifically abrogates MAPK protein expression and kinase activity, inhibited proliferation of B9 cells and MM patient cells triggered by IL-6. In contrast, neither p38 nor JNK1 kinase activities in B9 cells

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³ Abbreviations used in this paper: MM, multiple myeloma; BMSC, bone marrow stromal cell; Erk2, extracellular signal-related kinase 2; Grb2, growth factor receptor-bound protein 2; GST, glutathione S-transferase; JAK, Janus kinase; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, threonine-tyrosine kinase activator of MAPK or Erk2; ODN, oligonucleotide; Shc, Src homology 2/ α -collagen related; SI, stimulation index; Sos1, son of sevenless 1.

were altered by treatment with IL-6 or MAPK antisense ODN. These data suggest that IL-6-triggered proliferation of MM cells and B9 cells can be mediated via the MAPK cascade.

Materials and Methods

MM cells and MM-derived cell lines

MM cells (99% CD38⁺CD45RA⁺) were obtained from the peripheral blood of a patient with plasma cell leukemia (MM1), and from a malignant pleural effusion in a patient with multiple myeloma (MM2). Mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation, washed, and resuspended in RPMI 1640 media (Mediatech, Washington, DC) containing 10% FBS (PAA Laboratories, Newport Beach, CA), L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Hs Sultan (40), RPMI 8226 (41), ARH77 (42), IM9 (43), and U266 (44) human MM-derived cell lines were obtained from American Type Culture Collection (Rockville, MD). The U1958 cell line (45) was kindly provided by Dr. Kenneth Nilsson (Uppsala University, Uppsala, Sweden). Cells were cultured in RPMI 1640 media containing 10% FBS, L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. The OCI-My5 human MM cell line (46) (kindly provided by Dr. H. A. Messner, Ontario Cancer Institute, Toronto, Canada) was maintained in Iscove's modified Dulbecco's medium containing 10% FBS, L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. The B9 murine IL-6-dependent hybridoma/plasmacytoma cell line (47) (a kind gift of Dr. Lucien Aarden, The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) was maintained in RPMI 1640 media supplemented with 10% FBS, 50 μ M 2-ME (Sigma Chemical Co., St. Louis, MO), L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 1 ng/ml rIL-6 (Genetics Institute, Cambridge, MA).

Reagents

The anti-gp130, JAK1, Tyk2, Sos1, and anti-phosphotyrosine 4G10 Abs were obtained from Upstate Biotechnology (Lake Placid, NY). The anti-JAK2, Shc, Erk1, and Erk2 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-STAT3 Ab (22) was kindly provided by Dr. Shizuo Akira (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan).

Assays of DNA synthesis

DNA synthesis of MM cells stimulated by IL-6 was measured by tritiated thymidine (³H]TdR) incorporation. Briefly, 1×10^4 cells in 200 μ l of RPMI 1640 media without FBS were cultured for 48 h in 96-well culture plates in the presence or absence of 100 ng/ml of IL-6. Cells were labeled with 1 μ Ci/well of [³H]TdR (sp. act., 603.1 GBq/mmol) (DuPont, Wilmington, DE) during the last 6 h of culture, harvested onto glass filters with the aid of the HARVESTAR 96 MACH II (Tomtec, Orange, CT), and counted on a 1205 BETAPLATE (Wallac, Finland). Proliferation was defined by the stimulation index (SI): [³H]TdR uptake of sample in media plus IL-6/[³H]TdR uptake of control sample in media alone.

Immunoprecipitation and immunoblotting

For immunoprecipitation experiments, 2×10^7 cells were cultured for 1 h in the absence of serum and growth factors. After 1-h incubation with 1 mM sodium *o*-vanadate (Sigma Chemical Co.), cells were stimulated with 100 ng/ml of IL-6 for 30 min at 37°C. Cells were then washed twice with ice-cold Tris-buffered saline containing 1 mM of sodium orthovanadate and resuspended for 30 min at 4°C in 1 ml of lysis buffer: 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 2 mM sodium *o*-vanadate (Na₃VO₄), 1 mM PMSF, 5 μ g aprotinin/ml, and 1 mM NaF. Cell lysates were centrifuged (10,000 $\times$ g at 4°C) and then precleared for 1 h with protein A Sepharose CL-4B or protein G Sepharose 4FF (Pharmacia, Uppsala, Sweden). The precleared supernatant was then immunoprecipitated overnight at 4°C with specific Abs and pelleted by protein A Sepharose CL-4B or protein G Sepharose 4FF. The immunoprecipitates were washed with lysis buffer three times and then boiled for 5 min, using a modified Laemmli's method, in loading buffer: 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, and 0.001% Bromophenol Blue. The supernatants were analyzed by SDS-PAGE and subsequent immunoblotting with 4G10 anti-phosphotyrosine mAb using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

ODN treatment of cells

Antisense strategy for depleting MAPK was described previously (48). Antisense ODN with the sequence 5'-GCC GCC GCC GCC AT-3',

Table I. IL-6 triggering proliferation of MM patient cells as well as MM-derived cell lines^a

	IL-6 (-) cpm	IL-6 (+) cpm	SI ^b
U1958	18,189 $\pm$ 1,763	17,621 $\pm$ 583	1.0
Hs Sultan	6,394 $\pm$ 621	6,281 $\pm$ 425	1.0
RPMI8226	11,684 $\pm$ 1,808	14,182 $\pm$ 1,460	1.1
B9	5,252 $\pm$ 1,009	36,158 $\pm$ 1,665	6.9
ARH77	2,438 $\pm$ 321	2,589 $\pm$ 183	1.1
IM9	9,382 $\pm$ 628	10,321 $\pm$ 1,235	1.1
OCI-My5	29,121 $\pm$ 457	35,820 $\pm$ 1,148	1.2
U266	9,798 $\pm$ 140	13,512 $\pm$ 741	1.4
MM1	2,831 $\pm$ 432	3,692 $\pm$ 526	1.3
MM2	2,154 $\pm$ 521	7,114 $\pm$ 724	3.3

^a Cells (1×10^5) were cultured for 72 h in the presence or absence of IL-6. Cells were labeled with 1 μ Ci/well of [³H]TdR during the last 12 h of culture, harvested onto glass filters, and then counted by beta counter.

^b SI: [³H]TdR uptake of sample in media plus IL-6/[³H]TdR uptake of control sample in media alone.

and sense ODN with the sequence 5'-ATG GCG GCG GCG GC-3' were synthesized on an automated DNA synthesizer (DFCI Molecular Biology Core Facilities, Boston, MA). Two hundred microliters of either sense or antisense ODN (1 μ M) were added to RPMI 1640 (170 μ l) and Lipofectin solution (30 μ l) (Life Technologies, Grand Island, NY), and incubated at room temperature for 15 min. This mixture was added to the cells for 5 h at 37°C in the presence of 5% CO₂. Then, cells were washed and suspended in media with ODN, but without Lipofectin solution, and ODN replenished every 24 h. Total cell lysates of B9 and MM2 cells after 24-h incubation were analyzed for Erk2 expression using anti-Erk2 Ab (Transduction Laboratories, Lexington, KY). For proliferation assays, 1×10^4 cells/200 μ l were cultured with serial doses of IL-6 for 48 h. Cells were pulsed during the last 12 h of 48-h cultures with 1 μ Ci of [³H]TdR/well, harvested onto glass filters, and counted on a scintillation counter.

Immune complex kinase assays

In vitro kinase assays were performed as previously described (49). Lysates were precleared by incubating with 5 mg/ml of rabbit anti-mouse IgG for 1 h at 4°C, and then for an additional 30-min incubation with protein A Sepharose CL-4B. The supernatants were incubated with preimmune rabbit serum or specific Abs for MAPK, p38, or JNK1 kinase for 2 h at 4°C before the addition of protein A Sepharose CL-4B. The immune complexes were washed three times with lysis buffer and once with kinase buffer, and resuspended in kinase buffer containing [γ -³²P]ATP (3000 Ci/mmol; DuPont NEN, Boston, MA) and myelin basic protein, GST-activated transcription factor 2 (provided by Dr. Roger Davis, University of Massachusetts Medical School, Worcester, MA), or GST-Jun (2-100), as substrates. The reaction was incubated for 15 min at 30°C and terminated by the addition of SDS sample buffer. The phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography.

