

# Characterization of Signaling Cascades Triggered by Human Interleukin-6 *versus* Kaposi's Sarcoma-associated Herpes Virus-encoded Viral Interleukin 6<sup>1</sup>

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## ABSTRACT

Kaposi's sarcoma-associated herpes virus (KSHV) is associated with Kaposi's sarcoma, multicentric Castleman's disease, and body cavity-based lymphomas, settings in which human interleukin-6 (hIL-6) acts as a growth factor. The KSHV open reading frame K2 encodes for viral IL-6 (vIL-6), a protein with 25% amino acid identity to hIL-6, which can promote the growth of hIL-6-dependent cell lines. In the present study, we characterized biological sequelae and signaling cascades triggered by hIL-6 *versus* vIL-6 in the hIL-6-dependent MH60 and B9 cell lines. Both hIL-6 and vIL-6 induced significant increases ( $P < 0.01$ ) in DNA synthesis in these cell lines in a dose-dependent fashion. Neutralizing anti-hIL-6 antibody (Ab) inhibited DNA synthesis triggered by hIL-6, without similarly affecting proliferation in response to vIL-6. On the other hand, antimouse IL-6 receptor (mIL-6R) Ab blocked response to vIL-6, but not that to hIL-6. Both hIL-6 and vIL-6 activated gp130, Janus kinase 1, signal transducers and activators of transcription-3, and mitogen-activated protein kinase in both MH60 and B9 cells. Proliferation of these cell lines in response to both hIL-6 and vIL-6 was blocked by PD98059, an inhibitor of MEK1 activation. These data suggest that MEK1 activation mediates the proliferative response to both cytokines. Finally, both hIL-6 and vIL-6 also maintained viability of serum-starved MH60 and B9 cells and blocked dexamethasone-induced apoptosis of MM.1S human myeloma cells. Further characterization of the signaling cascades mediating the growth

and antiapoptotic effects of vIL-6 *versus* hIL-6 may help identify their unique roles in disease pathogenesis in Kaposi's sarcoma and other KSHV-associated neoplasms.

## INTRODUCTION

Recent studies have identified a new  $\gamma$  herpes virus, KSHV<sup>3</sup> or human herpesvirus-8, within Kaposi's sarcoma tumor cells, body cavity-based lymphomas, and multicentric Castleman's disease (1–5). Although controversial, it has also been associated with MM in some studies (6–9). Sequencing of the viral genome has revealed colinearity and a notable degree of sequence homology with herpes virus saimiri (5, 10). The KSHV genome encodes for numerous cytokine homologues, including IL-6 and macrophage inhibitory factors MIP1 $\alpha$  and MIP1 $\beta$  (11–14). Specifically, KSHV open reading frame K2 encodes for vIL-6 (11, 12), a protein with 24.8% amino acid sequence identity to hIL-6, which promotes the survival of IL-6-dependent cell lines (13–15).

hIL-6 is a multifunctional cytokine that induces normal B-cell differentiation (16). It is also an autocrine and paracrine growth factor (17–20) as well as an antiapoptotic factor (21–26) for some human MM cells. 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 (16). Binding of hIL-6 to IL-6R induces homodimerization of gp130 (27) and activation of JAK family tyrosine kinases, including JAK1, JAK2, and/or Tyk2 (28–31); activated JAK kinases phosphorylate gp130 (30). Following activation of these tyrosine kinases, three downstream pathways have been reported (16). First, phosphorylated gp130 binds STAT3, which is phosphorylated by JAK family kinases; homodimers of phosphorylated STAT3 migrate rapidly to the nucleus and bind to hIL-6 response elements on the promoter of IL-6-induced genes (31–36). Second, hIL-6 phosphorylates STAT1, and heterodimers of tyrosine-phosphorylated STAT1 and STAT3 bind the nuclear DNA sequence termed GAS (IFN-activated sequence) or sis-inducible element (31, 37–40). Finally, hIL-6 can also activate the Ras-dependent MAPK cascade with sequential activation of Src homology 2/a collagen related, Grb2, son of sevenless 1, Ras, Raf, MEK, and MAPK; this cascade ultimately leads to activation of transcription factors NF-IL-6 or AP-1 complex (Jun/Fos; Refs. 41–44). We and others have begun to characterize signaling cascades that mediate hIL-6-dependent growth

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<sup>3</sup> The abbreviations used are: KSHV, Kaposi's sarcoma-associated herpes virus; MM, multiple myeloma; vIL-6 and hIL-6, viral and human interleukin 6; IL-6R, IL-6 receptor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; Dex, dexamethasone; Ab, antibody.

of MM and other cell lines on the one hand (45, 46) *versus* hIL-6-mediated antiapoptotic effects on the other (21–26). Although recent studies have suggested that vIL-6 can induce proliferation of IL-6-dependent cell lines (13–15), signaling pathways mediating these effects have not been characterized extensively. Moreover, results to date are inconsistent as to whether IL-6R is used in vIL-6 signaling (14, 15, 47).

In the present study, we compared the functional sequelae and signaling cascades triggered by hIL-6 *versus* vIL-6 in the MH60 and B9 hIL-6-dependent cell lines. Both cytokines induced proliferation, which was associated with activation of gp130, JAK, and STAT kinases; in both cases, growth was blocked by inhibition of MEK1. Moreover, both hIL-6 and vIL-6 rescued B9 and MH60 cells from serum starvation and MM.1S from Dex-induced apoptosis. Ongoing studies are determining the cascades whereby hIL-6 *versus* vIL-6 may mediate growth and antiapoptotic effects to define their roles in the pathogenesis of KSHV-associated diseases.

## MATERIALS AND METHODS

**Cell Lines and Culture.** The B9 murine hIL-6-dependent cell line (a kind gift of Dr. Lucien Aarden, The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; Ref. 48) and the MH60 murine hIL-6-dependent hybridoma cell line (provided by Dr. Kishimoto, Osaka University, Osaka, Japan; Ref. 49) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2  $\mu$ M L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and hIL-6 (Genetics Institute, Cambridge, MA) at 1 ng/ml for B9 cells and 0.1 ng/ml for MH60 cells. The MM.1S human MM cell line (kindly provided by Dr. Steven Rosen, Northwestern University, Chicago, IL) was also maintained in the same medium without hIL-6.

