ROLE OF HUMAN HERPESVIRUS 8 VIRAL INTERLEUKIN-6 SIGNALING IN VIRUS BIOLOGY

by
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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
December 2014

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ABSTRACT

Human herpesvirus 8 (HHV-8) is associated with B cell and endothelial tumors. HHV-8 encodes a viral homolog of interleukin 6 (vIL-6), which promotes the proliferation and survival of virally infected and neighboring cells and induces angiogenesis, suggesting that vIL-6 contributes to HHV-8 malignant pathogenesis. Furthermore, vIL-6 is expressed during viral latency and lytic replication, allowing vIL-6 to function in viral latency maintenance and virus productive replication.

Prior to this dissertation research, little was known regarding the role of vIL-6 interactions with its signal transducer (gp130) within the endoplasmic reticulum (ER), where vIL-6 predominantly localizes. High levels of phosphorylated STAT3, a product of vIL-6 signaling, had been observed in HHV-8+ primary effusion lymphomas (PEL), but the mechanism behind STAT3 activation was unclear. The effects of vIL-6 on virus replication were limited to one published study, which failed to detect any influence of vIL-6 in a model culture system. Mechanistically, vIL-6 is distinct from its cellular counterparts because it can signal from the ER, but the biological functions of ER-localized vIL-6/gp130 signaling were unknown. Therefore, the goals of this dissertation were to further characterize ER-localized vIL-6/gp130 interactions, investigate the mechanism(s) responsible for increased STAT3 activation in PEL cells, decipher the roles of gp130-activated STAT and ERK signaling in PEL cell maintenance, and evaluate the role of vIL-6 and the vIL-6/gp130 interaction in HHV-8 replication.

Studies performed during this dissertation work demonstrated that ER-localized vIL-6/gp130 signaling in PEL cells contributes to high levels of active STAT3, that vIL-6/gp130 signaling also activates STAT1 and ERK1/2 in PEL cells, that ER-localized vIL-6/gp130 signaling can sustain PEL cell growth and viability, and that STAT3 and ERK1/2 are crucial for cell growth and survival. In addition to promoting the growth and survival of latently infected PEL cells, vIL-6 and gp130 also promote productive virus replication in
PEL and endothelial cells. In PEL cells, vIL-6 pro-replication effects require its interaction with gp130 in the ER, where vIL-6/gp130-mediated STAT signaling, and not MAPK signaling, is critical. These studies have expanded our understanding of how vIL-6 functions in normal virus biology and have opened new avenues for targeted HHV-8 therapy.

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ACKNOWLEDGEMENTS

Many individuals have supported me during my graduate training at the Johns Hopkins University School of Medicine. First and foremost, I must acknowledge my mentor, John Nicholas. My development as a scientist can be attributed to John’s patience and guidance throughout my time in his laboratory. John taught me how to design experiments with proper controls, how to write scientific articles and grant applications, and how to develop a story worthy of publication. Additionally, John fostered a collaborative environment in the laboratory in which techniques, reagents, and expertise were shared. I would also like to thank the other members of the Nicholas lab for their guidance, instruction, and friendship: Daming Chen, Young Choi, Gordon (Sandy) Sandford, Yang Gao, and Kihyun Yoo. Additionally, much of my dissertation research was funded by a Ruth L. Kirschstein F31 fellowship (5F31CA171933), and I am grateful for the support of this program.

Prior to joining the Nicholas laboratory, several other scientists taught and encouraged me. My first foray into research occurred during my undergraduate studies at Clemson University. My mentor at Clemson, Michael Sehorn, is passionate about science (and college football), and his continued support and guidance throughout my scientific training has been incredibly valuable. Additionally, my research rotations with Joel Pomerantz and Kathryn Wagner were both fun and instructive; Joel and Kathryn are both gifted scientists, and they have graciously advised me throughout my time at Hopkins.

Furthermore, I owe my gratitude to members of my thesis committee, whose time and expertise were crucial to the development of my thesis project. Young Choi, Diane Hayward, Kuan-Teh Jeang, and Gary Ketner were instrumental in the design and execution of my research projects. Interactions with my thesis committee encouraged me to ask the right research questions, to determine the best method for answering my
research questions, and to think about my career after graduation. Their help and support cannot be overstated.

The training environment within the Graduate Training Program in Cellular and Molecular Medicine encourages collaboration and friendship from the very beginning. I would like to thank all of my classmates for making graduate school enjoyable. Afternoon coffee breaks with friends and fellow students were not only stress-relieving, but these discussions often yielded new approaches for circumventing experimental failures. Thank you, Adam Moyer, Laurene Cheung, Laura Gottschalk, Sara Sinclair, deMauri Mackie, Emily Chang, Kate Laws, and Justin Glenn.

I also want to acknowledge and thank my family for their love and support throughout this journey. I attribute my love of science to my parents, Shawn and Susan Gaunt, who have spent their careers in the medical field. They have always encouraged me and my siblings (Patrick, Benjamin, Christine, and Leah) to study hard, help others, and explore new things.

