

Human Herpesvirus 8 Viral Interleukin-6 Signaling through gp130 Promotes Virus Replication in Primary Effusion Lymphoma and Endothelial Cells

Emily Cousins, Yang Gao, Gordon Sandford, John Nicholas

Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

The contributions of human herpesvirus 8 (HHV-8) viral interleukin-6 (vIL-6) to virus biology remain unclear. Here we examined the role of vIL-6/gp130 signaling in HHV-8 productive replication in primary effusion lymphoma and endothelial cells. Depletion and depletion-complementation experiments revealed that endoplasmic reticulum-localized vIL-6 activity via gp130 and gp130-activated signal transducer and activator of transcription (STAT) signaling, but not extracellular signal-regulated kinase (ERK) activation, was critical for vIL-6 prereplication activity. Our data significantly extend current understanding of vIL-6 function and associated mechanisms in HHV-8 biology.

Human herpesvirus 8 (HHV-8) is associated with multicentric Castleman's disease, primary effusion lymphoma (PEL), and Kaposi's sarcoma (1–3). HHV-8-encoded viral interleukin-6 (vIL-6) promotes cell growth and survival, inflammation, and angiogenesis and is implicated in pathogenesis (4–9). However, its role in normal virus biology remains unclear. Viral IL-6 and its human IL-6 (hIL-6) homolog share ~25% sequence identity, fold into similar three-dimensional structures, and initiate signaling through similar complexes (10–15). Human IL-6 forms hexameric signaling complexes comprised of two gp80 receptor subunits, two gp130 signal transducers, and two hIL-6 molecules (gp80₂-gp130₂-IL-6₂) (10). Viral IL-6 can also form signaling hexamers at the cell surface, but its unique signaling from the endoplasmic reticulum (ER) occurs via tetrameric complexes (gp130₂-vIL-6₂) exclusively (8, 12, 15, 16). Viral IL-6 can be expressed during latent stages, in addition to lytic stages, of the viral life cycle (8, 17). Latently infected PEL cell viability is maintained in part via vIL-6/gp130 signaling from the ER and also through its interaction with vitamin K epoxide reductase complex subunit 1 variant 2 (VKORC1v2) (7, 18); the latter contributes to productive replication as well (19). Upon vIL-6-induced gp130 dimerization, phosphorylation of specific gp130 tyrosine residues initiates either signal transducer and activator of transcription 1 and 3 (STAT1/3) (Y₇₆₇, Y₈₀₄, Y₉₀₅, and Y₉₁₅) or mitogen-activated protein kinase (MAPK) (Y₇₅₉) signaling (20–23). Activated STAT3 and extracellular signal-regulated kinase 1 and 2 (ERK1/2) have been shown to promote growth and survival of PEL cells, and vIL-6/gp130 signaling contributes (7, 24). The goal of this study was to determine the role of vIL-6/gp130 signaling in HHV-8 lytic replication in PEL and endothelial cells.

gp130 and vIL-6 promote HHV-8 replication in PEL and endothelial cells. The gp130 signal transducer was depleted in TRExBCBL1-replication and transcription activator (RTA) and JSC-1 PEL cells (26, 27) and in TIME-TRE/RTA endothelial cells (28) latently infected with HHV-8 r219 (29) using previously established techniques (7, 8). Following depletion, lytic replication was induced with doxycycline (Dox) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (NaB). HHV-8 was collected from PEL and endothelial cell culture media, and infectious viral titers were determined as undertaken previously (19).

Depletion of gp130 led to decreased HHV-8 titers in PEL (~3-fold) and endothelial cell (>8-fold) cultures (Fig. 1A, B, and C). Similarly, vIL-6 depletion in endothelial cells reduced viral titers by ~3-fold (Fig. 1D). Rates of reactivation, measured by ORF59 antigen immunofluorescence (PEL cells; data not shown) and red fluorescent protein (RFP) imaging (Fig. 1C and D, bottom), were equivalent in depleted and control cultures. Combined, these data identify the importance of gp130 for productive replication in PEL and endothelial cells and the involvement of vIL-6 in support of productive replication in endothelial cells in addition to BCBL-1 and JSC-1 cells, which we previously reported (19). In contrast to effects on reactivated productive replication, gp130 or vIL-6 depletion had no substantial effect on latency titers (measured 3 days postinfection) following *de novo* infection of endothelial cells (Fig. 1E), a process known to involve a burst of expression of a subset of lytic genes, including vIL-6, prior to rapid latency establishment (30, 31).