**Reagents.** Recombinant hIL-6 (specific activity,  $5.1 \times 10^5$  units/mg) was provided by Genetics Institute (Cambridge, MA). The following Abs were used either for blocking in cell culture experiments or for immunoprecipitation and immunoblotting: rat neutralizing antimouse IL-6R (mIL-6R; CD126) monoclonal Ab (Genzyme, Cambridge, MA); rabbit antihuman gp130 Ab and antiphosphotyrosine (RC20) Ab (Upstate Biotechnology, Lake Placid, NY); anti-JAK1, -JAK2, -Tyk2, -STAT1, -STAT3, and -ERK2 Abs (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-phospho-MAPK and anti-phospho-STAT3 Abs (New England Biolabs, Beverly, MA). The MEK1 inhibitor PD98059 was obtained from New England Biolabs.

**vIL-6.** Dr. John Nicholas (Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD) kindly provided plasmid containing vIL-6 sequences in the sense (pvIL-6) and antisense (pvIL-6 neg) orientation expressed under the control of HCMV promoter-enhancer in the pcDNA.3 vector, and plasmid pSVvIL-6, containing the vIL-6 open reading frame expressed under the control of the SV40 promoter in the vector pSG5 (14). Cos cells were transfected with 2  $\mu$ g of either pvIL-6 or pvIL-6 neg by calcium phosphate coprecipitation. Cos supernatants were harvested 3 days after transfection and immunoblotted with anti-vIL-6 Ab (kindly provided by Dr. Nicholas). The titer of vIL-6 was measured in a bioassay using hIL-6-dependent B9 and MH60 cell lines.

**DNA Synthesis.** DNA synthesis of B9 and MH60 cells stimulated by hIL-6 or vIL-6 was measured by [ $^3$ H]thymidine incorporation, as described previously (45). Briefly, MH60 and B9 cells were washed four times in medium without hIL-6. Cells ( $1 \times 10^4$  in 100  $\mu$ l of RPMI 1640/well in 96-well round-bottomed culture plates; Costar, Corning, NY) were cultured in the presence or absence of hIL-6 or vIL-6 for 48 h. Cells were labeled with [ $^3$ H]thymidine (New England Nuclear, Boston, MA) at 0.5  $\mu$ Ci/well during the last 8 h of culture, harvested using the Harvester 96 Mach II (Tomtec, Inc., Orange, CT), and counted on a 1205 Betaplate  $\beta$  counter (Wallac, Helsinki, Finland). The incorporation of [ $^3$ H]thymidine of cells cultured with hIL-6 or vIL-6 was compared with that of cells in medium alone. Values represent the mean  $\pm$  SD [ $^3$ H]thymidine incorporation (cpm) of triplicate cultures.

**Immunoprecipitation and Immunoblotting.** For immunoprecipitation,  $2\text{--}3 \times 10^7$  cells were washed six times with RPMI 1640 and cultured for 6 h in the absence of fetal bovine serum and growth factors. Cells were next stimulated with hIL-6 (100 ng/ml) or Cos vIL-6 transfectant supernatants (diluted 1:1 in medium) for 10–30 min at 37°C, and then washed with ice-cold Tris-buffered saline containing 1 mM  $\text{Na}_3\text{VO}_4$ . Cells were treated for 45 min with 1 ml of lysis buffer [1% NP40, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM DTT, 2 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mM leupeptin, and 5  $\mu$ g/ml aprotinin]. Cell lysates were centrifuged ( $16,000 \times g$  for 20 min at 4°C), and the supernatants were immunoprecipitated overnight at 4°C with specific Abs and protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden). The immunoprecipitates were washed three times with lysis buffer, boiled for 5 min, and subjected to SDS-PAGE. After electrophoresis, proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) and analyzed by subsequent immunoblotting with antiphosphotyrosine monoclonal Ab, using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

**Inhibition of Signaling Triggered by hIL-6 and vIL-6.** Both B9 and MH60 cells were harvested and washed three times with serum-free RPMI 1640. The cells were preincubated in serum-free RPMI 1640 supplemented with the MEK1 inhibitor PD98059 (50) at 10 and 50  $\mu$ M for 1 h at 37°C. These cells subsequently were cultured for 48 h in the presence of medium, hIL-6, Cos control, or vIL-6. DNA synthesis was determined by [ $^3$ H]thymidine incorporation, as described above.

To confirm the inhibitory effect of PD98059 on tyrosine phosphorylation of MAPK, MH60 cells were cultured in the absence of serum and hIL-6 for 18 h. These cells were then incubated with 50  $\mu$ M PD98059 for 1 h at 37°C prior to stimulation with medium, hIL-6, Cos control, or vIL-6 for 30 min. After stimulation, cells were lysed and subjected to SDS-PAGE. Tyrosine phosphorylation of MAPK was determined by immunoblotting with anti-phospho-MAPK Ab.

**Determination of Apoptosis.** B9 or MH60 cells ( $2 \times 10^6$ ) were washed and cultured in serum-free medium for 48 h at 37°C in the presence or absence of hIL-6 (50 ng/ml) or vIL-6 (1:2 dilution of vIL-6 Cos supernatant). MM.1S MM cells were treated with 20  $\mu$ M Dex for 48 h at 37°C in the presence or absence of hIL-6 (50 ng/ml) or vIL-6 (1:2 dilution of vIL-6 Cos supernatant). After incubation, genomic DNA was extracted

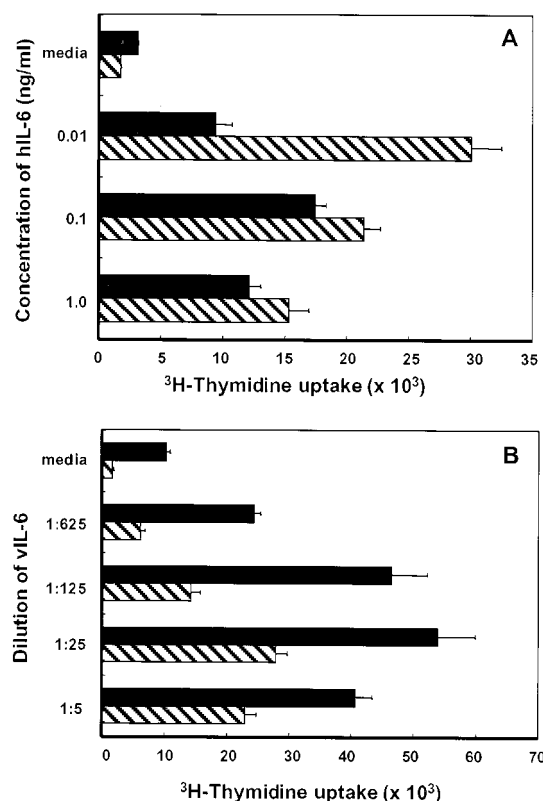


Fig. 1 Effect of hIL-6 and vIL-6 on proliferation of hIL-6-dependent MH60 and B9 cells. MH60 (▨) and B9 (■) cells ( $1 \times 10^4$ /well in 96-well plates) were cultured with medium plus hIL-6 (0.01–1.0 ng/ml; A), as well as medium plus vIL-6 (vIL-6 Cos supernatants diluted 1:5 to 1:625 in medium; B). [ $^3$ H]Thymidine uptake was measured during the last 8 h of 48-h cultures. Values represent the mean; bars, SD. [ $^3$ H]Thymidine incorporation (cpm) of triplicate cultures.

using a genomic DNA purification kit (Promega, Madison, WI), electrophoresed for 2 h at 90 V on a 2% agarose gel containing 5  $\mu$ g/ml ethidium bromide, and analyzed under UV light for DNA fragmentation, as in prior studies (24).