Lastly, I could not have completed my PhD without the love, encouragement, and support of my husband, Matthew Cousins. Having a spouse understand not only the time commitment of research but also one that you can bounce scientific ideas off of is incredibly helpful. Learning how to be parents to Mark may be our biggest challenge yet, but I have no doubt that we will succeed together.
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<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CatD</td>
<td>cathepsin D</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>deoxynucleotide triphosphate</td>
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<td>dithiothreitol</td>
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<td>Epstein-Barr virus (HHV-4)</td>
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<td>ER</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FLICE</td>
<td>FADD-like interleukin-1 beta-converting enzyme</td>
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<td>gB</td>
<td>glycoprotein B</td>
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<td>GC</td>
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<td>GSK3β</td>
<td>glycogen synthase kinase-3 beta</td>
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<td>HAART</td>
<td>highly active antiretroviral therapy</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>IL6R</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<td>KICS</td>
<td>KSHV inflammatory cytokine syndrome</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>KS</td>
<td>Kaposi's sarcoma</td>
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<td>KSHV</td>
<td>Kaposi's sarcoma-associated herpesvirus (HHV-8)</td>
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<td>LANA</td>
<td>latency-associated nuclear antigen</td>
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<td>LB</td>
<td>Luria broth</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MCD</td>
<td>multicentric Castleman's disease</td>
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<td>MCMV</td>
<td>murine cytomegalovirus</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MIR</td>
<td>modulator of immune recognition</td>
</tr>
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<td>miRNA</td>
<td>micro ribonucleic acid</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>N</td>
<td>asparagin</td>
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<td>NaB</td>
<td>sodium butyrate</td>
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<td>NK</td>
<td>natural killer</td>
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<td>ORF</td>
<td>open reading frame</td>
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<td>OX-2</td>
<td>orexin receptor 2 (CD 200)</td>
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<td>PAN RNA</td>
<td>polyadenylated nuclear ribonucleic acid</td>
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<td>PBS</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PEL</td>
<td>primary effusion lymphoma</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PPMO</td>
<td>peptide-conjugated phosphorodiamidate morpholino oligomer</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma protein</td>
</tr>
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<td>PS</td>
<td>phosphatidylserine</td>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>Rta</td>
<td>replication and transcriptional activator</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<td>ROS</td>
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<td>SCID</td>
<td>severe combined immunodeficiency</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>shRNA</td>
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<td>SOCS3</td>
<td>suppressor of cytokine signaling 3</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>STAT3D</td>
<td>dominant negative signal transducer and activator of transcription</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
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<tr>
<td>TIME</td>
<td>telomerase-immortalized microvascular endothelial</td>
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<tr>
<td>TK</td>
<td>thymidine kinase</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
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<td>TR</td>
<td>terminal repeat</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
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<td>UTR</td>
<td>untranslated region</td>
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<td>vCCL-2</td>
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<tr>
<td>vFLIP</td>
<td>viral FLICE-inhibitory protein</td>
</tr>
<tr>
<td>vGPCR</td>
<td>viral G-protein-coupled receptor</td>
</tr>
<tr>
<td>xi</td>
<td></td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>vIRF</td>
<td>viral interferon regulatory factor</td>
</tr>
<tr>
<td>VKORC1v1</td>
<td>vitamin K epoxide reductase complex subunit 1 variant 1</td>
</tr>
<tr>
<td>VKORC1v2</td>
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</tr>
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<td>VKORC1v3</td>
<td>vitamin K epoxide reductase complex subunit 1 variant 3</td>
</tr>
<tr>
<td>VSV/G</td>
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<td>vIL-6</td>
<td>viral interleukin 6</td>
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<td>VZV</td>
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<td>western blot</td>
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<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
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<td>tyrosine</td>
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CHAPTER 1: INTRODUCTION
CLINICAL SIGNIFICANCE OF HUMAN HERPESVIRUS 8

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma-associated herpesvirus (KSHV), has been linked to three diseases in humans: Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). HHV-8 infection is required for the development of these neoplasias; however, disease progression is rare in immunocompetent hosts. These diseases are most prevalent in populations that are immunocompromised, such as those with acquired immunodeficiency syndrome (AIDS) and those who are immunosuppressed due to organ transplant or treatment of unrelated disease. It is believed that immunosuppression allows the virus to replicate and for latently infected cell populations to expand in the absence of host-immune pressures, leading to more rapid and aggressive disease progression. While KS, PEL, and MCD share an etiologic cause in HHV-8, they differ in target cell types, clonality of lesions, and host cell responses to viral infection.

KS is a distinctive condition that inspired one of the alternative names given to HHV-8, KSHV. Four forms of KS have been described: classical, AIDS-associated, endemic, and iatrogenic [1-8]. These classifications are based on geographical prevalence, disease progression, and other factors. Classical KS was first described by dermatologist Dr. Moritz Kaposi in 1872 [9]. This form of the disease is characterized by cutaneous lesions of the skin, which are commonly observed in the extremities [9]. Classical KS is most common in elderly men of eastern European and Mediterranean descent [10], but with the emergence of HIV/AIDS in Africa beginning in the 1980s, the AIDS-associated form of KS became more prevalent in this geographical region [11]. The prevalence of AIDS-associated KS has mirrored the prevalence of HIV infection in Africa, and the advent of highly active antiretroviral therapy (HAART) has led to a rapid decline in both HIV incidence and AIDS-associated KS [12]. AIDS-associated KS is the most aggressive form of KS and has the highest prevalence of the four subtypes [11,13].
Endemic KS is most commonly observed in African children and is characterized by high mortality rates [14]. Finally, iatrogenic KS is observed in transplant recipients that have undergone immunosuppressive therapy [15].

Nearly all KS lesions contain cells infected with HHV-8, though the fraction of virus-positive cells within each lesion varies widely (partially dependent on the stage of disease) [16,17]. HHV-8 infects endothelial cells, which subsequently become spindloid and proliferate rapidly [17]. Spindle cells have a distinct morphology and a unique gene expression profile; these cells express a mixture of blood and lymphatic endothelial cell markers [18-20]. KS lesions are generally polyclonal in nature, though clonal KS has also been observed [21,22]. Lesions are reddish, brown, or purple in color due to the induction of angiogenesis and extravasation of erythrocytes from malformed microvasculature.