ER-localized vIL-6/gp130 signaling contributes to productive replication. Dox-inducible TRExBCBL1-RTA PEL cells were used to test the ability of gp130 signaling-competent and ER-retained vIL-6 to rescue the vIL-6 depletion phenotype. As before, lentiviral vectors encoding either control (NS) or vIL-6 mRNA-directed short hairpin RNAs (shRNAs) were used for transduction. Depletion of vIL-6 led to reduced virus production (luciferase control), and this phenotype could be rescued with lentiviral vector-transduced vIL-6.KDEL (ER-retained [8]) but not with vIL-6.W₁₆₇G.KDEL (ER-retained and gp130 dimerization-incompetent in the ER [7, 32]) (Fig. 2). These data identify the involvement of ER-localized vIL-6/gp130 signaling in support of HHV-8 productive replication.

gp130-mediated STAT signaling, but not ERK signaling, supports HHV-8 replication. To determine the significance of gp130-

Received 17 June 2014 Accepted 27 July 2014

Published ahead of print 30 July 2014

Editor: R. M. Longnecker

Address correspondence to John Nicholas, nichojo@jhmi.edu.

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doi:10.1128/JVI.01751-14

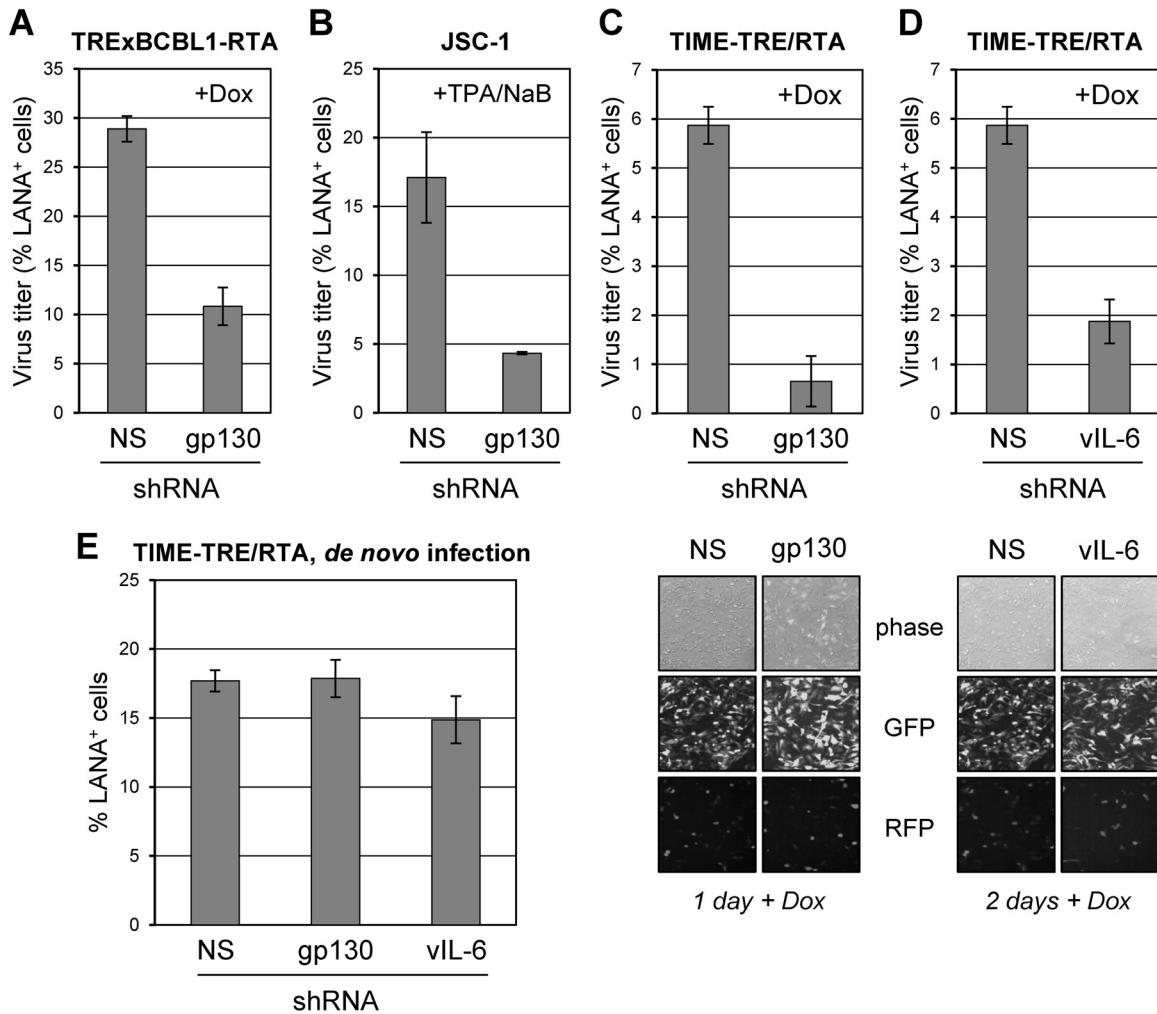


FIG 1 Involvement of gp130 and vIL-6 in promotion of HHV-8 productive replication. (A) HHV-8 titers following gp130 depletion in TRExBCBL1-RTA cells. The gp130 signal transducer was depleted in TRExBCBL1-RTA cells using lentivirus-encoded shRNAs (nonsilencing [NS] control or gp130-specific) described previously (7). Two days posttransduction of lentiviral vectors, cell numbers were normalized and cultures were treated with doxycycline (1 $\mu\text{g}/\mu\text{l}$) to induce RTA expression and lytic reactivation. Media were collected following 4 days of induction, and virus was pelleted by ultracentrifugation. Aliquots of harvested virus were applied to naive TIME-TRE/RTA cells to determine infectious virus titers by staining for latency-associated nuclear antigen (LANA) as described previously (19). Multiple random fields were assessed to quantify the number of LANA-positive (LANA⁺) cells in the total [4',6'-diamidino-2-phenylindole (DAPI)-positive] population. Densities of viable (trypan blue-excluding) TRExBCBL1-RTA cells at the time of medium harvest were not negatively affected by gp130 transduction (1.31×10^6 and 1.41×10^6 cells per ml for NS shRNA-transduced duplicate cultures versus 1.75×10^6 and 1.83×10^6 cells per ml for gp130 