## RESULTS

**Effect of hIL-6 and vIL-6 on Proliferation of hIL-6-dependent MH60 and B9 Cells.** Culture supernatants of vIL-6 Cos transfectants (diluted 1:5, 1:25, 1:125, and 1:625 in medium) were tested for their ability to trigger proliferation of hIL-6-dependent MH60 and B9 cell lines. As shown in Fig. 1, induction of DNA synthesis was dose dependent for both hIL-6 (Fig. 1A) and vIL-6 (Fig. 1B). Peak proliferation of MH60 cells was observed at 0.01 ng/ml hIL-6 and with the 1:25 dilution of vIL-6 Cos supernatant in medium. For B9 cells, peak proliferation occurred at 0.1 ng/ml hIL-6 and with the 1:25 dilution of vIL-6 Cos supernatant. hIL-6 triggered a 12- and 8-fold increase in [ $^3$ H]thymidine uptake in MH60 and B9 cells, respectively ( $n = 5$ ;  $P < 0.01$ ), relative to cells cultured in medium alone (Fig. 1A). vIL-6 induced a 12- and 6-fold increase in DNA synthesis in MH60 and B9 cells, respectively ( $n = 5$ ;  $P < 0.01$ ; Fig. 1B).

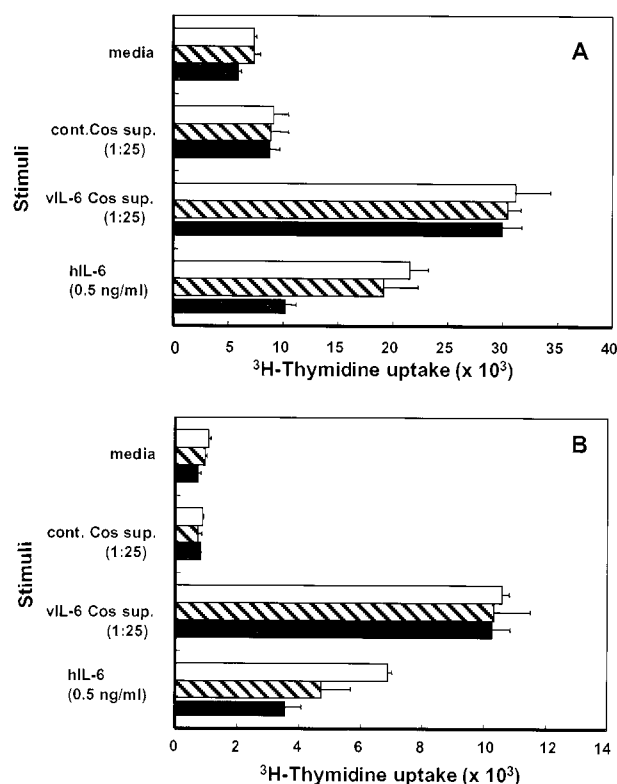
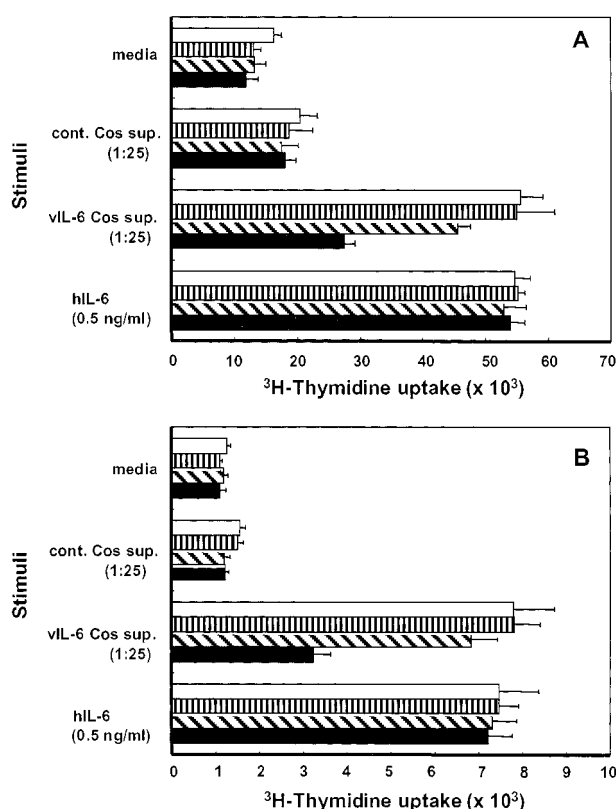


Fig. 2 Effect of neutralizing anti-hIL-6 Ab on hIL-6- and vIL-6-induced proliferation of MH60 and B9 cells. MH60 (A) and B9 (B) cells ( $1 \times 10^4$ /well in 96-well plates) were cultured with medium, control Cos transfectant supernatant (cont. Cos sup.), vIL-6 (vIL-6 Cos sup.; vIL-6 Cos transfectant supernatant diluted 1:25 in medium), or hIL-6 (0.5 ng/ml) in the absence (□) or presence of neutralizing anti-hIL-6 Ab at 5  $\mu$ g/ml (■) or 1  $\mu$ g/ml (▨). [ $^3$ H]Thymidine uptake was measured during the last 8 h of 48-h cultures. Values represent the mean; bars, SD. [ $^3$ H]Thymidine incorporation (cpm) of triplicate cultures.