Cytokine dysregulation has been implicated as a cause of KS. Pro-inflammatory factors promote the infiltration of immune cells into infected tissues. Cultured KS cells require basic fibroblast growth factor (bFGF), interleukins 1 and 6 (IL-1, IL-6), and platelet-derived growth factor (PDGF) for growth, and cells found in KS lesions express IL-6 and PDGF receptors [23-27]. Furthermore, cells in KS lesions secrete IL-1β, tumor necrosis factor alpha (TNFα), and IL-6 [23]. Virus infection drives this cytokine-rich environment, which promotes virus replication and the infection of new host cells that secrete additional cellular and viral cytokines, further exacerbating the infection.

HHV-8 is also the causative agent of PEL, a B-cell malignancy that involves the pleural, peritoneal, and pericardial spaces [28,29]. Infected B-cells express both B-cell and plasma cell markers (CD138, CD38, and CD23), suggesting that the HHV-8-infected cells are in fact immature B-cells that were in the process of differentiating into plasma cells when the virus promoted their proliferation and halted their differentiation [30-33]. PEL tumors are monoclonal, and each infected B-cell contains many (approximately 50) copies of the viral genome maintained as circular episomes [34,35]. PEL is generally
diffuse with no detectable mass, but solid tumors have also been reported [36]. Cases of PEL are most commonly observed in HIV-positive patients or other immunocompromised individuals; the median survival of patients with this aggressive cancer is only six months [37]. The initiation of HAART prior to PEL diagnosis is thought to improve clinical prognosis in patients co-infected with HIV [37].

Finally, HHV-8 is the etiologic agent of MCD, which is characterized by viral infection of plasmablastic B-cells within the mantle zone of B-cell follicles [38]. Unlike PEL, MCD is generally polyclonal, though cases of clonal MCD have been reported [39]. MCD progression in HIV-infected individuals is rapid; the disease is driven by the elevated expression of pro-inflammatory cytokines, including IL-6, IL-10, and vascular endothelial growth factor (VEGF) [40-43]. Furthermore, levels of IL-6 in MCD patients were inversely correlated with patient prognosis [40]. Recently, it was reported that six patients coinfected with HIV and HHV-8 presented with pathologies and symptoms similar to MCD, but these patients could not be classified as having MCD and did not develop MCD during follow-up of 3-60 months [44]. These patients had elevated levels of HHV-8-encoded viral IL-6 (vIL-6) (similar to levels observed in patients diagnosed with MCD) compared to patients with mild or severe KS [44]. Furthermore, those with MCD-like inflammatory disease also had significantly increased levels of serum human IL-6 (hIL-6), serum IL-10, and HHV-8 viral load compared to control patients with KS [44]. These levels were comparable to serum hIL-6 levels, IL-10 levels, and HHV-8 viral loads in MCD patients [44]. This MCD-like disease has since been termed KSHV inflammatory cytokine syndrome (KICS) [45]. In an AIDS patient presenting with KICS, circulating HHV-8 viral load was calculated as 5,300,000 copies per ml; this HHV-8 viral load is the highest value reported to date [46]. This patient also had high HHV-6A viremia, though the significance of HHV-6A infection as it relates to KICS is unclear [46]. KICS is thought to arise from the dysregulation of pro-inflammatory cytokines in the context of increased lytic replication of HHV-8 [45].
HHV-8 causes neoplasia in the context of an immunocompromised host. This virus has been linked to three malignancies that complicate the management of infectious and induced immunodeficiencies. vIL-6 is believed to contribute to all three diseases via its pro-proliferative, anti-apoptotic, pro-angiogenic, and pro-inflammatory activities. Though HHV-8 is unique in its possession of vIL-6 and other virus-specific genes, this virus shares many characteristics with other human herpesviruses.

HUMAN HERPESVIRUSES

Herpesviruses are ancient (approximately 180-220 million years old) [47]. Members of the family Herpesviridae can infect a variety of hosts, including humans, birds, pigs, mice, elephants, and oysters [47]. Each of the herpesviruses has a linear, double-stranded, deoxyribonucleic acid (DNA) genome, a lipid bilayer envelope surrounding the tegument, and an icosahedral capsid architecture (triangulation T=16) [48]. Herpesvirus genomes range in size from approximately 125-225 kilobases (kb) and contain 70-200 open reading frames (ORFs) [48]. Mature virions measure approximately 120-260 nanometers in diameter [49]. Of the greater than 100 identified members of the herpesvirus family, eight infect humans [50]. The eight known human herpesviruses fall into three subfamilies: Alphaherpesvirinae (α), Betaherpesvirinae (β), and Gammaherpesvirinae (Ɣ) [51].

Alphaherpesviruses include herpes simplex virus 1 (HSV-1; also called HHV-1), herpes simplex virus 2 (HSV-2; also called HHV-2), and varicella-zoster virus (VZV; also called HHV-3). HSV-1 and HSV-2 are responsible for cold sores and genital warts, respectively. During outbreaks, HSV-1 and HSV-2 are reactivated from host neuronal cells, and virions are shed from the oral or genital mucosa. VZV is associated with both chicken pox, a primary infection characterized by a vesicular skin rash, and shingles, a reactivation of latent VZV from neural ganglia. The alphaherpesviruses are characterized
by accelerated replication in culture, efficient cell lysis of infected host cells, and diverse cell tropism compared to members of Betaherpesvirinae and Gammaherpesvirinae [48].