Results

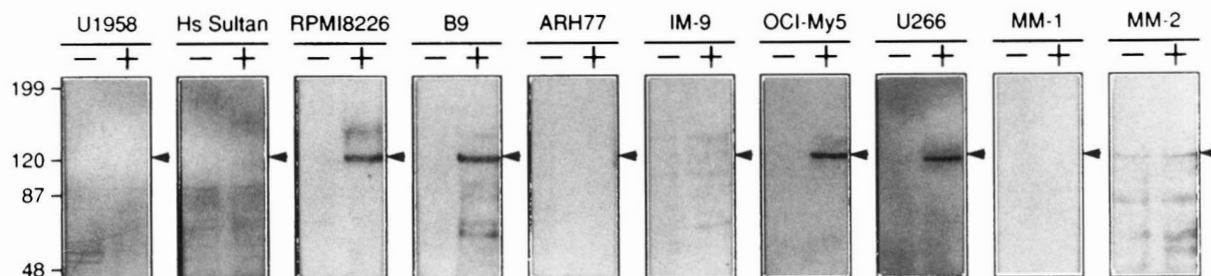
Effects of IL-6 on proliferation of patient MM cells, as well as MM-derived cell lines

The effect of IL-6 on DNA synthesis by MM patient cells, as well as MM-derived cell lines, was analyzed by [³H]TdR uptake. As shown in Table I, B9 IL-6-dependent cell line showed significant increases in proliferation (SI 6.9, $p < 0.005$) in response to IL-6 (100 ng/ml). MM2 patient cells similarly proliferated to IL-6 (SI 3.3, $p < 0.005$). No significant change in DNA synthesis of U1958, Hs Sultan, RPMI 8226, ARH77, IM9, OCI-My5, U266, or MM1 patient cells was noted in response to IL-6.

Effects of IL-6 on tyrosine phosphorylation of JAK family (JAK1, JAK2, and Tyk2) protein kinases

To determine whether IL-6 induces tyrosine phosphorylation of JAK family kinases, MM patient cells, as well as MM-derived cell lines, were cultured in the presence or absence of IL-6 for 30 min. Cell lysates were immunoprecipitated with Abs to JAK1, JAK2,

A) Phosphotyrosine Blot



B) JAK1 Blot

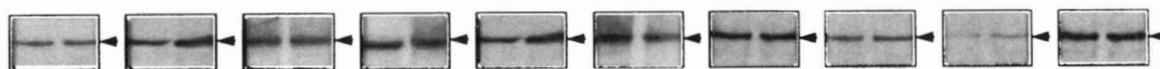
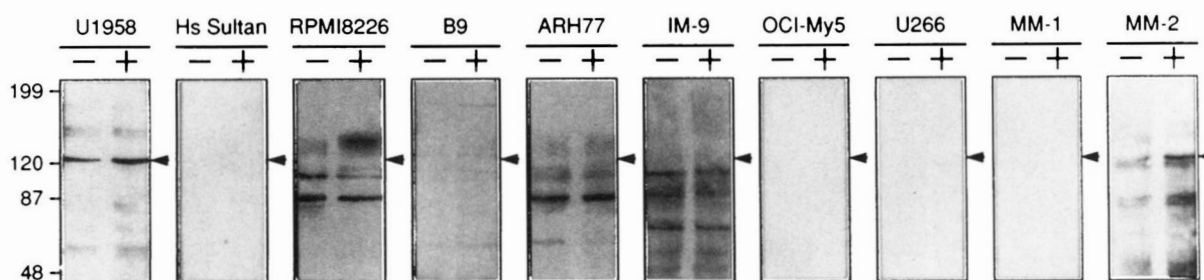


FIGURE 1. Effects of IL-6 on JAK1 phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-JAK1 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and phosphorylation of JAK1 was detected by anti-phosphotyrosine Ab. *B*, The blot was stripped and reprobed with anti-JAK1 Ab.

A) Phosphotyrosine Blot



B) JAK2 Blot

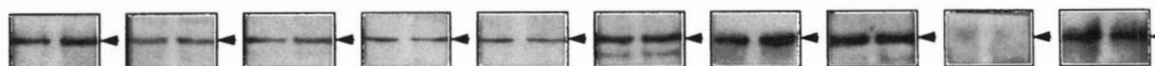


FIGURE 2. Effects of IL-6 on JAK2 phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-JAK2 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and phosphorylation of JAK2 was detected by anti-phosphotyrosine Ab. *B*, The blot was stripped and reprobed with anti-JAK2 Ab.

and Tyk2, and tyrosine-phosphorylated proteins were detected using the 4G10 anti-phosphotyrosine mAb (Figs. 1–3). JAK1 protein was present in all cell lines studied (Fig. 1*B*). JAK2 (Fig. 2*B*) and Tyk2 (Fig. 3*B*) proteins were present in all cell lines studied, except for MM1 patient cells. IL-6 up-regulated phosphorylation of JAK1 in RPMI 8226, B9, OCI-My5, and U266 cells (Fig. 1*A*). JAK2 phosphorylation was stimulated by IL-6 in MM2 patient cells (Fig. 2*A*). Finally, IL-6 triggered phosphorylation of Tyk2 in RPMI 8226 and B9 cells (Fig. 3*A*). In IM9 cell lines, JAK1 and Tyk2 were also phosphorylated slightly by IL-6. In these experiments, reprobing the blots with anti-JAK1, anti-JAK2, and anti-Tyk2 Abs, respectively, showed equal amounts of protein in both IL-6-treated and nontreated cells of each type studied (Fig. 1*B*, 2*B*, and 3*B*).

Effects of IL-6 on tyrosine phosphorylation of gp130

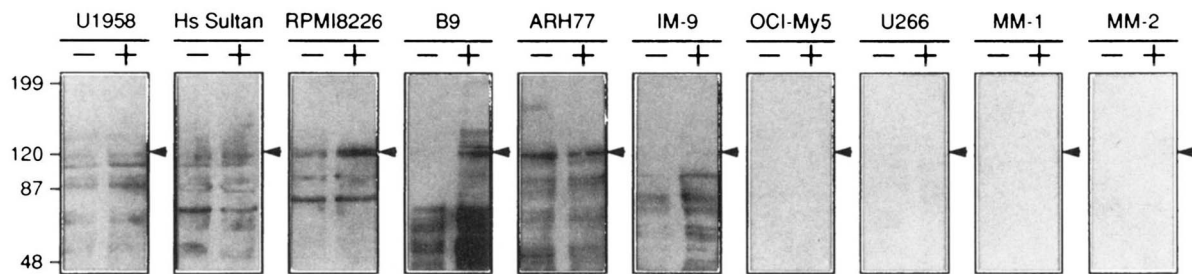
gp130 is phosphorylated by activated JAK family kinases; we therefore next examined whether gp130 phosphorylation was triggered by IL-6. gp130 protein was present in all cell lines studied

(Fig. 4*B*). Tyrosine phosphorylation of gp130 was markedly up-regulated by IL-6 in RPMI 8226, B9, OCI-My5, U266, and IM9 cells (Fig. 4*A*), in which at least one of the JAK family tyrosine kinases was also phosphorylated in response to IL-6. Although IL-6 induced phosphorylation of JAK2 in MM2 patient cells, it did not trigger phosphorylation of gp130. Finally, in Hs Sultan, ARH77, and MM1 patient cells, IL-6 did not trigger phosphorylation of either JAK kinases or gp130 (Fig. 4*A*). Reprobing of the blot with anti-gp130 confirmed equivalent protein amounts in both IL-6-treated and nontreated cells of each type (Fig. 4*B*).