shRNA-transduced cells). Frequencies of reactivation, as determined by immunostaining for ORF59-encoded early antigen, were unaffected (data not shown). (B) Importance of gp130 in HHV-8 replication in JSC-1 cells. JSC-1 cells were depleted of gp130 (as described for panel A) and induced with sodium butyrate (NaB; 0.5 μM) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 20 ng/ml) for 12 h and then continuously treated with TPA for 3 days. Released virus was pelleted from culture media and quantified as before. (C and D) Influence of gp130 and vIL-6 on HHV-8 replication in endothelial cells. TIME-TRE/RTA cells were first infected with HHV-8 r219 virus (29) at a multiplicity of infection yielding an infection rate (determined by the proportion of cells positive for green fluorescent protein [GFP]) of $\sim 90\%$; these cells were negative for RFP, confirming that they were latently infected. Cells were then transduced with shRNA-encoding lentiviral vectors (NS [control], gp130-specific, or vIL-6-specific [8]) 2 days post-HHV-8 infection and induced with doxycycline (1 $\mu\text{g}/\text{ml}$) 1 day post-lentiviral transduction. Lentiviral vector-encoded GFP, expressed above levels arising from latent r219 HHV-8 genomes, was used to monitor lentiviral infection, achieved in $>90\%$ of cells. HHV-8 reactivation was monitored by visualizing RTA-induced RFP expression from r219 viral genomes via fluorescence after doxycycline treatment (examples shown). Media containing virus were collected for 6 days and pooled prior to virus concentration and titration. TIME-TRE/RTA cells were visibly unaffected by gp130 or vIL-6 depletion, and rates of apoptosis (percent annexin V-Cy3⁺ cells) were equivalent in gp130- and vIL-6-depleted and NS shRNA-transduced cultures (data not shown). (E) NS, gp130, and vIL-6 shRNA-transduced TIME-TRE/RTA cells were infected with a subsaturating infectious dose ($\sim 50\%$ GFP⁺) of HHV-8 r219 2 days post-lentiviral transduction to determine possible effects of gp130 and vIL-6 depletion on *de novo* infection and establishment of latency, as determined by LANA staining 3 days post-HHV-8 r219 infection. For all panels, data were derived by counting LANA⁺ cells in multiple (>6) fields, together comprising 200 to 300 cells, to obtain the percentage of LANA⁺/DAPI⁺ cells. Average values from duplicate cultures are shown; error bars represent deviations of individual values from the means.