The betaherpesvirus subfamily includes human cytomegalovirus (HCMV; formally HHV-5), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7). HCMV infects cells of the salivary glands, causing characteristic cytomegaly [48]. HCMV has the largest genome of the eight human herpesviruses at 235 kb and encodes more than 200 ORFs [52]. HCMV infection is generally asymptomatic in immunocompetent individuals; however, HCMV infection of immunocompromised individuals can result in pneumonia, dyspepsia, meningitis, and pericardial effusions [53-56]. Though highly prevalent in children, HHV-6 (95% seropositive by age 3) and HHV-7 (90% seropositive by age 5) have minor clinical significance [57,58]. HHV-6 causes roseola infantum, and HHV-7 is often associated with concurrent HHV-6 infection, though HHV-7 infection alone can also cause roseola infantum [59-61]. Roseola infantum, also known as exanthema subitum, is characterized by high fevers and a morbilliform rash that first appears on the trunk and later on the face and extremities [62]. Viruses within the betaherpesvirinae subfamily undergo slow replication and spread in culture and have asynchronous lytic cycles [48].

The gammaherpesviruses include Epstein-Barr virus (EBV; formally HHV-4) and HHV-8 (also called KSHV). According to the 2013 release from the International Committee on the Taxonomy of Viruses, EBV can be further classified as a member of the lymphocryptovirus (gamma-1) genus while HHV-8 has been placed within the rhadinovirus (gamma-2) genus. EBV is associated with infectious mononucleosis, Burkitt’s lymphoma, Hodgkin’s lymphoma, and nasopharyngeal carcinoma. As noted previously, HHV-8 is the causative agent of KS, MCD, and PEL [28,38,63]. Gammaherpesviruses exhibit latent tropism for lymphocytes (with EBV and HHV-8 also infecting epithelial and endothelial cells, respectively), have transforming capabilities, and are more likely to be oncogenic in
immunocompromised hosts. HIV-positive individuals and those having received organ transplants are particularly susceptible to EBV- and HHV-8-associated cancers [64].

**HUMAN HERPESVIRUS GENOME ORGANIZATION**

Though each of the human herpesviruses has a complement of unique genes, these viruses also share sets of genes encoded in conserved gene blocks. Though the relative orientations and genomic locations of the gene blocks differ between the herpesvirus subfamilies (α, β, and γ), the genes encoded within a specific gene block are maintained in the same relative arrangement. Many of these conserved genes (>40) are involved in viral entry, replication, virion packaging, virion maturation, and egress [65]. Enzymes involved in basic metabolism, including thymidine kinase (TK), ribonucleotide reductase, and uracil-DNA glycosylase, are also encoded within the genomes of all human herpesviruses. Many viral structural proteins that make up the virion capsid are also conserved within the human herpesviruses; these capsid proteins include the major capsid protein, small capsid protein, triplex monomer, portal protein, and portal capping protein [65].

A number of the human herpesviruses encode “accessory” (non-core) cellular gene homologs. Frequently, these genes are specific to one or a few members of Herpesviridae. These genes encode proteins that function as chemokines (CXC chemokines in HCMV, CC chemokines in HHV-8), cytokines (vIL-10 in EBV and vIL-6 in HHV-8), and cell cycle regulators (vCyc in HHV-8) [66-73]. Several anti-apoptotic proteins are also encoded by human herpesviruses (e.g., vBcl-2 in EBV and HHV-8 and viral FLICE-inhibitory protein [vFLIP] in HHV-8) [74-78]. Additionally, microRNAs (miRNAs) are encoded by several herpesviruses, including HSV-1, HSV-2, HCMV, HHV-6, EBV, and HHV-8 [79].
HISTORY AND UNIQUE FEATURES OF HHV-8

HHV-8, the eighth human herpesvirus to be identified, was discovered in 1994 using representational difference analysis [63]. HHV-8 DNA sequences were found in KS lesions from an HIV-positive patient but not in adjacent unaffected tissue [63]. Shortly thereafter, the virus was also causally linked to PEL and MCD [38,80].

HHV-8 encodes at least 90 ORFs within its genome of approximately 170 kb (Figure 1.1) [34,63]. Guanine-cytosine (GC)-rich terminal repeat (TR) sequences are present at both ends of the genome and comprise about 30 kb of genomic sequence [81]. The HHV-8 ORFs that are shared with herpesvirus saimiri (the first-sequenced gamma-2 herpesvirus) are designated ORF1 to ORF75 starting at the left end of the genome, and the HHV-8-unique ORFs are designated K1-K15 and are located at particular regions of gammaherpesvirus genome divergence (Table 1.1) [82]. The HHV-8-specific ORFs are also numbered from left to right within the genome. In addition to these protein-encoding ORFs, 12 microRNAs (miR-K12-1 to miR-K12-12) have recently been identified within the HHV-8 genome (Tables 1.2). The majority (10) of these miRNAs are located between ORF71 and K12, while the other two miRNAs are located within the 3’ untranslated region (UTR) and coding sequence of K12 [83,84]. These miRNAs facilitate host immune system evasion, cell cycle regulation, latency maintenance, inhibition of apoptosis, promotion of angiogenesis, growth signaling, and chromatin modification [83,84].