Effects of IL-6 on the tyrosine phosphorylation of STAT1 and STAT3

We next assayed the phosphorylation state of STAT1 and STAT3 kinases in control and IL-6-treated MM cells. As shown in Figure 5*B*, STAT1 protein was present in all cell lines studied. IL-6 triggered phosphorylation of both STAT1 proteins (p91 and p84) in RPMI 8226, OCI-My5, U266, and IM9 MM cells (Fig. 5*A*). p84

A) Phosphotyrosine Blot



B) Tyk2 Blot

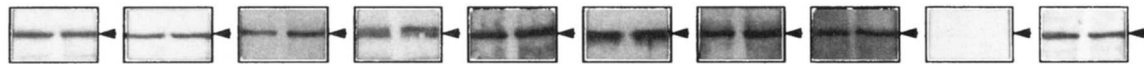
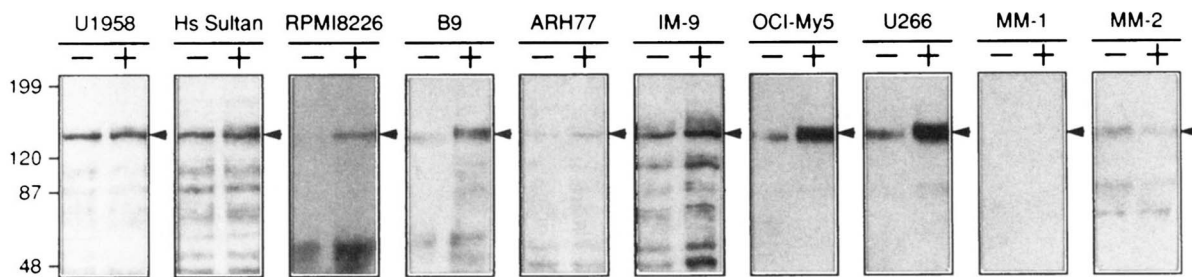


FIGURE 3. Effects of IL-6 on Tyk2 phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-Tyk2 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and phosphorylation of Tyk2 was detected by anti-phosphotyrosine Ab. *B*, The blot was stripped and reprobed with anti-Tyk2 Ab.

A) Phosphotyrosine Blot



B) gp130 Blot

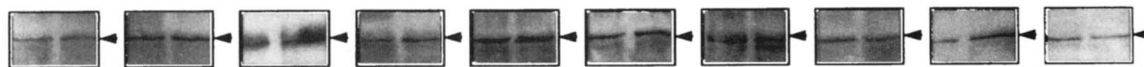


FIGURE 4. Effects of IL-6 on gp130 phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-gp130 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and phosphorylation of gp130 was detected by anti-phosphotyrosine Ab. *B*, The blot was stripped and reprobed with anti-gp130 Ab.

phosphorylation was weaker than that of p91 in RPMI 8226, OCI-My5, and U266 cells. Stripping and reprobing of the blot with anti-STAT1 Ab demonstrated equal protein amounts in both IL-6-treated and nontreated cells of each cell type (Fig. 5*B*).

IL-6 induced phosphorylation of STAT3 in RPMI 8226, B9, OCI-My5, and U266 MM cell lines (Fig. 6*A*). Two phosphorylated bands were observed on the immunoblots of B9 and OCI-My5 cells, both of which reacted with anti-STAT3 Ab. Cytokines that activate gp130 can induce two distinct forms of STAT3 (p88 STAT3f and p90 STAT3s) (50), and the two phosphorylated STAT3 bands induced by IL-6 in B9 and OCI-My5 cell may represent STAT3s and STAT3f. Reprobing of the blot with anti-STAT3 Ab showed equal protein amounts in both IL-6-treated and nontreated cells of each cell type (Fig. 6*B*).

Effects of IL-6 on the tyrosine phosphorylation of Shc, association of phosphorylated Shc with Sos1, and tyrosine phosphorylation of MAPK

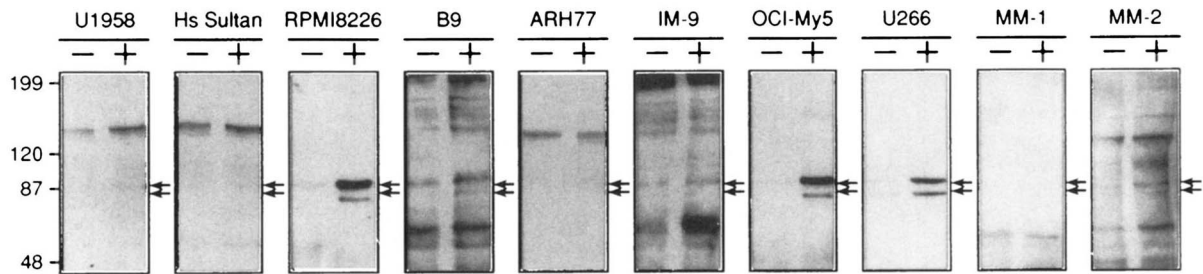
To determine whether Ras-dependent MAPK cascade is activated by IL-6 stimulation, we probed for activation of three proteins

(Shc, Sos1, and MAPK) in this pathway. Characteristic Shc bands (p52 and p46) were intrinsically phosphorylated in U1958, Hs Sultan, ARH77, OCI-My5, and U266 cells, as well as MM1 and MM2 patient cells (Fig. 7*A*). IL-6 triggered phosphorylation of p52 in B9, IM9, and MM2 patient cells. Reprobing of the blot with anti-Shc Ab showed equal protein amounts in both IL-6-treated and nontreated cells of each type (Fig. 7*B*).

In this cascade, Sos1 associates with the adapter Grb2 protein, and the Grb2-Sos1 complex then associates with phosphorylated Shc (51). To determine whether Sos1 is associated with phosphorylated Shc, cell lysates were immunoprecipitated with anti-Sos1 Ab and then probed with anti-Shc Ab. Up-regulation of the association of Sos1 and Shc was triggered by IL-6 only in B9 and MM2 patient cells (Fig. 8*A*). Sos1 and Shc coimmunoprecipitation were not observed in IM9 cells, in spite of Shc phosphorylation, suggesting a blockade in signaling between Shc and Sos1. Sos1 protein was present in all cell lines studied, except for U266 and MM1 patient cells (Fig. 8*B*).

To further confirm MAPK cascade activation, we probed for tyrosine phosphorylation of MAPK (Erk1 and Erk2), which is

A) Phosphotyrosine Blot



B) stat1 Blot

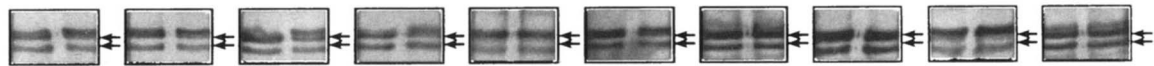
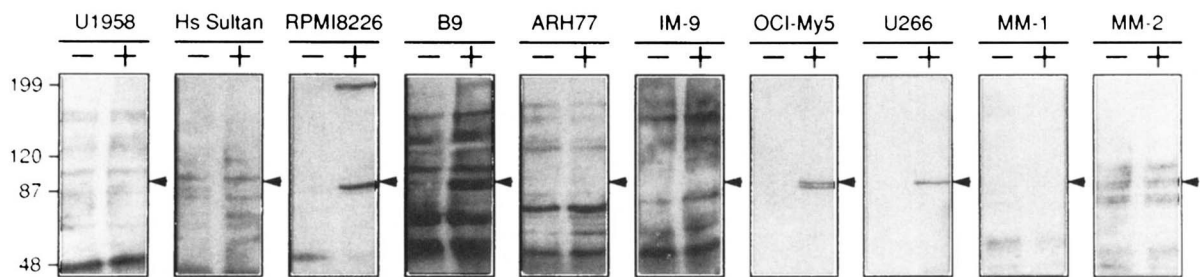


FIGURE 5. Effects of IL-6 on STAT1 phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-STAT1 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and phosphorylation of STAT1 was detected by anti-phosphotyrosine Ab. *B*, The blot was stripped and reprobed with anti-STAT1 Ab.

A) Phosphotyrosine Blot



B) stat3 Blot

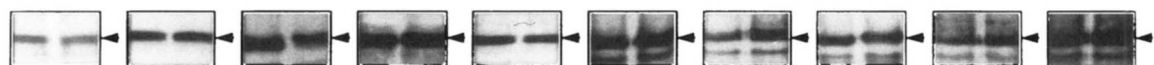


FIGURE 6. Effects of IL-6 on STAT3 phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-STAT3 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and phosphorylation of STAT3 was detected by anti-phosphotyrosine Ab. *B*, The blot was stripped and reprobed with anti-STAT3 Ab.

phosphorylated on threonine and tyrosine residues following mitogen stimulation (32, 52). Cell lysates were immunoprecipitated with Ab to Erk2, which cross-reacts with Erk1, and tyrosine-phosphorylated proteins were detected using the 4G10 anti-phosphotyrosine mAb. Two bands were immunoprecipitated: Erk1 (p44) and Erk2 (p42). Up-regulation of tyrosine phosphorylation of Erk2 induced by IL-6 was observed only in B9 and MM2 patient cells (Fig. 9A), confirming that the MAPK cascade was fully activated in IL-6-responsive B9 cells and MM2 patient cells (Table II). Reprobing of the blot with anti-Erk2 Ab showed equal protein amounts in both IL-6-treated and nontreated cells of each type (Fig. 9B). Similar experiments utilizing Erk1-specific Ab demonstrated that IL-6 treatment of B9 cells does not induce tyrosine phosphorylation of Erk1 (data not shown).