activated STAT versus ERK signaling in HHV-8 replication, we employed signaling-altered gp130 variants in gp130 depletion-rescue experiments. Four C-tail tyrosines of gp130 initiate STAT1/3 signaling, and one induces MAPK signaling (20–23). Lentiviral vector-

cloned gp130 open reading frames (ORFs) for complementation encoded wild-type gp130 (WT), gp130.STAT-Ys (Y₇₅₉F mutated; ERK1/2 signaling-null), gp130.ERK-Y (Y₇₅₉ only; STAT1/3 signaling-null), and gp130. Δ Ys (inactive). Functional testing in transfected

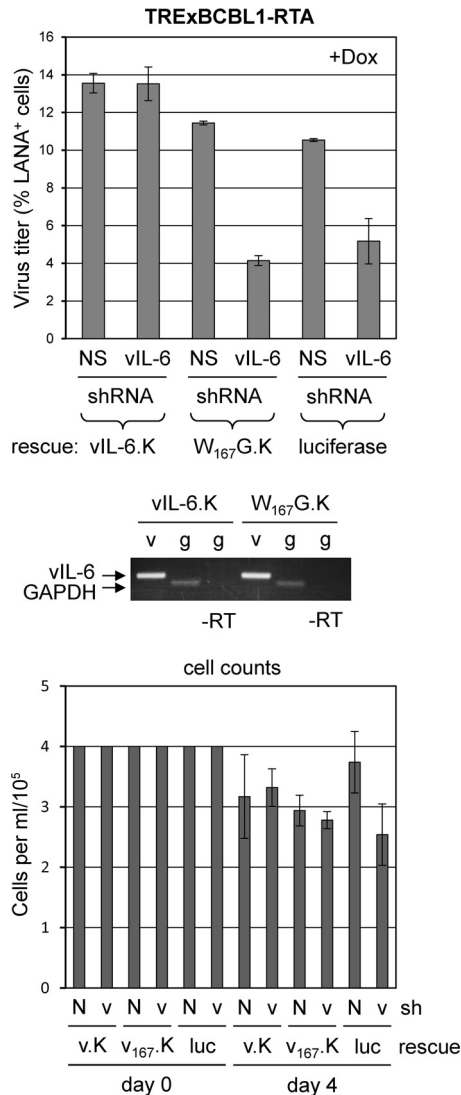


FIG 2 Importance of ER-localized vIL-6/gp130 interaction for HHV-8 replication. TRExBCBL1-RTA cells were transduced with lentiviral expression vectors encoding KDEL motif-tagged (ER-retained) and shRNA-resistant forms of vIL-6 (vIL-6.K) or vIL-6.W₁₆₇G (W₁₆₇G.K) (7, 8) or with lentivirus encoding luciferase (negative control). Two days posttransduction, cells were transduced with control (NS) shRNA-encoding lentivirus (N) or lentivirus expressing vIL-6-specific shRNA (v). Dually transduced cells were then normalized for cell density and induced the following day with doxycycline. Virus was collected and titers were determined as before (see Fig. 1 legend). RNA was harvested from a subset of cells, and cDNA was amplified for vIL-6.KDEL (7) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used for normalization and positive control) mRNA to verify expression of the introduced vIL-6 genes (v, vIL-6 reverse transcription [RT]-PCR; g, GAPDH RT-PCR; -RT, no reverse transcriptase). Densities of viable cells at the time of Dox treatment (day 0, normalized) and at virus harvest (day 4) are shown (bottom panel). v.K, vIL-6-KDEL; v₁₆₇.K, vIL-6.W₁₆₇G-KDEL; luc, luciferase.

cells verified that in the presence of vIL-6, introduced WT and gp130.STAT-Ys induced STAT3 phosphorylation above levels supported by endogenous gp130, and gp130.ERK-Y exclusively induced phospho-ERK levels (Fig. 3A). Unexpectedly, WT gp130 did not support detectable ERK signaling in response to vIL-6, but the same result was seen for hIL-6 in the presence of overexpressed gp80 (data not shown). In TRExBCBL1-RTA-