**Effect of Neutralizing Anti-hIL-6 Ab on hIL-6- and vIL-6-induced Proliferation of MH60 and B9 Cells.** To characterize the effect of neutralizing anti-hIL-6 Ab on [ $^3$ H]thymidine uptake of MH60 and B9 cells stimulated by hIL-6 or vIL-6, both cell lines were cultured with hIL-6 (0.5 ng/ml) or vIL-6 (1:25 dilution of vIL-6 Cos supernatant) in the presence or absence of anti-hIL-6 Ab (1 and 5  $\mu$ g/ml). As shown in Fig. 2A, anti-hIL-6 Ab (5  $\mu$ g/ml) blocked DNA synthesis of MH60 cells triggered by hIL-6 (75% inhibition;  $n = 3$ ;  $P < 0.01$ ) but did not alter proliferation of MH60 cells induced by vIL-6. The blocking effect of anti-hIL-6 Ab on [ $^3$ H]thymidine uptake of B9 cells triggered by hIL-6 was less than that observed in MH60 cells (50% inhibition;  $n = 3$ ;  $P < 0.01$ ; Fig. 2B); again anti-hIL-6 Ab did not alter proliferation induced by vIL-6. These results suggest that hIL-6 and vIL-6 may differ in upstream signaling or may be related to the specificity of anti-hIL-6 Ab.

**Effect of Neutralizing Anti-mIL-6R Ab on hIL-6- and vIL-6-induced Proliferation of MH60 and B9 Cells.** To determine whether neutralizing anti-mIL-6R (CD126) Ab was able to inhibit proliferation of MH60 and B9 cells stimulated with hIL-6 or vIL-6, these cells were washed four times in medium before incubation with anti-mIL-6R Ab for 30 min at



**Fig. 3** Effect of neutralizing anti-mIL-6R Ab on hIL-6- and vIL-6-induced proliferation of MH60 and B9 cells. MH60 (A) and B9 (B) cells ( $1 \times 10^4$ /well in 96-well plates) were cultured with medium, control Cos transfectant supernatant (*cont. Cos sup.*), vIL-6 (*vIL-6 Cos sup.*; vIL-6 Cos transfectant supernatant diluted 1:25 in medium), or hIL-6 (0.5 ng/ml) in the absence ( $\square$ ) or presence of neutralizing anti-mIL-6R Ab at 2  $\mu$ g/ml ( $\blacksquare$ ), 0.5  $\mu$ g/ml ( $\boxtimes$ ), and 0.2  $\mu$ g/ml ( $\boxplus$ ). [ $^3$ H]Thymidine uptake was measured during the last 8 h of 48-h cultures. Values represent the mean; bars, SD. [ $^3$ H]Thymidine incorporation (cpm) of triplicate cultures.

37°C. Cells were subsequently cultured in the presence or absence of hIL-6 (0.5 ng/ml) or vIL-6 (1:25 dilution of vIL-6 Cos supernatant) for 48 h. As shown in Fig. 3A, anti-mIL-6R Ab blocked proliferation of MH60 cells triggered by vIL-6 (60% inhibition;  $n = 3$ ;  $P < 0.01$ ) without altering the proliferation of these cells triggered by hIL-6. The inhibitory effect of anti-mIL-6R Ab on [ $^3$ H]thymidine uptake of B9 cells triggered by vIL-6 was similar that in MH60 cells (60% inhibition;  $n = 3$ ;  $P < 0.01$ ; Fig. 3B); again anti-mIL-6R Ab did not alter the proliferation triggered by hIL-6.

**Effect of hIL-6 and vIL-6 on Tyrosine Phosphorylation of gp130 and JAK Family Kinases.** We first determined whether vIL-6 induced phosphorylation of gp130 in MH60 and B9 cells. As shown in Fig. 4, low level intrinsic tyrosine phosphorylation of gp130 was evident in MH60 cells, and gp130 phosphorylation was induced 30 min after triggering with hIL-6 (100 ng/ml) and vIL-6 (1:1 dilution of vIL-6 Cos supernatant). In contrast, no gp130 phosphorylation was triggered in MH60 cells cultured with control Cos transfectant supernatants. In B9 cells, tyrosine phosphorylation of gp130 was also induced

30 min after triggering with hIL-6 and vIL-6 (Fig. 4), albeit to a lesser degree than observed in MH60 cells. Again, no gp130 phosphorylation was induced in cultures of B9 cells by Cos control transfectant supernatants.

Because gp130 phosphorylation in response to hIL-6 is mediated via JAK kinases (31), we next determined whether vIL-6 induced tyrosine phosphorylation of JAK1, JAK2, and/or Tyk2 protein kinases. As shown in Fig. 5, low level constitutive phosphorylation of JAK2 and Tyk2, but not of JAK1, was observed in MH60 and B9 cells. In MH60 cells, both vIL-6 (1:1 dilution of vIL-6 Cos supernatant) and hIL-6 (100 ng/ml) triggered phosphorylation of JAK1, but not JAK2 or Tyk2. In B9 cells, vIL-6 induced phosphorylation of JAK1, but not JAK2 or Tyk2; however, hIL-6 triggered phosphorylation of JAK1, JAK2, and Tyk2. These data suggest that both vIL-6 and hIL-6 activate JAK kinases in MH60 cells, and implicate JAK1 in vIL-6 signaling.

**Effect of hIL-6 and vIL-6 on Phosphorylation of STAT1 and STAT3.** To define downstream signaling, we next determined whether STAT1 and/or STAT3 are phosphorylated in MH60 and B9 cells in response to vIL-6. As shown Fig. 6, weak constitutive phosphorylation of STAT3 was observed in B9 cells, but not in MH60 cells; STAT1 was not intrinsically phosphorylated in either cell line. vIL-6 induced phosphorylation of STAT3, but not STAT1, in both MH60 and B9 cells. In contrast, low level STAT3 phosphorylation was induced in cultures of B9 cells by Cos control transfectant supernatants. hIL-6 triggered phosphorylation of STAT3 as well as STAT1 in these cell lines. These data further confirm STAT kinase activation by both hIL-6 and vIL-6, and suggest a role for STAT3 in vIL-6 signaling.

**Effect of hIL-6 and vIL-6 on Phosphorylation of ERK2.** We previously demonstrated activation of the ras-dependent MAPK cascade during proliferation of B9 cells in response to hIL-6 (45, 46). We therefore next assayed for phosphorylation of ERK2 in MH60 and B9 cells in response to vIL-6 (1:1 dilution of vIL-6 Cos supernatant) and hIL-6 (100 ng/ml). As shown in Fig. 7, no MAPK phosphorylation was induced in cultures of B9 cells by Cos control transfectant supernatants, whereas both vIL-6 and hIL-6 induced phosphorylation of ERK2 in both cell lines, confirming MAPK cascade activation.