Effects of MAPK sense and antisense ODN on IL-6-induced proliferation of B9 and patient MM2 cells

To further confirm the importance of the Ras-dependent MAPK cascade in IL-6-related proliferation, we examined the effect of

MAPK sense or antisense ODN on IL-6-triggered proliferation of B9 and patient MM2 cells. B9 and patient MM2 cells were cultured in media alone, as well as with MAPK sense or antisense ODN; DNA synthesis triggered by IL-6 was measured using [³H]TdR incorporation. IL-6 (100 ng/ml) induced a fivefold and fourfold increase in proliferation of B9 cells (Fig. 10A) and patient MM2 cells (Fig. 10B), respectively, compared with cells cultured either in media or with MAPK sense ODN. In contrast, IL-6 did not induce significant increases in proliferation of B9 cells or patient MM2 cells cultured with MAPK antisense ODN. MAPK antisense ODN, but not sense ODN, inhibited Erk2 protein expression in B9 cells (Fig. 10C) and patient MM2 cells (Fig. 10D) after 48-h incubation. In contrast, STAT1 and STAT3 expression were not changed by culturing with MAPK antisense ODN.

Effect of MAPK antisense ODN on p38 and JNK1 protein expression and kinase activities in B9 cells

Given the potential for interaction of proteins within the MAPK family, we next examined the kinase activities of p38, JNK, and

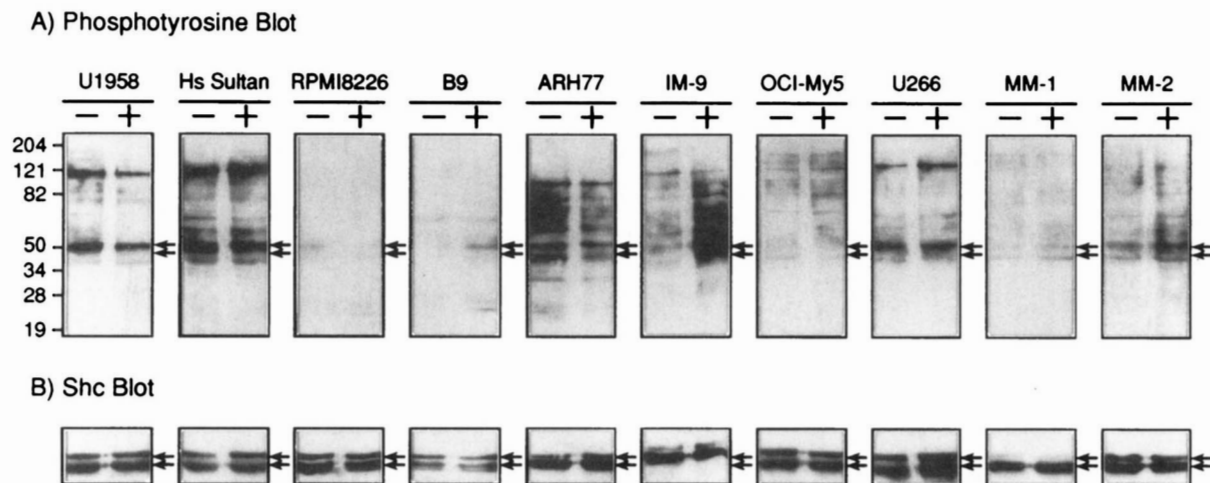


FIGURE 7. Effects of IL-6 on Shc phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM-derived cell lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-Sos1 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and Shc coimmunoprecipitation with Sos1 was detected by anti-Shc Ab. *B*, The blot was stripped and reprobed with anti-Sos1 Ab.

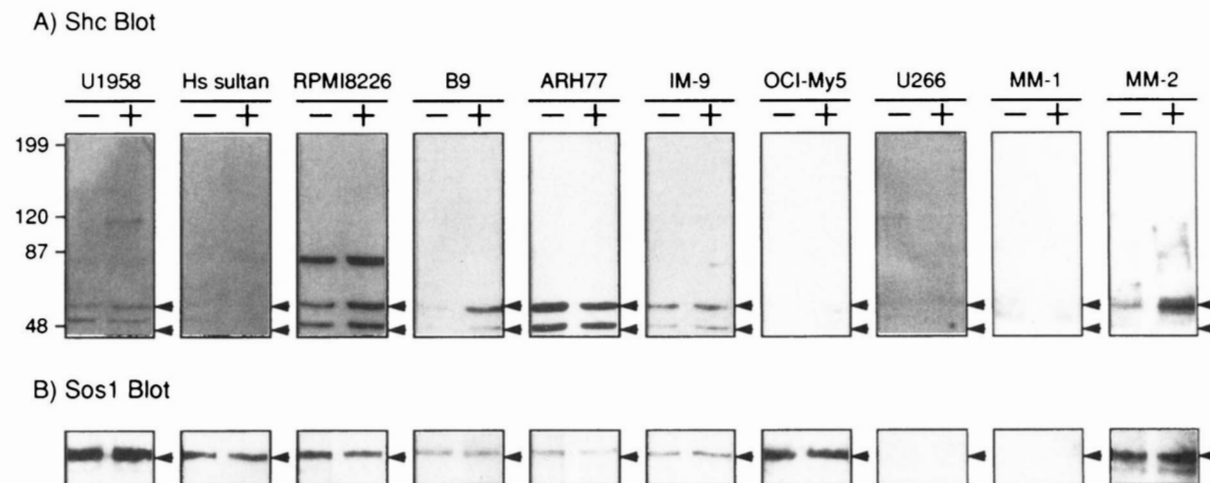


FIGURE 8. Effects of IL-6 on association of Shc and Sos1 in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-Sos1 Ab. *A*, The precipitates were resolved by 5% SDS-PAGE, and Shc coimmunoprecipitation with Sos1 was detected by anti-Shc Ab. *B*, The blot was stripped and reprobed with anti-Sos1 Ab.

MAPK in IL-6-responsive B9 cells. As seen in Figure 11A (*lower panel*), MAPK kinase activity was increased in B9 cells cultured with IL-6. However, IL-6 did not trigger increased p38 or JNK kinase activities in B9 cells (Fig. 11A, *upper and middle panels*). Moreover, treatment with MAPK antisense ODN did not alter either p38 and JNK1 kinase activities (Fig. 11B) or protein expression (Fig. 11C) in B9 cells.

Discussion

In this study, we characterized IL-6 signaling pathways mediating cell growth in patient MM cells and MM-derived cell lines that both proliferated to IL-6 and those that did not, as well as B9 IL-6-dependent cells. We harvested cells at 30 min after IL-6 treatment, since our prior studies demonstrated activation of gp130, PTP1D, Shc, and Sos in B9 cells under these conditions (53). Although IL-6 triggered phosphorylation of JAK family kinases, gp130, STAT1, and/or STAT3 in both MM cells that proliferated

to IL-6 and those that did not, IL-6 activated the MAPK cascade only in B9 cells and MM2 patient cells that proliferated to IL-6 (Table II). Moreover, proliferation of B9 cells and MM2 patient cells in response to IL-6 was inhibited by MAPK antisense (but not sense) ODN. These studies suggest that IL-6 can trigger growth of cells via the Ras-dependent MAPK cascade.