HHV-8 LYTIC CYCLE

Immediately upon primary infection of a cell by HHV-8, a burst in lytic gene expression is triggered and quickly aborted in favor of latency. Under normal conditions, the virus remains latent until stimulated to reactivate. The mechanisms governing this latent-to-lytic switch are unclear, though latently infected cultured cells can be experimentally reactivated by the addition of phorbol esters or sodium butyrate (NaB)
In addition to chemically-induced reactivation, the lytic cycle can be reactivated by biological stressors, including interferon-γ, toll-like receptor 7 and 8 (TLR7 and TLR8) agonists, reactive oxygen species (ROS), and the ER stress-activated X-box binding protein 1 (XBP1) transcription factor [88-91].

Following reactivation or primary infection, lytic genes are transcribed and expressed. These lytic genes are transcribed in a specific order and are categorized as immediate-early, delayed-early, and late genes [92]. Immediate-early genes do not require protein synthesis for their expression, whereas delayed-early genes require protein synthesis but not DNA replication. Late genes require DNA replication and the expression of immediate-early and delayed-early gene products for expression. Nine viral genes have been classified as being expressed very early after butyrate induction, and 27 have been classified as late genes [93-95].

The major immediate-early gene product, Rta (replication and transcriptional activator, encoded by ORF50), is required for lytic replication and the induction of downstream genes (delayed-early and late genes). Rta alone is sufficient to initiate the lytic cascade, and inhibition of Rta prohibits lytic reactivation [96-99]. Ectopic expression of Rta in PEL cells leads to the transcription of viral lytic genes [100]. Rta contains both a DNA-binding domain and a transactivation domain and is detected within 4 hours (hrs) of n-butyrate induction [92,101]. The DNA-binding domain of Rta allows the viral protein to bind to viral DNA promoter sequences and to recruit cellular transcription factors, such as RBP-Jk, via its transactivation domain [101]. Rta can also induce genes lacking Rta-binding cis sequences via association with promoter-bound transcription factors including C/EBPα; C/EBPβ and Rta cooperatively activate the Rta and PAN promoters [101]. Rta functions as a tetramer and can be observed as decamers in solution [102]. Genes that are transcribed soon after Rta expression include ORF45, K3, K5, polyadenylated nuclear (PAN) ribonucleic acid (RNA), vIL-6, and viral chemokine (C-C motif) ligand 2 (vCCL-
2/vMIP II); these genes can be detected within 13 hrs of n-butyrte induction [92,93].

Delayed-early genes include TK synthase, vCCL-1/vMIP I, viral G-protein-coupled receptor (vGPCR), viral B-cell lymphoma 2 (vBcl-2), K12, and dihydrofolate reductase (DHFR) [92]. The late genes are maximally expressed following viral DNA replication.

Lytic viral DNA replication occurs from two lytic origins (OriLyt-L and OriLyt-R) and proceeds via a rolling circle mechanism [103]. Like other herpesviruses, HHV-8 encodes a viral polymerase, polymerase processivity factor, primase, primase-associated factor, helicase, and single-strand binding protein [82]. When these six gene products were co-transfected into Vero cells, globular pseudo-replication complexes were observed in the nucleus [104]. DNA replication is required for the expression of HHV-8 late genes such as capsid proteins (e.g. ORF65) and other structural proteins found in the tegument [95]. Following expression of late genes, which occurs more than 30 hrs after n-butyrte induction, viral capsid proteins are assembled, and viral DNA is loaded through the viral portal protein [92]. Capsid assembly and loading occurs in the host cell nucleus, necessitating a nuclear escape mechanism for efficient virion release. The nuclear egress complex, which assists with virion egress from the nucleus, is comprised of the ORF67 and ORF69 gene products [105,106]. The process of virion envelopment and maturation is complex, and HHV-8 encoded viral glycoprotein B (gB) plays an important role in this process [107-109]. The virion gains a primary envelope and some tegument proteins as it traverses the inner nuclear membrane [109]. The viral envelope then fuses with the outer nuclear membrane as the virus moves across the perinuclear space; the virus particle then deenvelopes, and the particle traverses the outer nuclear leaflet [109]. Reenvelopment and the acquisition of tegument proteins occur as the virus particle traverses membranes of the trans-Golgi network [109]. Vesicles containing enveloped and tegumented virus particles then fuse with the plasma membrane, releasing mature virions [109]. Studies conducted using wild-type and gB-deleted HHV-8 bacmids demonstrated
that gB is required for the complete maturation of HHV-8 virions; cells infected with gB-deleted HHV-8 bacmid did not produce completely enveloped viral particles [107]. After escaping the cell, progeny virus can infect naïve cells, and the original host cell undergoes apoptosis.

HHV-8 LATENCY

Viral productive replication is required for the infection of naïve cells and the expansion of the viral reservoir. However, most infected cells remain latently infected following primary HHV-8 infection. In B-cells, the virus is nearly completely latent within hrs of de novo infection, as only a small subset of cells continues to produce virions. In a study of KS, approximately 3% of cells were found to be lytic, while the remaining infected cells were latent [92]. The latent virus is maintained as a circular episome within the host cell nucleus [34,80,110,111]. During latency, a small number of viral genes are expressed, allowing the virus to evade host immune responses that target viral gene products. Latent gene products include latency-associated nuclear antigen (LANA), vFLIP, vCyclin, viral microRNAs, and kaposins A, B, and C [112]. These latency products are encoded in a cluster within the viral genome known as the latency locus. Two promoters are responsible for the expression of these latency genes, the LANA promoter (drives expression of LANA, vCyclin, and vFLIP) and the kaposin promoter (regulates expression of the kaposins and viral microRNAs) [113,114]. Recently, studies have demonstrated that vIL-6 is also expressed at low levels by latently infected PEL cells; expression of viral interferon regulatory factors (vIRFs) 1, 2, and 3 have also been reported in latently infected PEL cells [115-119].