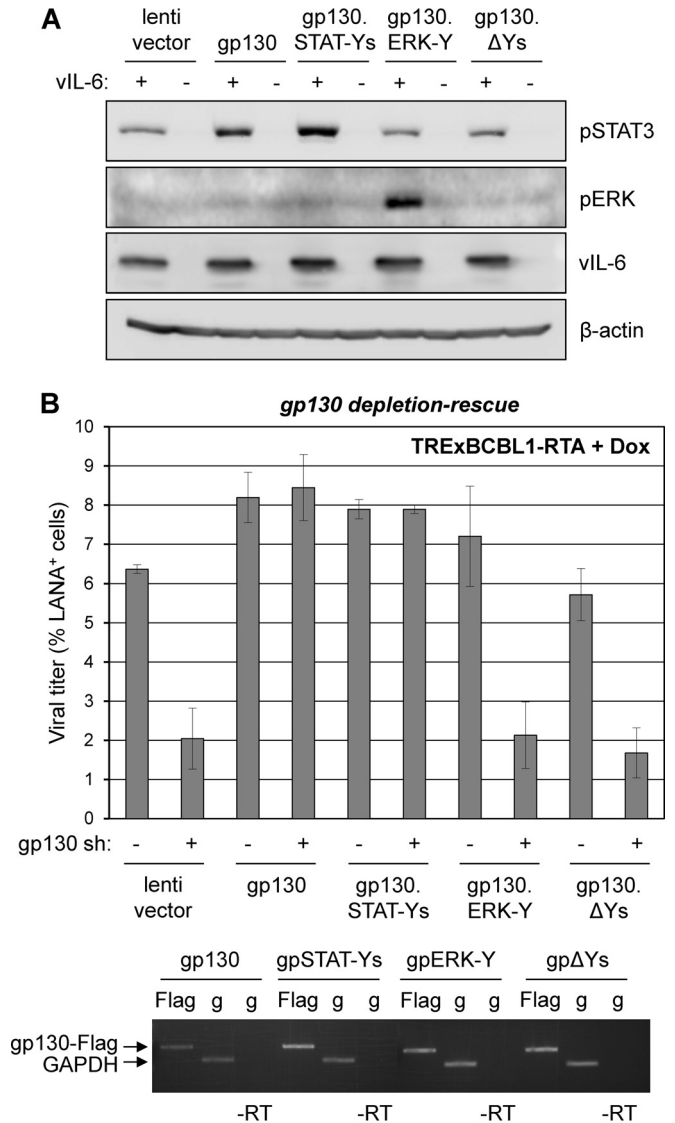


FIG 3 Contributions of gp130-mediated STAT and ERK signaling to HHV-8 productive replication. (A) Functional assessment of gp130 signaling-tyrosine variants. HEK293T cells were cotransfected with empty vector (-) or vIL-6 plasmid (+) together with empty lentiviral vector or lentiviral vectors expressing wild-type gp130, gp130.STAT-Ys (Y₇₅₉F-mutated), gp130.ERK-Y (Y₇₅₉ only; other C-tail tyrosines mutated to F), or gp130.ΔYs (pan-Y-to-F-mutated). Two days posttransduction, cells were lysed for Western blot analysis to detect phospho-STAT3 (pSTAT3) and pERK as indicators of gp130-activated STAT and MAPK pathways. (B) Role of STAT and ERK signaling in vIL-6/gp130 promotion of HHV-8 replication. TRExBCBL1-RTA PEL cells were transduced with empty lentiviral vector (pDUET001 [39]) or vectors expressing Flag-tagged wild-type gp130, gp130.STAT-Ys, gp130.ERK-Y, or gp130.ΔYs. Two days posttransduction, cells were transduced with either NS (control) or gp130-specific shRNA-encoding lentiviral vectors. Following doxycycline-induced HHV-8 reactivation for 4 days, virus was collected and titers were determined as described previously. To verify appropriate expression of wild-type gp130 and the tyrosine-mutated gp130 variants, we lysed a subset of cells in TRIzol for preparation of RNA samples. RNA was reverse transcribed and amplified using 3'-Flag- and 5'-gp130-directed primers (TA GATGGCGGTGATGGTATTT and CTTGTCATCGTCGCTTGTGA) to assess expression of transduced gp130-Flag mRNAs. GAPDH-specific primers were used for normalization and to provide a positive control. Flag, gp130-Flag RT-PCR; g, GAPDH RT-PCR; -RT, no reverse transcriptase.