IL-6 initially triggers phosphorylation of JAK family kinases (JAK1, JAK2, Tyk2) that bind to box1 region of gp130; activated JAK family kinases then phosphorylate gp130 (19). Previous studies have shown that IL-6 induces phosphorylation of JAK1 in a wide spectrum of cell types: BAFm130 gp130-transfected murine proB cells; ES mouse embryonic stem cells; M1 mouse myeloid leukemia cells; EW-1 Ewing tumor cells; U266 human MM cells; B9E IL-11-dependent derivatives of B9 cells; ANBL6 IL-6-dependent human MM cells; OCI-My4 IL-6-responsive human MM cells; HT1080 human fibrosarcoma cells; HepG2 hepatoma cells; and AF10 IL-6-dependent derivative of U266 cells (14–18, 28,

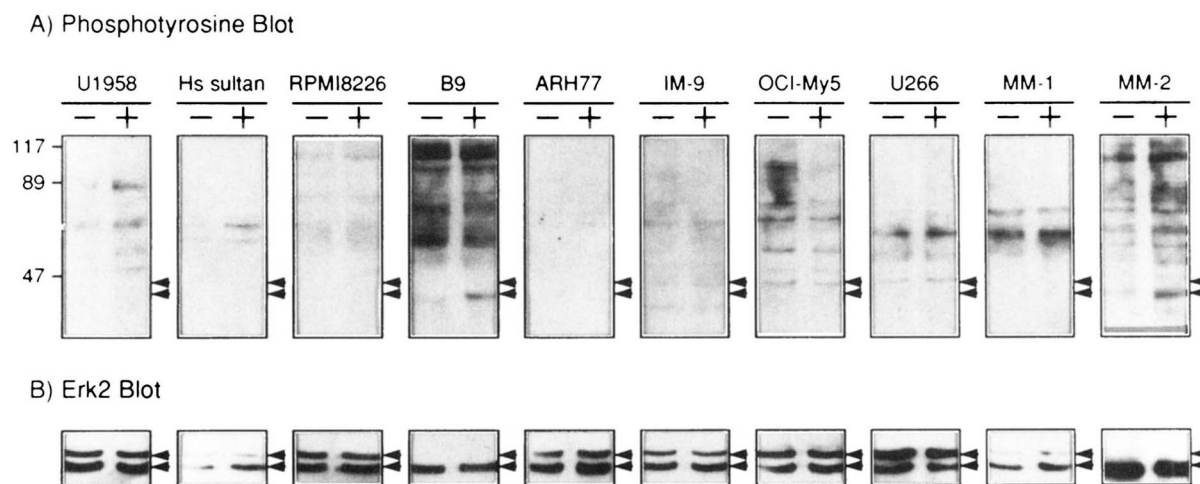


FIGURE 9. Effects of IL-6 on MAPK phosphorylation in MM patient cells, as well as MM-derived cell lines. Patient MM cells, as well as MM cell-derived lines, were cultured either in media alone (–) or with (+) 100 ng/ml of IL-6 for 30 min. Cell lysates from control and IL-6-treated cells were immunoprecipitated with anti-Erk2 Ab. Both Erk1 (p44) and Erk2 (p42) were immunoprecipitated by anti-Erk2 Ab. A, The precipitates were resolved by 10% SDS-PAGE, and phosphorylation of MAPK was detected by anti-phosphotyrosine Ab. B, The blot was stripped and reprobed with anti-Erk2 Ab.

Table II. Activation of IL-6 signaling pathways in MM patient cells as well as MM-derived cell lines

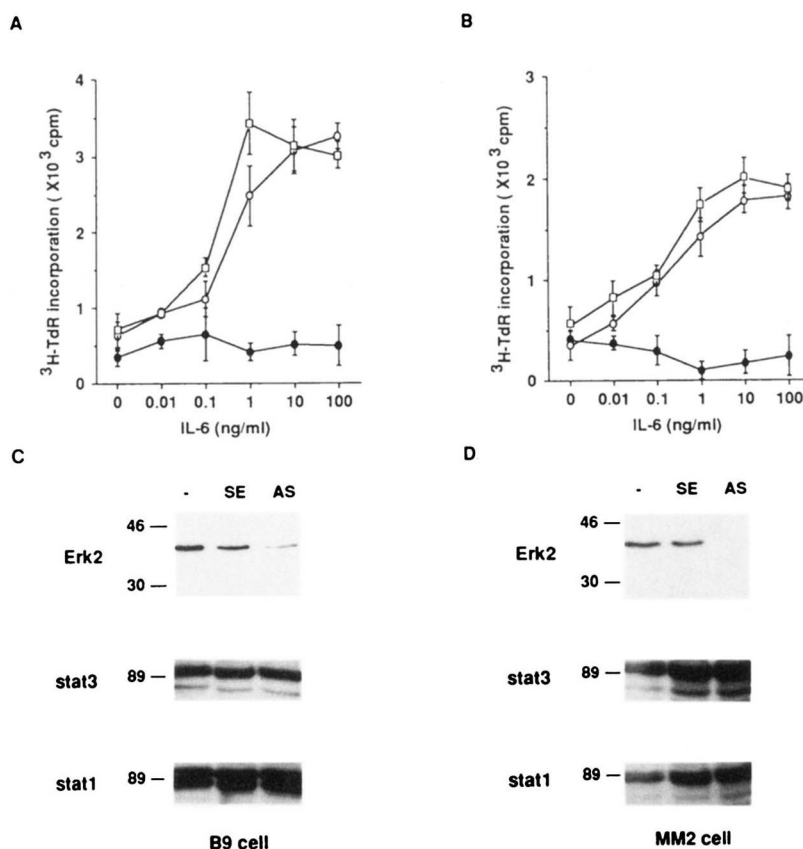
Cells	Protein Kinases								
	JAK1	JAK2	Tyk2	gp130	STAT1	STAT3	Shc	Sos	MAPK
U1958	– ^a	–	–	–	–	–	–	–	–
Hs Sultan	–	–	–	–	–	–	–	–	–
RPMI8226	+	–	+	+	+	+	–	–	–
B9	+	–	+	+	–	+	+	+	+
ARH77	–	–	–	–	–	–	–	–	–
IM9	±	–	±	±	±	–	+	–	–
OCI-My5	+	–	–	+	+	+	–	–	–
U266	+	–	–	+	+	+	–	–	–
MM1	–	–	–	–	–	–	–	–	–
MM2	–	+	–	–	–	–	+	+	+

^a –, no activation by IL-6; +, significant activation by IL-6; ±, slight activation by IL-6.

33). In contrast, IL-6 does not trigger phosphorylation of JAK1 in cells of multiple lineages: SK-MES squamous cell lung carcinoma cells; T10D cells, an IL-11-dependent derivative of IL-6-dependent T1105 murine plasmacytoma cells; Y6 murine hemopoietic cells; 3T3-L1 mouse preadipocytes; TF1 multifactor-dependent human erythroleukemia cells; and SKW6.4 IgM-producing cells responsive to IL-6 (15–17, 33). Phosphorylation of JAK2 in response to IL-6 also varies in different cell types: JAK2 phosphorylation was triggered by IL-6 in ES, M1, BAFm130, EW1, B9E, ANBL6, Y6, HT1080, 3T3-L1, TF1, and SKW6.4 cells (14–18, 33), but not in U266, OCI-My4, T10D, and AF10 cells (15, 16, 33). Finally, phosphorylation of Tyk2 induced by IL-6 was demonstrated in SK-MES, U266, B9E, ANBL6, HT1080, and HepG2 cells (14, 15, 18, 28), but not evident in EW1, OCI-My4, and T10D cells (15, 16). In our study, marked heterogeneity of JAK family kinase activation in MM cells in response to IL-6 was also observed: JAK1, Tyk2, and gp130 were phosphorylated in RPMI 8226, B9, and IM9 cells; JAK1 and gp130 were phosphorylated in OCI-My5 and U266 cells; and only JAK2 was phosphorylated in MM2 patient cells. Of note, gp130 was phosphorylated in response to IL-6 only in the cells in which JAK family kinases were also activated by IL-6 (RPMI 8226, U266, OCI-My5, B9, and IM9). These studies suggest that JAK family kinases are differentially activated by IL-6 in cells of various lineages, and that all JAK family kinases can phosphorylate gp130.