LANA is a crucial protein of latency that functions to tether the viral episome to the host chromosome through its interactions with TR regions of the HHV-8 genome and host cell histone H2A/H2B proteins [120-123]. This tethering allows the HHV-8 genome to be
replicated during host cell DNA replication and ensures appropriate segregation of viral genomes to daughter cell nuclei [120,124,125]. Plasmids containing TR regions can be replicated in the presence of LANA but are not replicated in the absence of LANA, indicating that LANA is required for episomal replication and maintenance [123,124,126,127].

LANA expression regulates both host and viral gene transcription. Overexpression of LANA results in the upregulation of DNA methyltransferase Dnmt3a, which is responsible for methylating specific promoters, repressing the transcription of these promoter-driven genes (e.g. cadherin 13) [128]. One study found 186 cellular genes that were subject to greater than 2-fold positive or negative regulation by LANA; several of these LANA-responsive genes are involved in the p53 and retinoblastoma protein (pRB) pathways [129]. Moreover, LANA binds to and inactivates both the p53 and pRb tumor suppressors, repressing apoptosis and driving E2F-dependent gene transcription [130,131]. LANA also autoregulates its own transcription and the expression of Rta; silencing of Rta by LANA inhibits lytic reactivation and promotes latency [132-134].

LANA achieves its pro-growth and survival functions by targeting multiple cellular pathways in addition to p53 and pRb. LANA enhances levels of β-catenin via sequestration of glycogen synthase kinase 3β (GSK-3β), ultimately leading to increased expression of cyclin D and c-Myc [135]. Cellular Myc is an oncogene and a transcription factor associated with growth, proliferation, and apoptosis [136]. LANA stabilizes c-Myc expression by repressing the phosphorylation and ubiquitination of the transcription factor [137,138]. Telomere length is also maintained by LANA, increasing the lifespan of virally-infected cells [139]. Furthermore, LANA appears to be involved in the initial stages of lymphogenesis. A transgenic mouse model expressing LANA driven by the LANA promoter showed increased germinal center formation and an increased incidence of
lymphoma [140]. Thus, LANA is critical for the maintenance and replication of HHV-8 episomes, the promotion of infected cell growth, and likely contributes to lymphogenesis.

**VIRAL INTERLEUKIN 6 AND PATHOGENESIS**

Prior to the discovery of HHV-8, elevated levels of IL-6 were observed in KS and MCD patients, and disease severity was correlated with IL-6 levels [25,40]. Moreover, the growth of cells derived from an HIV-positive KS patient could be suppressed (reduced by ~67%) by the inhibition of IL-6 via antisense oligomers, indicating potential IL-6 involvement in the pathogenesis of KS [25]. Soon after the discovery of HHV-8, one of the 15 unique ORFs of the virus was found to encode a homolog of interleukin 6 [67,69,141]. Cellular IL-6 is involved in many normal physiologic processes, including hematopoiesis, inflammation, cell growth, and cell survival. Early studies demonstrated that the vIL-6 homolog could substitute for hIL-6 in some experimental systems. vIL-6 could prevent murine plasmacytoma B9 cell apoptosis and promote B9 cell proliferation; B9 cells are IL-6-dependent [67,69]. Furthermore, antibodies specific to the IL-6 receptor inhibited hIL-6- and vIL-6-mediated growth of B9 cells in culture while control antibodies specific for IL-2 had no effect on cell growth [69]. hIL-6 is also known to induce the expression of acute-phase proteins in hepatocytes [142]. Thus, cell culture-based methods were employed to determine whether vIL-6 could similarly promote acute-phase protein production [69]. The Hep3B hepatocyte cell line was transfected with plasmids encoding vIL-6, hIL-6, or control sequences, and Northern blots were conducted using harvested RNA from the transfected cells [69]. Both human and viral cytokines could induce the messenger RNA (mRNA) expression of α1 acid glycoprotein, a prototypical acute-phase protein [69]. Thus, at the time of its discovery, many surmised that vIL-6 might play a role in the development and progression of HHV-8-associated disease [67-69].
Following the discovery of vIL-6, clinical and other data were reported that implicated vIL-6 as an important factor in the progression of KS, PEL, and MCD. Elevated levels of serum vIL-6 were correlated with episodes of acute inflammation in MCD patients [143]. Additionally, high levels of both hIL-6 and vIL-6 were observed in PEL tumors, and these cytokines were required for PEL cell growth [115, 144]. Depletion of vIL-6 via a short hairpin RNA (shRNA)-encoding lentiviral vector and inhibition of vIL-6 using neutralizing antibodies led to decreased PEL growth in culture [115, 144]. The engraftment of PEL tumors in severe combined immunodeficiency (SCID) mice could be inhibited by the inhibition of vIL-6 via morpholino oligomers specific to vIL-6 [145]. In KS tumors, vIL-6 can induce the expression of angiogenic factors (including VEGF), which are critical in the development of KS [146]. In these studies, vIL-6-expressing NIH3T3 fibroblasts were injected into nude mice, and these mice were compared to control mice that received injections of NIH3T3 cells expressing control plasmid; vIL-6-expressing mice exhibited increased hematopoiesis in specific lineages (myeloid, erythroid, and megakaryocytic), greater vascularization, and polyclonal hypergammaglobulinemia [147]. In NIH3T3 cells expressing vIL-6, VEGF expression could be detected by immunofluorescence staining; in control cells, VEGF was not detected [147]. In a mouse model of peritoneal inflammation, vIL-6 was found in enhance CCL-2 secretion and inhibit CXCL-8 expression, ultimately blocking the recruitment of neutrophils to the site of inflammation [148]. Finally, vIL-6 can also enhance the secretion of hIL-6 [149]. Plasmids encoding vIL-6 or the reverse vIL-6 sequence were transfected into cells, and culture medium was collected for use in experimental assays [149]. Collected culture medium was then added to several B and T cell lines, and hIL-6 levels were assessed; the introduction of vIL-6 led to significant increases (up to 30-fold) in hIL-6 expression in five cell lines (MT-4, THP-1, U937, Raji, and CESS) [149]. Additionally, vIL-6-containing culture medium was capable of inducing a 2-fold increase in hIL-6 secretion in cell lines derived from MCD patients [149].
Other studies have further characterized the role of vIL-6 in HHV-8 pathogenesis. vIL-6 is expressed maximally during lytic replication, though it is also expressed at low, though functional, levels during PEL latency [115,116]. vIL-6 transcripts were identified via limiting dilution reverse transcription-polymerase chain reaction (RT-PCR) and array-based methods in HHV-8 latently infected SLK cells (KS-derived, HIV) [116]. Immunofluorescence staining of HHV-8-positive PEL cells for vIL-6 indicates that the viral cytokine is expressed during latency; vIL-6-specific immunofluorescence staining in PEL cells was detected above background staining in co-mixed HHV-8-negative Akata cells [115]. Depletion of vIL-6 via shRNA-mediated lentiviral transduction led to decreased growth and survival of PEL cells in culture [115]. Following vIL-6 depletion, PEL cell growth could be rescued by the addition of fully ER-retained, shRNA-resistant vIL-6 [115]. Similar results were observed when vIL-6 was neutralized using an ER-directed vIL-6-specific antibody (MAV) or peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) directed to vIL-6 [150,151]. These studies are significant because they indicate that vIL-6 plays an important role in cell growth and viability during latency when the protein is expressed at much lower levels than during lytic reactivation.