**Effect of PD98059 MEK1 Inhibitor on hIL-6- and vIL-6-induced Proliferation and MAPK Activation.** We previously showed in B9 cells that blocking of MAPK activation, using MAPK antisense oligonucleotide, abrogates DNA synthesis in response to hIL-6 (45). We therefore next determined whether inhibition of MAPK activation triggered by vIL-6 also blocks the proliferative response. As shown in Fig. 8A, proliferation in response to vIL-6 (1:25 dilution of vIL-6 Cos supernatant) was blocked in MH60 cells (Fig. 8A; 60% inhibition;  $n = 3$ ;  $P < 0.01$ ) by preincubation of these cells with the MEK1 inhibitor PD98059 (50  $\mu$ M for 1 h). Proliferation in response to hIL-6 (0.1 ng/ml) was also blocked by PD98059 (65% inhibition;  $n = 3$ ;  $P < 0.01$ ) in MH60 cells. Preincubation of B9 cells with PD98059 similarly blocked vIL-6- and hIL-6-induced proliferation (70% inhibition;  $n = 3$ ;  $P < 0.01$ ; data not shown).

The specific inhibitory effect of PD98059 on phosphorylation of MAPK was confirmed by immunoblotting of lysates



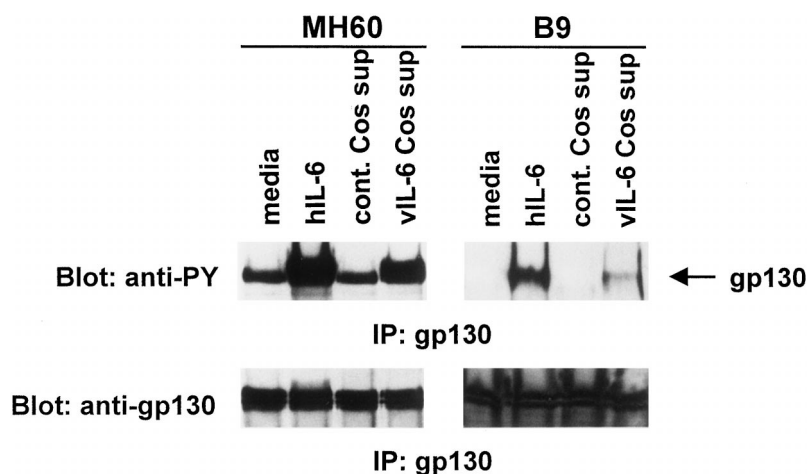


Fig. 4 Effect of hIL-6 and vIL-6 on tyrosine phosphorylation of gp130. MH60 and B9 cells were cultured with medium, hIL-6 (100 ng/ml), control Cos supernatant diluted 1:1 in medium (cont. Cos sup), or vIL-6 (vIL-6 Cos sup; vIL-6 Cos supernatant diluted 1:1 in medium) for 30 min. Cell lysates from medium-, hIL-6-, Cos control-, and vIL-6-treated cells were immunoprecipitated with anti-gp130 Ab. The precipitates were resolved by 6% SDS-PAGE, and phosphorylation of gp130 was detected by antiphosphotyrosine (anti-PY) Ab. The blots were stripped and reprobed with anti-gp130 Ab. IP, immunoprecipitate.

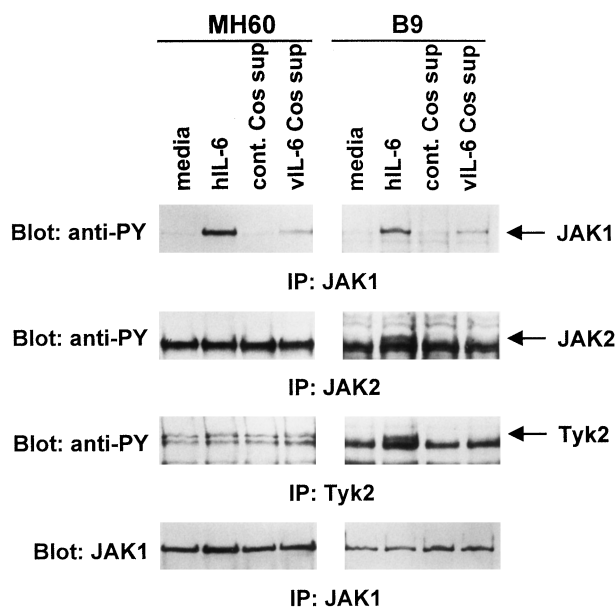


Fig. 5 Effect of hIL-6 and vIL-6 on tyrosine phosphorylation of JAK family kinases. MH60 and B9 cells were cultured with medium, hIL-6 (100 ng/ml), control Cos supernatant diluted 1:1 in medium (cont. Cos sup), or vIL-6 (vIL-6 Cos sup; vIL-6 Cos supernatant diluted 1:1 in medium) for 10 min. Cell lysates from medium-, hIL-6-, Cos control-, and vIL-6-treated cells were immunoprecipitated with anti-JAK1, JAK2, and Tyk2 Abs. The precipitates were resolved by 6% SDS-PAGE, and phosphorylation of JAK1, JAK2, and Tyk2 was detected by antiphosphotyrosine (anti-PY) Ab. The blots were stripped and reprobed with anti-JAK1 Ab. IP, immunoprecipitate.

obtained from MH60 cells triggered by hIL-6 (100 ng/ml) and vIL-6 (1:1 dilution of vIL-6 Cos supernatant), with or without PD98059 pretreatment. As shown in Fig. 8B, both hIL-6 and vIL-6 triggered phosphorylation of MAPK in non-PD98059-treated MH60 cells. Importantly, MAPK phosphorylation triggered by these cytokines was nearly completely inhibited by pretreatment of MH60 cells with PD98059 (50  $\mu$ M for 1 h). Similar effects were observed in B9 cells (data not shown).

**Effect of hIL-6 and vIL-6 on Viability of Serum-starved B9 Cells.** To determine whether vIL-6 could support the long-term growth of hIL-6-dependent cells, B9 cells were cultured in serum-free medium in the presence or absence of vIL-6 (vIL-6 Cos supernatant diluted 1:10 to 1:40). Although B9 cells cultured in the absence of vIL-6 did not survive beyond 5 days (<5% viable), the cells cultured in the presence of vIL-6 remained 85–90% viable at 10 days, as assessed by trypan blue exclusion (Fig. 9A). The effect of vIL-6 on viability was dose dependent, with peak effect at 1:10 to 1:20 dilutions of vIL-6. Cultures with hIL-6 served as positive controls. vIL-6 similarly maintained the viability of MH60 cells in serum-free medium (data not shown).