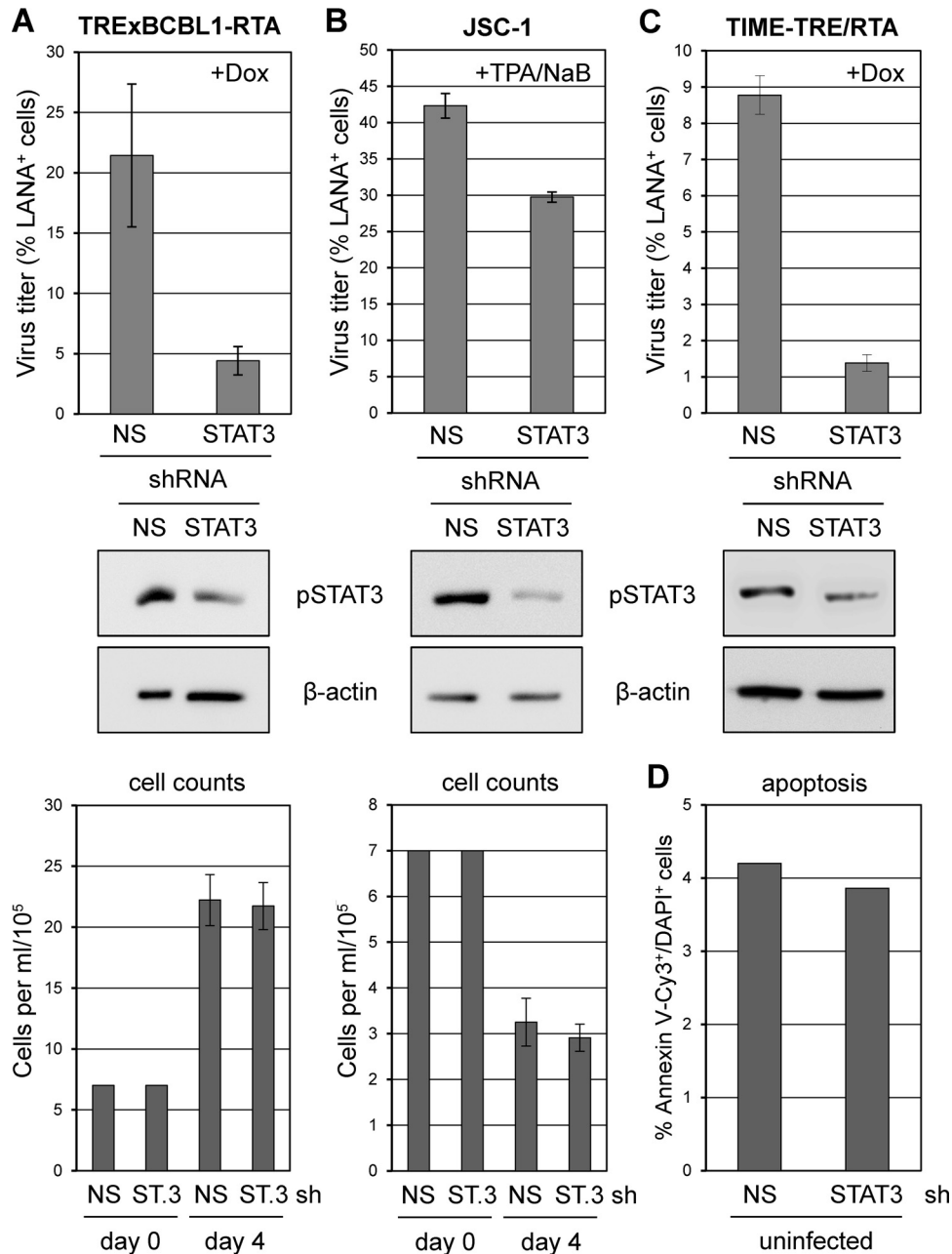


FIG 4 Role of STAT3 in HHV-8 replication in PEL and endothelial cells. (A) TRExBCBL1-RTA cells were transduced with control (NS) and STAT3 (ST.3)-specific shRNA-encoding lentiviral vectors. After 2 days, lytic reactivation was induced with doxycycline for 4 days, and virus was then collected and concentrated from culture media for titration. Experimental procedures and data collection and calculations were as outlined in the legend to Fig. 1. Subsets of transduced cells were harvested at the time of induction and lysed for Western blot analysis to verify STAT3 depletion. Densities of viable (trypan blue-excluding) cells were normalized at the time of lytic induction (day 0) and determined at the end of the experiment (day 4) (bottom panel). (B) The same analyses were undertaken for JSC-1 cultures induced with TPA/NaB and harvested after 4 days for virus titration. A subset of cells was lysed for Western blotting at the time of induction to verify STAT3 depletion. Densities of viable cells at the start and end of the experiment are shown (bottom). (C) Doxycycline-induced TIME-TRE/RTA cultures were analyzed in the same way except that virus was harvested following 6 days of doxycycline treatment; parallel cultures were harvested at the time of induction for Western blot analysis. Cultures were not visibly affected by STAT3 depletion during the course of infection (not shown). (D) Rates of apoptosis (% annexin V-Cy3⁺/DAPI⁺ cells) in similarly transduced TIME-TRE/RTA cultures were unaffected by STAT3 depletion.

based depletion-complementation experiments, WT and gp130. STAT-Ys, but not gp130.ERK-Y or negative controls gp130.ΔY and empty vector, fully rescued replication (Fig. 3B), indicating that STAT signaling alone is sufficient for vIL-6/gp130-mediated support of HHV-8 replication.

Contribution of STAT3 to HHV-8 replication. As previous reports have demonstrated the importance of STAT3 for latent PEL cell proliferation and viability (7, 24), we examined its role in productive replication. STAT3 depletion led to reduced HHV-8 titers induced from both PEL and endothelial cell cultures, al-

though effects in TPA/NaB-induced JSC-1 cultures were modest despite robust STAT3 depletion (Fig. 4). The latter may reflect different PEL cell-specific thresholds of STAT3 functionality or different dependencies on STAT3 for support of HHV-8 replication. It is important to note that viable cell densities at the end of the experiment were equivalent in NS and STAT3-directed shRNA-transduced cultures (Fig. 4A and B, bottom), indicating that proliferative and antiapoptotic activities of STAT3 operating in latently infected PEL cells (7, 24) do not account directly