vIL-6 is known to activate both mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) signaling (discussed in more detail below), and high levels of active (phosphorylated) STAT3 have been observed in PEL cells [152]. Several PEL cells lines, including BC-1, BCBL-1, and VG-1, express constitutively phosphorylated STAT3 [152]. Furthermore, inactivation of STAT3 (via treatment with JAK2 pharmacological inhibitor AG490 or the overexpression of dominant-negative STAT3 [STAT3D]) led to decreased PEL cell growth, increased apoptosis, and decreased expression of survivin, a protein that is required for PEL cell survival [152]. STAT3D contains a mutation within the DNA binding domain of STAT3; thus, STAT3D can dimerize with wild-type STAT3 but cannot bind STAT3-responsive DNA elements to
Initiate transcription, effectively acting as a competitive inhibitor of wild-type STAT3 [152,153]. Survivin is an inhibitor of apoptosis (IAP) family member, and its inhibition has been implicated in several cancers [154]. Forced overexpression of survivin in VG-1 PEL cells was capable of rescuing AG490-mediated apoptosis and STAT3 inhibition, confirming the importance of STAT3 and survivin for PEL cell viability [152]. Importantly, STAT3 can also be activated by VEGF in bovine aortic endothelial cells, and VEGF is upregulated by vIL-6 in other cell culture models [147,155]. Thus, vIL-6 can be linked to pro-growth and survival as well as pro-angiogenic pathways, further implicating the viral cytokine in HHV-8 malignant pathogenesis.

**VIRAL INTERLEUKIN 6 CHARACTERISTICS**

hIL-6 and vIL-6 share approximately 25% amino acid sequence identity and 63% similarity [67-69]. While the primary sequences of the homologs are quite divergent, both cytokines fold into similar three-dimensional structures consisting of 4-alpha helical bundles (Figure 1.2) [156]. This structural similarity allows both cytokines to engage the IL-6 α receptor (IL6R or gp80) and gp130 (β subunit) and initiate signaling, leading to the induction of MAPK and STAT signaling (Figure 1.3) [156-160]. Phosphorylation of tyrosine (Y) 759 (Y759) of gp130 results in MAPK signaling, while the phosphorylation of Y767, Y804, Y905, or Y915 leads to the initiation of STAT signaling [156,161-163]. Ultimately, hIL-6 and vIL-6 signaling lead to cell growth and survival, and both cytokines can sustain IL-6-dependent B9 cells in culture [67,141]. While both proteins can initiate signaling via interactions with gp80 and gp130, hIL-6 and vIL-6 have different requirements for signaling induction. hIL-6-containing signaling complexes comprise two molecules each of hIL-6, gp80, and the gp130 signal transducer (hIL-62-gp802-gp1302) [156-160]. vIL-6 can also form identical hexameric complexes but is unique among IL-6 proteins in that it can signal in the absence of gp80 via tetrameric complexes (vIL-62-gp1302) [158,164-166]. While
gp80 is not required for vIL-6-initiated signaling, the presence of gp80 facilitates a more sustained signaling event [159,167,168]. Hexameric (gp80-containing) signaling complexes are found at the plasma membrane (PM), while (unique) vIL-6 signaling from the ER occurs exclusively through tetrameric (gp80-devoid) complexes [115,169]. vIL-6-containing tetrameric complexes may also form at the cell surface. Thus, hIL-6 can signal at the cell surface, while vIL-6 can signal from the cell surface and intracellularly within the ER. These distinct signaling mechanisms allow vIL-6 to signal in both a paracrine and strictly autocrine (intracellular) manner.