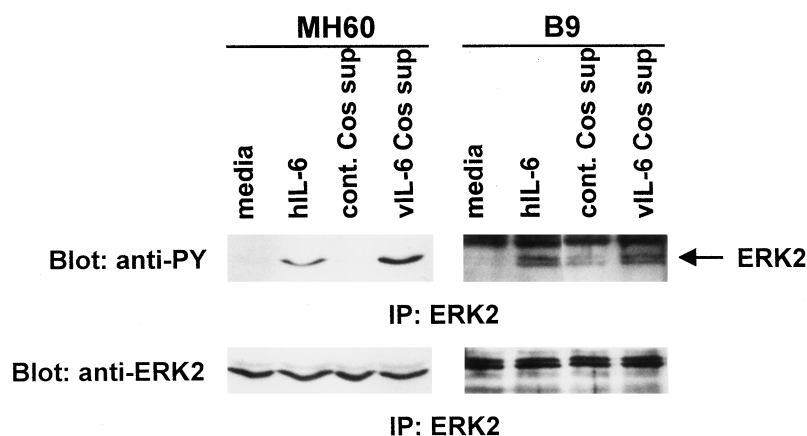
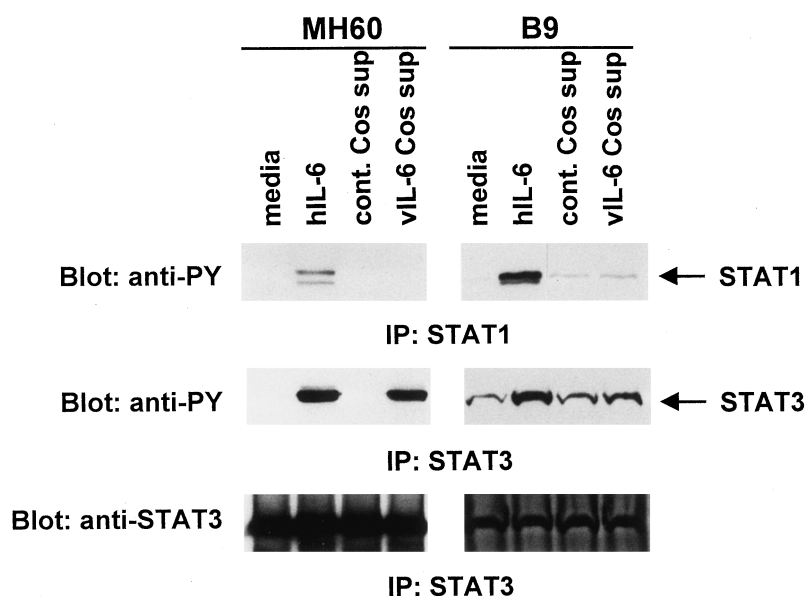
To confirm an antiapoptotic effect of vIL-6, serum-starved B9 cells were cultured in serum-free medium for 48 h in the presence of hIL-6 (50 ng/ml) or vIL-6 (1:2 dilution of vIL-6 Cos supernatant), and assayed for apoptosis using DNA fragmentation. Control B9 cells were cultured in serum containing medium supplemented with IL-6. As can be seen in Fig. 9B, apoptosis was observed in serum-free cultures in the absence of vIL-6 or hIL-6, but was not evident in cultures with either of these cytokines. Similar results were observed in MH60 cells (data not shown). These data suggest that vIL-6, like hIL-6, prevents apoptosis induced by serum starvation in both B9 and MH60 cells.

**Effect of vIL-6 on Apoptosis of MM.1S MM Cells Induced by Dex.** We previously reported that hIL-6 inhibits apoptosis in MM.1S cells induced by Dex (24), and next similarly examined the antiapoptotic effect of vIL-6. Significant apoptosis of MM.1S cells, evidenced by DNA fragmentation, was induced by Dex; importantly, either hIL-6 (50 ng/ml) or vIL-6 (1:2 dilution of vIL-6 Cos supernatant) abrogated Dex-triggered apoptosis (Fig. 10). These data further confirm that vIL-6, like hIL-6, is a survival factor for human MM cells.

## DISCUSSION

The novel  $\gamma$  herpes virus KSHV, or human herpesvirus-8, has been associated with Kaposi's sarcoma, multicentric Castleman's disease, and body cavity-based B-cell lymphoma (1–5). Although controversial, investigators at the University of Cali-

**Fig. 6** Effect of hIL-6 and vIL-6 on phosphorylation of STAT1 and STAT3. MH60 and B9 cells were cultured with medium, hIL-6 (100 ng/ml), control Cos supernatant diluted 1:1 in medium (*cont. Cos sup*), or vIL-6 (vIL-6 *Cos sup*; vIL-6 Cos supernatant diluted 1:1 in medium) for 30 min. Cell lysates from medium-, hIL-6-, Cos control-, and vIL-6-treated cells were immunoprecipitated with anti-STAT1 and anti-STAT3 Abs. The precipitates were resolved by 6% SDS-PAGE, and phosphorylation of STAT1 and STAT3 was detected by antiphosphotyrosine (*anti-PY*) Ab. The blots were stripped and reprobed with anti-STAT3 Ab. *IP*, immunoprecipitate.