After phosphorylation of JAK family kinases and gp130, IL-6 signaling proceeds via the STAT3 homodimer, STAT1-STAT3 heterodimer, and Ras-dependent MAPK pathways. Previous reports showed that IL-6 induced phosphorylation of STAT3 in HepG2, M1, National Institutes of Health3T3, B9, and HT1080 cells (18, 21–23), but not in PX63AG8 MM cells (24). On the other hand, IL-6 triggered phosphorylation of STAT1 in ANBL6, HeLa, HepG2, SK-N-MC (human neuroblastoma cell line), M1, and HT1080 cells (16, 18, 25, 27, 28, 30), but not in B9E, T10D, and OCI-My4 cells (16). In our study, STAT1 phosphorylation triggered by IL-6 was demonstrated in RPMI 8226, U266, and OCI-My5 cells, whereas STAT3 was phosphorylated in these cell lines, as well as in B9 cells. These results suggest that IL-6 activates the STAT3 homodimer pathway in B9 cells and the STAT3 homodimer and/or STAT1-STAT3 heterodimer pathways in RPMI 8226, OCI-My5, U266, and IM9 cells. As noted above, IL-6 stimulated phosphorylation of gp130 in all of these cells, suggesting that phosphorylation of gp130 is essential for activation of the STAT3 homodimer or STAT1-STAT3 heterodimer pathways. In our studies, STAT1 and STAT3 phosphorylation were induced by IL-6, even in RPMI 8226, OCI-My5, and U266 cells, which do not proliferate to IL-6 and are therefore judged to be IL-6 nonresponsive. These data suggest that these cell lines have a normal STAT signaling pathway, and that STAT3 homodimer and/or STAT1-STAT3 heterodimer pathways may be activated in MM cells growing independently of IL-6. Recent reports have demonstrated that truncated form

FIGURE 10. Effect of MAPK antisense ODN on IL-6-induced B9 cell and patient MM2 cell proliferation. After incubation with MAPK antisense ODN (●), MAPK sense ODN (□), or media alone (○), B9 cells (A) or patient MM2 cells (B) were cultured for 48 h with serial doses of IL-6. Cells were pulsed during the last 12 h with 1 μ Ci of [3 H]TdR/well. Cell lysates from B9 cells (C) or patient MM2 cells (D) cultured with MAPK antisense ODN (AS), sense ODN (SE), and media alone (–) were immunoblotted with anti-Erk2 Ab, anti-STAT3 Ab, and anti-STAT1 Ab.



of gp130 (box3 region deletion, IC65) induced DNA synthesis without gp130 and STAT3 phosphorylation (19). This further supports the view that gp130, as well as STAT1 and STAT3 kinase phosphorylation, may not be required for IL-6-dependent DNA synthesis.

In previous reports, IL-6 induced activation of the Ras-dependent MAPK cascade in PC12 (rat pheochromocytoma cell line), AF10, EW-1, and National Institutes of Health3T3 cells (30–35, 36), but not in SKW6.4 and MAH (sympathoadrenal progenitor cell line) cells (30, 33). The SH2 domain of the Grb2 adapter protein binds to tyrosine-phosphorylated proteins, including receptor tyrosine kinases and Shc protein, while the SH3 domains bind to the Ras guanine nucleotide exchanger factor, Sos1 (51). In the current study, IL-6 induced both Shc phosphorylation and its association with Sos1, as well as Erk2 phosphorylation only in B9 and MM2 patient cells, which proliferated in response to IL-6. On the other hand, IL-6 triggered Shc phosphorylation without its association with Sos1 and Erk2 phosphorylation in IL-6-nonresponsive IM9 cells, suggesting a blockade in signaling between Shc and Sos1. Although constitutive Shc-Sos1 binding was evident in the IL-6-nonresponsive U1958, RPMI 8226, ARH77, and IM9 cell lines, neither Shc phosphorylation nor increased Shc-Sos1 binding was induced by IL-6. In the present and prior studies (53), IL-6 triggered Shc-Sos1 binding and phosphorylation of Shc only in B9 and MM2 cells, which proliferate in response to IL-6. These data demonstrate the importance of Ras-dependent MAPK cascade activation in IL-6-dependent cell growth. Inhibition of IL-6-induced B9 cell and patient MM2 cell proliferation by MAPK antisense ODN further supports this view.

Given the potential for interaction of kinases within the MAPK family, we also examined p38 and JNK1 kinases during IL-6-dependent proliferation of B9 cells. IL-6 triggered up-regulation of MAPK kinase activity without associated changes in p38 and JNK1 kinase activities. To further assess the potential interactive

role of these kinases, we also assayed p38 and JNK1 protein expression and kinase activity in B9 cells cultured with MAPK antisense ODN. Treatment of B9 cells with MAPK antisense ODN inhibited MAPK protein expression and proliferation to IL-6; however, the protein expression and kinase activities of p38 and JNK1 were not altered under these conditions. These data confirm the importance of MAPK signaling in growth, and further suggest that p38 and JNK1 do not play a complementary role.

Other reports also support a critical role for the Ras-dependent MAPK cascade in IL-6-dependent growth. For example, cAMP is known to be an inhibitor of MAPK cascade by inhibiting Raf1 activation (54), and increased cAMP inhibits IL-6-stimulated growth of IL-6-dependent 7TD1 plasmacytoma cells and IL-6-dependent subclone of U266 cells (55). In addition, transfection of mutated Ras into IL-6-dependent ANBL6 MM cell confers IL-6-independent growth (35). Finally, Hirano and colleagues most recently demonstrated, using a series of pro-B cell lines expressing chimeric receptors composed of the extracellular domain of the granulocyte CSF receptor and the transmembrane and cytoplasmic domains of gp130, that MAPK activation and STAT3 activation were important in mitogenesis and apoptosis, respectively (39).

In the present studies of patient MM2 cells, we demonstrated JAK2, Shc, Sos1, and MAPK activation without an associated increase above constitutive levels of gp130 phosphorylation. In earlier studies using deletion mutant IC65 of gp130, Kishimoto and colleagues have similarly demonstrated JAK1, JAK2, and Tyk2 phosphorylation and cell proliferation in response to IL-6, without associated phosphorylation of gp130 (19). Moreover, in mouse IL-6-dependent T10D plasmacytoma cells, none of the JAK family kinases is phosphorylated during IL-6-induced proliferation (16). These data suggest that JAK kinase and gp130 phosphorylation may not be essential for activation of the Ras-dependent MAPK cascade.

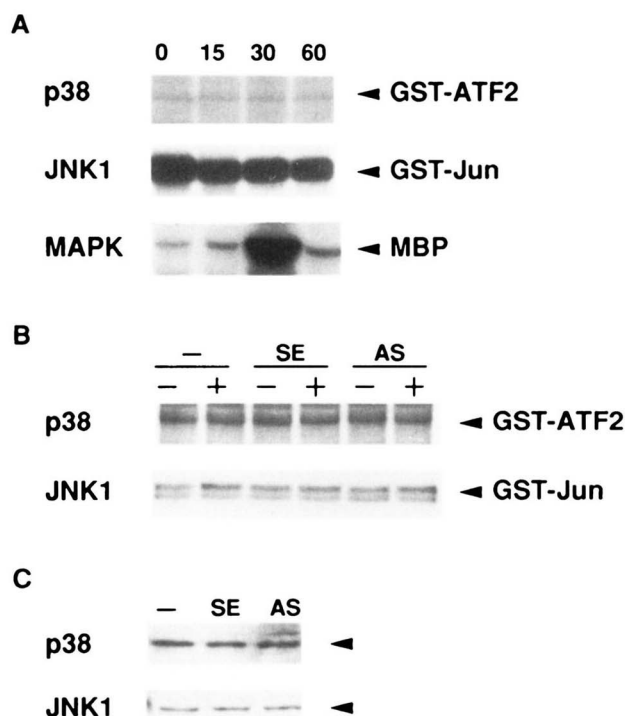


FIGURE 11. Effects of IL-6 and MAPK antisense ODN on p38 and JNK1 kinase activities and protein expression in B9 cells. **A**, B9 cells were cultured for the indicated times with 100 ng/ml of IL-6; p38, JNK1, and MAPK kinase activities were measured using GST-activated transcription factor 2, GST-Jun, and myelin basic protein, respectively, as substrates. **B**, B9 cells were treated for 48 h with MAPK antisense ODN (AS) or sense ODN (SE) before culture of cells for 30 min in media alone (–) or with 100 ng/ml of IL-6 (+). Cell lysates were assayed for p38 and JNK1 kinase activities. **C**, Cell lysates from B9 cells cultured with MAPK antisense ODN (AS), sense ODN (SE), and media alone (–) for 48 h were immunoblotted with anti-p38 Ab and anti-JNK1 Ab.