for diminished replication resulting from STAT3 depletion in reactivated cultures. Also, STAT3 depletion in TIME-TRE/RTA cells had no detectable effect on cell viability (Fig. 4D). Our data show that STAT3 supports HHV-8 replication, and together with the vIL-6 and gp130 depletion-rescue data (Fig. 2 and 3) indicate that vIL-6/gp130 signaling via STAT3 contributes significantly to the prereplication activities of vIL-6 and gp130.

The likely role of vIL-6 in pathogenesis has been described in numerous reports (4–9). However, investigation of the role of vIL-6 in HHV-8 replication is restricted to one study of vIL-6-null virus replication in (HHV-8-negative) BJAB cells, which noted no phenotype (33), and our own preliminary vIL-6 depletion experiments in PEL cells (19). The present study significantly extends these previous reports, demonstrating that vIL-6 promotes HHV-8 productive replication in endothelial cells in addition to PEL cells, that gp130 contributes significantly to HHV-8 replication in these cell types, that vIL-6 activity via gp130 can be mediated largely or exclusively through ER-localized signaling via tetrameric complexes, and that STAT3 signaling, but not gp130-activated MAPK signaling, is likely critical for vIL-6/gp130-enhanced replication. While it is possible that gp130-activated STAT1 signaling could also contribute, the lack of detectable effects of STAT1 depletion on PEL cell growth (7) coupled with the well-established role of STAT1 in promotion of antiviral interferon and apoptotic signaling (34) indicates that this is unlikely. It is noteworthy that previous studies have reported the importance of STAT3 signaling for the replication of viruses, including varicella-zoster virus (VZV) and hepatitis C virus (35, 36). For VZV, STAT3-activated survivin was found to be involved in prereplication activity, reflecting the prosurvival activities of STAT3 via survivin reported for PEL cells (24). However, human cytomegalovirus blocks STAT3 phosphorylation, though it requires it for optimal replication, and mouse cytomegalovirus induces phosphorylation of STAT3 but not STAT3-responsive cellular genes, suggesting novel, virus-redirection activities of the transcription factor (37, 38). Our findings of vIL-6/gp130 and STAT3 involvement in HHV-8 productive replication could facilitate the development of therapeutic interventions for the treatment of HHV-8-associated diseases, to which productive replication contributes significantly.

ACKNOWLEDGMENTS

We thank Daming Chen for technical and scientific advice.

This work was supported by NIH grant R01-CA76445 to J.N. and NIH Ruth Kirschstein Fellowship F31-CA171933 to E.C.

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