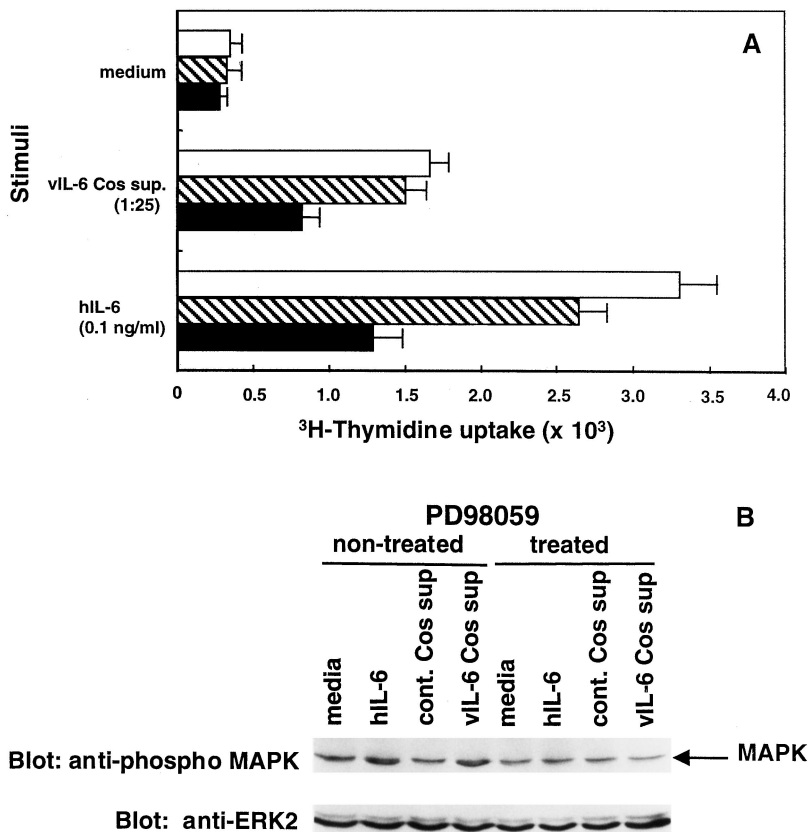


**Fig. 7** Effect of hIL-6 and vIL-6 on phosphorylation of ERK2. MH60 and B9 cells were cultured with medium, hIL-6 (100 ng/ml), control Cos supernatant diluted 1:1 in medium (*cont. Cos sup*), or vIL-6 (vIL-6 *Cos sup*; vIL-6 Cos supernatant diluted 1:1 in medium) for 10 min. Cell lysates from medium-, hIL-6-, Cos control-, and vIL-6-treated cells were immunoprecipitated with anti-ERK2 Ab. The precipitates were resolved by 10% SDS-PAGE, and phosphorylation of ERK2 was detected by antiphosphotyrosine (*anti-PY*) Ab. The blots were stripped and reprobed with anti-ERK2 Ab. *IP*, immunoprecipitate.

fornia at Los Angeles (6, 7) and in our laboratory (8, 9) have also found KSHV gene sequences in MM bone marrow cells expressing dendritic cell lineage antigens. The KSHV genome encodes for a number of homologues of human cytokines: the K2 open reading frame of KSHV encodes for vIL-6, a homologue of hIL-6 (11, 12). Given that hIL-6 is a growth and survival factor for some, but not all, tumor cells in the above clinical settings, it is of particular interest to assess the biological repertoire and mechanism of action of vIL-6 compared with hIL-6. This study demonstrated that vIL-6, like hIL-6, triggers proliferation of MH60 and B9 cells, which is associated with MAPK activation and blocked by MEK inhibitors. We also showed that vIL-6, like hIL-6, can protect B9 and MH60 cells from serum starvation, and MM.1S cells from Dex-induced apoptosis. These studies provide the framework for determining the relative roles of vIL-6 versus hIL-6 in the pathophysiology of KSHV-associated diseases.

In this study, we first demonstrated that both hIL-6 and vIL-6 significantly increased DNA synthesis and promoted the

long-term growth of the hIL-6-dependent MH60 and B9 cell lines. Previous studies have also shown that vIL-6 promotes proliferation of B9 cells (14) and hIL-6-dependent INA-6 human MM cells (15), as well as inducing signaling in HepG2 cells (47). As a first attempt to define the pathways mediating vIL-6 responsiveness, we and others have used neutralizing Abs to block vIL-6 versus hIL-6 responsiveness. In this study, anti-hIL-6 Ab blocked the response to hIL-6 without affecting the response to vIL-6; and conversely, anti-mIL-6R Ab blocked vIL-6 responsiveness without altering the response to hIL-6. This result suggests that hIL-6 and vIL-6 may bind either to different receptor sites or to distinct sites on a shared receptor on B9 and MH60 cells. Nicholas *et al.* (14) used a distinct anti-mIL-6R Ab and demonstrated blocking of DNA synthesis, also in B9 cells, to both hIL-6 and vIL-6. Burger *et al.* (15) showed that anti-hIL-6 Ab blocked the proliferation response of INA-6 cells to hIL-6 without altering their response to vIL-6; in that study as in our current study, anti-hIL-6R Ab blocked the response to hIL-6 but not to vIL-6. Finally, signaling triggered



**Fig. 8** Effect of PD98059 MEK1 inhibitor on hIL-6- and vIL-6-induced proliferation and MAPK activation. **A**, MH60 cells were incubated in medium (□) and with 10 μM (▨) or 50 μM (■) MEK1 inhibitor PD98059 for 1 h, and then cultured in the presence of either hIL-6 (0.1 ng/ml) or vIL-6 (vIL-6 Cos sup; 1:25 dilution of vIL-6 Cos supernatant) for 48 h. [<sup>3</sup>H]Thymidine uptake was measured during the last 8 h of 48-h cultures. Values represent the mean; bars, SD. [<sup>3</sup>H]Thymidine incorporation (cpm) of triplicate cultures. **B**, MH60 cells were cultured in the absence of serum and hIL-6 for 18 h. These cells were then incubated in the presence or absence of PD98059 (50 μM for 1 h) before culture with medium, hIL-6 (100 ng/ml), Cos control supernatant (cont. Cos sup), or vIL-6 (vIL-6 Cos sup; 1:1 dilution of vIL-6 Cos supernatant) for 10 min, lysed, and subjected to SDS-PAGE. Phosphorylation of MAPK was determined by immunoblotting with anti-phospho-MAPK Ab. Blots were stripped and reprobed with anti-ERK2 Ab.

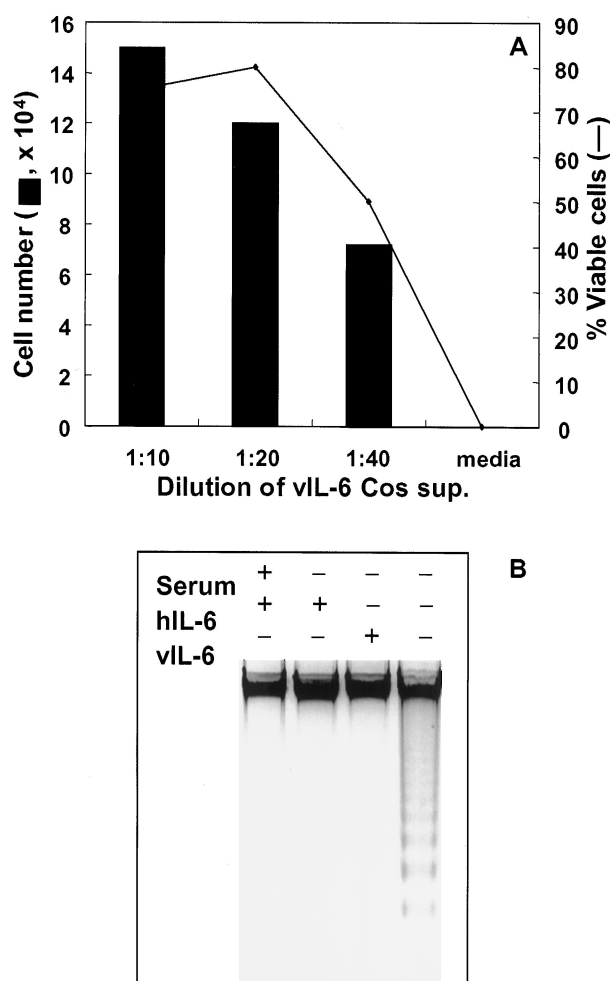
by vIL-6 in HepG2 cells was unaffected by anti-hIL-6R (47). These results demonstrate that neutralizing Abs to hIL-6 and hIL-6R do not abrogate responsiveness to vIL-6. Consistent with this view are the data indicating that a transfected cell line expressing gp130 without IL-6R remained responsive to vIL-6 (47). These studies suggest that vIL-6 and hIL-6 have similar biological activities, with differences in cell surface signaling suggesting agonistic effects.