Recent reports have demonstrated that the Sak, Hck, Fes, Btk, and Tec kinases are also involved in IL-6-induced signaling pathways in multiple cell types (56–59), and it is likely that pathways mediating IL-6 signaling will be further elucidated. Moreover, in human MM, IL-6 is not only a growth factor, but also inhibits tumor cell apoptosis (10–12, 49). Since it has been reported recently that differences in gp130-induced signaling may have distinct biologic sequelae, i.e., growth via the MAPK cascade and anti-apoptosis via the STAT3 pathway (39), it is possible in MM that IL-6 may trigger growth and anti-apoptotic effects via differential signaling. Previous reports have demonstrated activation of NF-IL-6 by IL-6 (34); however, those transcription factors activated during IL-6-induced MM cell growth or anti-apoptosis remain to be delineated. Future studies in MM cells, in conjunction with the present report, may therefore suggest novel therapeutic strategies to inhibit growth or promote apoptosis based upon interruption of IL-6 signaling in tumor cells (60). Finally, other cytokines that also utilize gp130 signaling, including oncostatin M, leukemia-inhibitory factor, and ciliary neurotrophic factor, may also be growth factors for MM cells (61–63); this suggests the possibility of interrupting tumor cell growth related to several cytokines by blocking shared signaling pathways.

References

- Kawano, M., T. Hirano, T. Matsuda, T. Taga, Y. Horii, K. Iwato, H. Asakura, B. Tang, O. Tanabe, H. Tanaka, A. Kuramoto, and T. Kishimoto. 1988. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature* 332:83.
- Klein, B., X. G. Zhang, M. Jourdan, J. Content, F. Houssiau, L. Aarden, M. Piechaczyk, and R. Bataille. 1989. Paracrine rather than autocrine regulation of myeloma cell growth and differentiation by interleukin-6. *Blood* 73:517.
- Westendorf, J. J., G. J. Ahmann, R. J. Armitage, M. K. Spriggs, J. A. Lust, P. R. Greipp, J. A. Katzmman, and D. F. Jelinek. 1994. CD40 expression in malignant plasma cells: role in stimulation of autocrine IL-6 secretion by a human myeloma cell line. *J. Immunol.* 152:117.
- Urashima, M., D. Chauhan, H. Uchiyama, G. J. Freeman, and K. C. Anderson. 1995. CD40 ligand triggered interleukin-6 secretion in multiple myeloma. *Blood* 85:1903.
- Lichtenstein, A., J. Berenson, D. Norman, M. P. Chang, and A. Carlile. 1989. Production of cytokines by bone marrow cells obtained from patients with multiple myeloma. *Blood* 74:1266.
- Caligaris-Cappio, F., L. Bergui, M. G. Gregoret, G. Gaidano, M. Gaboli, M. Schena, A. Zallone, and P. C. Marchisio. 1991. Role of bone marrow stromal cells in the growth of human multiple myeloma. *Blood* 77:2688.
- Zhang, X. G., J. P. Gaillard, N. Robillard, Z. Y. Lu, Z. J. Gu, M. Jourdan, J. M. Boiron, R. Bataille, and B. Klein. 1994. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. *Blood* 83:3654.
- Uchiyama, H., B. A. Barut, A. F. Mohrbacher, D. Chauhan, and K. C. Anderson. 1993. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. *Blood* 82:3712.
- Ahmann, E. J., H. M. Lokhorst, A. W. Dekker, and A. C. Bloem. 1992. Lymphocyte function-associated antigen-1 expression on plasma cells correlates with tumor growth in multiple myeloma. *Blood* 79:2068.
- Lichtenstein, A., Y. Tu, C. Fady, R. Vescio, and J. Berenson. 1995. Interleukin-6 inhibits apoptosis of malignant plasma cells. *Cell. Immunol.* 162:248.
- Kawano, M., K. Mihara, N. Huang, T. Tsujimoto, and A. Kuramoto. 1995. Differentiation of early plasma cells on bone marrow stromal cells requires interleukin-6 for escaping from apoptosis. *Blood* 85:487.
- Hata, H., H. Matsuzaki, M. Takeya, M. Yoshida, T. Sonoki, A. Nagasaki, N. Kuribayashi, F. Kawano, and K. Takatsuki. 1995. Expression of Fas/Apo-1 (CD95) and apoptosis in tumor cells from patients with plasma cell disorders. *Blood* 86:1939.
- Akira, T., T. Taga, and T. Kishimoto. 1993. Interleukin-6 in biology and medicine. *Adv. Immunol.* 54:1.
- Narazaki, M., B. A. Witthuhn, K. Yoshida, O. Silvennoinen, K. Yasukawa, J. N. Ihle, T. Kishimoto, and T. Taga. 1994. Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. *Proc. Natl. Acad. Sci. USA* 91:2285.
- Stahl, N., T. G. Boulton, T. Farruggella, N. Y. Ip, S. Davis, B. A. Witthuhn, E. W. Quelle, O. Silvennoinen, G. Barbieri, S. Pellegrini, J. N. Ihle, and G. D. Yancopoulos. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 β receptor components. *Science* 263:92.
- Berger, L. C., T. S. Hawley, J. A. Lust, S. J. Goldman, and R. G. Hawley. 1994. Tyrosine phosphorylation of JAK-TYK kinases in malignant plasma cell line growth-stimulated by interleukin-6 and 11. *Biochem. Biophys. Res. Commun.* 202:596.
- Matsuda, T., Y. Yamasaki, and T. Hirano. 1994. Interleukin-6-induced tyrosine phosphorylation of multiple proteins in murine hematopoietic lineage cells. *Biochem. Biophys. Res. Commun.* 200:821.
- Guschin, D., N. Rogers, J. Briscoe, B. Witthuhn, D. Waltling, F. Horn, S. Pellegrini, K. Yasukawa, P. Heinrich, G. R. Stark, J. N. Ihle, and I. M. Kerr. 1995. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. *EMBO J.* 14:1421.
- Kishimoto, T., S. Akira, M. Narazaki, and T. Taga. 1995. Interleukin-6 family of cytokines and gp130. *Blood* 86:1243.
- Kishimoto, T., T. Taga, and S. Akira. 1994. Cytokine signal transduction. *Cell* 76:253.
- Wegenka, U. M., J. Buschmann, C. Lütticken, P. C. Heinrich, and F. Horn. 1993. Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level. *Mol. Cell. Biol.* 13:276.
- Akira, S., Y. Nishio, M. Inoue, X.-J. Wang, S. Wei, T. Matsusaka, K. Yoshida, T. Sudo, M. Naruto, and T. Kishimoto. 1994. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 77:63.
- Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: A stat family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264:95.
- Wegenka, U. M., C. Lütticken, J. Buschmann, J. Yuan, F. Lottspeich, W. Müller-Esterl, C. Schindler, E. Roeb, P. C. Heinrich, and F. Horn. 1994. The interleukin-6-activated acute-phase response factor is antigenically and functionally related to members of the signal transducer and activator of transcription (STAT) family. *Mol. Cell. Biol.* 14:3186.
- Sadowski, H. B., K. Shuai, J. E. Darnell, Jr., and M. Z. Gilman. 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* 261:1739.
- Stahl, N., T. G. Boulton, Z. Zhong, J. E. Darnell, Jr., and G. D. Yancopoulos. 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* 267:1349.
- Bonni, A., D. A. Frank, C. Schindler, and M. E. Greenberg. 1993. Characterization of a pathway for ciliary neurotrophic factor signaling to the nucleus. *Science* 262:1575.