We next examined the effects of vIL-6 on known hIL-6 signaling cascades, first determining whether vIL-6 induced phosphorylation of JAK kinases and gp130. Our data showed that both vIL-6 and hIL-6 trigger phosphorylation of gp130. Given that activated JAK kinases phosphorylate gp130 (31), we next determined whether vIL-6 triggered activation of JAK kinases. Our data showed that both vIL-6 and hIL-6 induce phosphorylation of JAK1 and that hIL-6 also activates JAK2. These data therefore demonstrate JAK kinase activation in association with proliferation triggered by both cytokines and implicate JAK1 in vIL-6 signaling. These data are consistent with those of Molden *et al.* (47), who demonstrated that vIL-6 triggered rapid induction of JAK1, but not JAK2 or Tyk2, in HepG2 cells.

To examine downstream signaling, we next determined whether MAPK, STAT1, and/or STAT3 were activated by vIL-6. In our study, both vIL-6 and hIL-6 induced activation of MAPK in MH60 and B9 cells. These data are consistent with our prior studies of hIL-6 signaling in B9 and patient MM cells

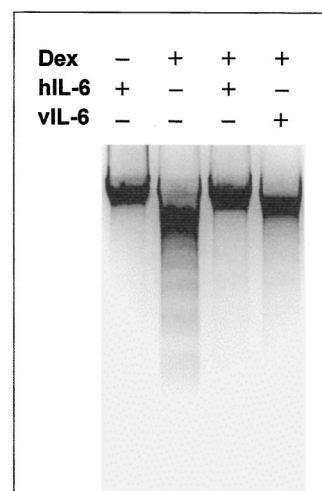
(45, 46). Together they suggest that the ras-raf/MAPK cascade can be triggered by both vIL-6 and hIL-6. As in our prior studies (45), both STAT1 and STAT3 were activated by hIL-6; the present studies also showed that vIL-6 triggers phosphorylation of STAT3. Molden *et al.* (47) have shown that vIL-6 induces DNA-binding complexes consisting of both STAT1 and STAT3 in HepG2 cells. Therefore, the current study, taken together with our (45, 46) and other (47) prior reports, suggests that vIL-6, like hIL-6, can induce both ras-raf MAPK and JAK/STAT pathways, and in particular implicate JAK1 and STAT3 in vIL-6 signaling in the MH60 and B9 cells examined in this study.

In our prior studies characterizing signaling in B9 and human MM cells, we confirmed the biological relevance of ras-raf/MAPK activation, using MAPK blockade (45). Specifically, inhibiting MAPK activation with MAPK antisense oligonucleotide abrogated the proliferative response to hIL-6. In the present study, we blocked MAPK activation using an alternative strategy, namely the MEK1 inhibitor PD98059 (50). Proliferation of both MH60 and B9 cells in response to vIL-6 and hIL-6 was inhibited by blockade of MAPK activation in this fashion, suggesting the importance of the ras-raf/MAPK cascade for proliferation induced by both of these cytokines. The present studies therefore demonstrate activation of the ras-raf/MAPK and JAK/STAT pathways by vIL-6. They are consistent with our prior studies demonstrating activation of both of these pathways by hIL-6 in B9 and human MM cells (45) as well as in PC12 cells (51).



**Fig. 9** Effect of vIL-6 on viability of serum-starved B9 cells. **A**, B9 cells ( $1 \times 10^4$ /ml) were washed and cultured for 10 days with medium or with vIL-6 (vIL-6 Cos sup.; vIL-6 Cos supernatant diluted 1:10 to 1:40 in medium) in the absence of serum. The number of cells (■) and the percentage of viable cells (—) at 10 days were determined by enumeration using trypan blue exclusion. **B**, B9 cells ( $2 \times 10^6$ ) were cultured in serum-free medium in the presence or absence of hIL-6 (50 ng/ml) or vIL-6 Cos supernatant (1:2 dilution) for 48 h. Control cultures were maintained in medium containing serum and supplemented with hIL-6. Genomic DNA was purified, electrophoresed on 2% agarose gel, and analyzed under UV light for DNA fragmentation. —, absent from culture medium; +, present in culture medium.

Our and other studies have shown that hIL-6 is also an antiapoptotic factor (21–26), and we therefore determined whether vIL-6 acted similarly. We showed that vIL-6, like hIL-6, confers protection from serum starvation (B9 and MH60 cells) or Dex-induced apoptosis (MM.1S human MM cells). Our previous studies demonstrated that Dex-induced apoptosis of MM.1S MM cells occurs independently of stress-activated protein kinase activation (24), is associated with down-regulation of MAPK and p70<sup>S6K</sup> (24), and is associated with mitochondrial cytochrome c release into the cytosol (25). Most recently, we have shown that Dex-induced apoptosis of MM.1S cells requires activation of related adhesion focal tyrosine kinase, and that



**Fig. 10** Effect of vIL-6 on apoptosis of MM.1S cells induced by Dex. MM.1S cells were incubated with 20  $\mu$ M Dex in the presence or absence of hIL-6 or vIL-6 Cos supernatant (1:2 dilution) for 48 h. Genomic DNA was purified, electrophoresed on 2% agarose gel, and analyzed under UV light for DNA fragmentation. —, absent from culture medium; +, present in culture medium.

hIL-6 blocks both activation by related adhesion focal tyrosine kinase and apoptosis of these cells triggered by Dex (26).

Catlett-Falcone *et al.* (52) recently demonstrated that constitutive activation of STAT3 in U266 human MM cells confers resistance to apoptosis, suggesting that STAT3 phosphorylation triggered by hIL-6 and vIL-6 in this study may also be protective. Ongoing studies are evaluating the signaling cascades whereby hIL-6, as well as vIL-6, mediate Dex resistance, to determine whether they act similarly and to design strategies to overcome drug resistance. Finally, because vIL-6 is a multifunctional cytokine that promotes hematopoiesis, plasmacytosis, and angiogenesis, future studies delineating the mechanisms mediating these effects may help characterize its role in the pathogenesis of KSHV-associated disorders (53, 54).

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