28. Lütticken, C., U. M. Wegenka, J. Yuan, J. Buschmann, C. Schindler, A. Ziemiecki, A. G. Harpur, A. F. Wilks, K. Yasukawa, T. Taga, T. Kishimoto, G. Barbieri, S. Pellegrini, M. Sendtner, P. C. Heinrich, and F. Horn. 1994. Association of transcription factor APRF and protein kinase JAK1 with the interleukin-6 signal transducer gp130. *Science* 263:89.
29. Feldman, G. M., E. F. Petricoin III, M. David, A. C. Lerner, and D. S. Finbloom. 1994. Cytokines that associate with the signal transducer gp130 activate interferon-induced transcription factor p91 by tyrosine phosphorylation. *J. Biol. Chem.* 269:10747.
30. Boulton, T. G., N. Stahl, and G. D. Yancopoulos. 1994. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J. Biol. Chem.* 269:11648.
31. Nakafuku, M., T. Satoh, and Y. Kaziro. 1992. Differentiation factors, including nerve growth factor, fibroblast growth factor, and interleukin-6, induce an accumulation of an active RAS-GTP complex in rat pheochromocytoma PC12 cells. *J. Biol. Chem.* 267:19448.
32. Daeipour, M., G. Kumar, M. C. Amaral, and A. E. Nel. 1993. Recombinant IL-6 activates p42 and p44 mitogen-activated protein kinases in the IL-6 responsive B cell line, AF10. *J. Immunol.* 150:4743.
33. Kumar, G., S. Gupta, S. Wang, and A. E. Nel. 1994. Involvement of Janus kinases, p52^{src}, Raf-1 and MEK-1 in the IL-6-induced mitogen-activated protein kinase cascade of a growth-responsive B cell line. *J. Immunol.* 153:4436.
34. Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira. 1993. Phosphorylation at threonine-235 by a *ras*-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL-6. *Proc. Natl. Acad. Sci. USA* 90:2207.
35. Billadeau, D., D. F. Jelinek, N. Shah, T. W. LeBien, and B. V. Ness. 1995. Introduction of an activated *N-ras* oncogene alters the growth characteristic of the interleukin 6-dependent myeloma cell line ANBL-6. *Cancer Res.* 55:3640.
36. Neumann, C., G. Zehentmaier, S. Danhauser-Riedel, B. Emmerich, and M. Hallek. 1996. Interleukin-6 induced tyrosine phosphorylation of the Ras activating protein Shc, and its complex formation with Grb2 in human multiple myeloma cell line LP-1. *Eur. J. Immunol.* 26:379.
37. Minami, M., M. Inoue, S. Wei, K. Takeda, M. Matsumoto, T. Kishimoto, and S. Akira. 1996. STAT3 activation is critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line. *Proc. Natl. Acad. Sci. USA* 93:3963.
38. Nakajima, K., Y. Yamanaka, K. Nakae, H. Kojima, M. Ichiba, N. Kiuchi, T. Kitaoka, T. Fukada, M. Hibi, and T. Hirano. 1996. A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. *EMBO J.* 15:3651.
39. Fukada, T., M. Hibi, Y. Yamanaka, M. Takahashi-Tezuka, Y. Fujitani, T. Yamaguchi, K. Nakajima, and T. Hirano. 1996. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of stat3 in anti-apoptosis. *Immunity* 5:449.
40. Harris, N. 1974. Plasma cell surface antigen on human blood lymphocytes. *Nature* 250:507.
41. Matsuoka, Y., G. Moore, Y. Yagi, and D. Pressman. 1967. Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. *Proc. Soc. Exp. Biol. Med.* 125:1246.
42. Burk, K., B. Drewinko, J. Trujillo, and M. Ahearn. 1971. Establishment of human plasma cell lines in vitro. *Cancer Res.* 38:2508.
43. Fahey, J., D. Buell, and H. Sox. 1971. Proliferation and differentiation of lymphoid cells: studies with human lymphoid cell lines and immunoglobulin synthesis. *Ann. NY Acad. Sci.* 190:221.
44. Nilsson, K., H. Bennich, S. Johansson, and J. Ponten. 1970. Established immunoglobulin producing myeloma (IgE) and lymphoblastic (IgG) cell lines from an IgE myeloma patient. *Clin. Exp. Immunol.* 7:477.
45. Jernberg, H., M. Pettersson, T. Kishimoto, and K. Nilsson. 1991. Heterogeneity in response to interleukin-6 (IL-6), expression of IL-6 and IL-6 receptor mRNA in a panel of established human multiple myeloma cell lines. *Leukemia* 5:255.
46. Hitzler, J. K., H. Martinez-Valdez, D. B. Bergsagel, M. D. Minden, and H. A. Messner. 1991. Role of interleukin-6 in the proliferation of human multiple myeloma cell lines OCI-MY 1 to 7 established from patients with advanced stage of the disease. *Blood* 78:1996.
47. Aarden, L. A., E. R. De Groot, O. L. Schaap, and P. M. Lansdorp. 1987. Production of hybridoma growth factor by human monocytes. *Eur. J. Immunol.* 17:1411.
48. Sale, E. M., P. G. P. Atkinson, and G. J. Sale. 1995. Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. *EMBO J.* 14:674.
49. Chauhan, D., S. M. Kharbanda, A. Ogata, M. Urashima, G. Teoh, M. Robertson, D. W. Kufe, and K. C. Anderson. 1997. Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. *Blood* 89:227.
50. Boulton, T. G., Z. Zhong, Z. Wen, J. E. Darnell, Jr., N. Stahl, and G. D. Yancopoulos. 1995. STAT3 activation by cytokines utilizing gp130 and related transducers involves a secondary modification requiring an H7-sensitive kinase. *Proc. Natl. Acad. Sci. USA* 92:6915.
51. Downward, J. 1994. The GRB2/Sem-5 adaptor protein. *FEBS Lett.* 338:113.
52. Fittahdieh, E., J. S. Sanghera, S. L. Pelech, D. Hess-Bienz, J. Watts, N. Shastri, and R. Aebersold. 1992. Tyrosyl phosphorylation and activation of MAP kinases by p56^{lck}. *Science* 255:853.
53. Ogata, A., D. Chauhan, M. Urashima, G. Teoh, S. P. Treon, and K. C. Anderson. 1997. Blockade of mitogen-activated protein kinase cascade signaling in interleukin-6 independent multiple myeloma cells. *Clinical Cancer Res.* 3:1017.
54. Marx, J. 1993. Two major signal pathways linked. *Science* 262:988.
55. Rezzonico, R., A. Loubat, D. Lallemand, C. M. Pfarr, D. F. Far, A. Proudfoot, B. Rossi, and G. Ponzio. 1995. Cyclic AMP stimulates a JunD/Fra-2 AP-1 complex and inhibits the proliferation of interleukin-6-dependent cell lines. *Oncogene* 11:1069.
56. Matsuda, T., and T. Hirano. 1994. Association of p72 tyrosine kinase with stat factors and its activation by interleukin-3, interleukin-6, and granulocyte colony-stimulating factor. *Blood* 83:3457.
57. Ernst, M., D. P. Gearing, and A. R. Dunn. 1994. Functional and biochemical association of Hck with the LIF/IL-6 receptor signal transducing subunit gp130 in embryonic stem cells. *EMBO J.* 13:1574.
58. Matsuda, T., T. Fukada, M. Takahashi-Tezuka, Y. Okuyama, Y. Fujitani, Y. Hanazono, H. Hirai, and T. Hirano. 1995. Activation of Fes tyrosine kinase by gp130, an interleukin-6 family cytokine signal transducer, and their association. *J. Biol. Chem.* 270:11037.
59. Matsuda, T., M. Takahashi-Tezuka, T. Fukada, Y. Okuyama, Y. Fujitani, S. Tsukada, H. Mano, H. Hirai, O. N. Witte, and T. Hirano. 1995. Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines. *Blood* 85:627.
60. Ogata, A., and K. C. Anderson. 1996. Therapeutic strategies for inhibition of interleukin-6 mediated multiple myeloma cell growth. *Leuk. Res.* 20:303.
61. Zhang, X.-Z., J. J. Gu, Z. Y. Lu, K. Yasukawa, G. D. Yancopoulos, K. Turner, M. Shoyab, T. Taga, T. Kishimoto, R. Bataille, and B. Klein. 1994. Ciliary neurotrophic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. *J. Exp. Med.* 177:1337.
62. Nishimoto, N., A. Ogata, Y. Shima, Y. Tani, H. Ogawa, M. Nakagawa, H. Sugiyama, K. Yoshizaki, and T. Kishimoto. 1994. Oncostatin M, leukemia inhibitory factor, and interleukin 6 induce the proliferation of human plasmacytoma cells via the common signal transducer, gp130. *J. Exp. Med.* 177:1343.
63. Chauhan, D., S. M. Kharbanda, A. Ogata, M. Urashima, D. Frank, N. Malik, D. W. Kufe, and K. C. Anderson. 1995. Oncostatin M induces association of Grb2 with Janus kinase JAK2 in multiple myeloma cells. *J. Exp. Med.* 182:1801.