In addition to differences in signaling complex composition, vIL-6 is mostly retained within the ER compartment, while hIL-6, like other cellular cytokines, is efficiently secreted from the cell [115,170,171]. Secretion kinetics indicate that vIL-6 is secreted approximately 8-fold slower than hIL-6 [170]. Within the ER, vIL-6 interacts with calnexin, which is involved in proper folding of the cytokine [171]. Depletion of calnexin led to reduced levels of intracellular vIL-6 but did not alter levels of secreted vIL-6; vIL-6 levels could be rescued by overexpression of gp130 in calnexin-depleted cells, but the underlying mechanism is unclear [171]. Furthermore, vIL-6 contains two potential asparagine glycosylation sites (asparagines [N] 78 and 89), and secreted vIL-6 is completely glycosylated [170,172]. Glycosylation of vIL-6 N89 is required for the induction of vIL-6-initiated signaling but not for its interaction with calnexin, for which N78 glycosylation is sufficient [171,172]. Thus, posttranslational modification differences between the human and viral cytokines may partially explain differences in secretion kinetics and interactions with ER-resident proteins.

vIL-6, but not hIL-6, was found to bind directly to a previously uncharacterized protein called vitamin K epoxide reductase complex subunit 1 variant 2 (VKORC1v2) in addition to calnexin [173]. The major isoform, VKORC1v1 (vitamin K epoxide reductase complex subunit 1 variant 1), plays a role in the vitamin K cycle and is the target of the
anticoagulant warfarin [174]. Evidence demonstrating that VKORC1v2 interacts with vIL-6 was the first reported function of VKORC1v2. VKORC1 variants 1 and 2 share the first 58 amino acids, and splicing gives rise to variant 2 [173]. Only VKORC1v2 interacts with vIL-6, though the interaction domain (vIL-6 binding domain [vBD], residues 31-39) of VKORC1v2 is shared with VKORC1v1 [173]. The difference in vIL-6-binding between the two variants can be explained by the opposing orientations of the two proteins in the ER membrane. Both VKORC1 variants contain a transmembrane domain between amino acids 11 to 29 [173]. For VKORC1v1, the N-terminus of the protein is located in the ER lumen, and amino acids 31-39 (vBD) are located in the cytoplasm [173]. Conversely, the N-terminus of VKORC1v2 is located in the cytoplasm, and amino acids 31-39 are located within the ER lumen and are accessible to vIL-6 [173]. A third VKORC1 variant, VKORC1v3, is similar to VKORC1v1 but has a deletion of amino acids 58-73 due to alternative splicing, and VKORC1v3 is also incapable of binding to vIL-6 [175]. Studies have shown that depletion of VKORC1v2, but not VKORC1v1, leads to reduced rates of PEL cell growth and increased rates of apoptosis in these cells, identifying the importance of VKORC1v2 for PEL cell viability [173]. Growth of VKORC1v2-depleted cells could be rescued by the addition of shRNA-resistant wild-type but not vBD-mutated VKORC1v2 [173]. Similarly, overexpression of vBD led to reduced growth and increased apoptosis in PEL cultures, presumably due to disruption of the vIL-6/VKORC1v2 interaction [173]. These findings indicate the importance of the vIL-6/VKORC1v2 interaction for PEL cell viability.

The interaction between vIL-6 and VKORC1v2 can enhance VKORC1v2 interactions with the proenzyme form of cathepsin D (CatD) [175]. CatD, in its mature, lysosomally localized form, is an aspartate protease, which can be released into the cytoplasm in response to certain stress signals [176]. Depletion of vIL-6 results in increased levels of ER-localized/transiting pro-CatD and mature CatD, leading to
decreased PEL cell viability in culture [175]. Introduction of vIL-6/VKORC1v2-disrupting vBD also led to increased levels of CatD and reduced PEL cell viability [175]. PEL cell apoptosis could be induced with the overexpression of CatD, suggesting that vIL-6 may suppress CatD function to maintain cell viability [175]. PEL cell viability is critical for efficient virus replication, and depletion of vIL-6 or introduction of vIL-6/VKORC1v2-disrupting vBD in lytically reactivated BCBL-1 and JSC-1 PEL cells led to increased levels of CatD and decreased HHV-8 viral titers [175]. In contrast, depletion of CatD in these PEL cell lines led to increased viral titers [175]. These results suggest a role for vIL-6 in HHV-8 replication via its interactions with VKORC1v2 and regulation of CatD.

**SIGNIFICANCE OF VIRAL IL-6 AND THIS PROJECT**

In order to develop therapeutic agents for the treatment of HHV-8-associated disease, it will be crucial to develop a better understanding of the mechanisms governing vIL-6-mediated pathogenesis. Several research questions must be addressed before beginning the process of selecting therapeutic targets. First, the role of ER-localized vIL-6/gp130 interactions must be investigated. vIL-6/gp130-mediated signaling likely activates STAT and MAPK pathways, though this has not been shown in the context of PEL. Furthermore, the mechanism(s) responsible for elevated levels of active STAT3 in PEL tumors should be elucidated. In addition to its proposed roles in HHV-8 latency, functions of the vIL-6/gp130 interaction in the context of virus replication are unclear. The focus of this dissertation project was to functionally characterize ER-localized vIL-6/gp130 interactions in the context of both viral latency and virus productive replication.
Table 1.1. Descriptions of HHV-8-specific open reading frames ("K" ORFs).

<table>
<thead>
<tr>
<th>ORF</th>
<th>Encoded Protein</th>
<th>Protein Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>VIP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>activation of NFκB; pro-angiogenic; downregulation of B-cell receptor; cytokine induction</td>
<td>[177-182]</td>
</tr>
<tr>
<td>K2</td>
<td>vIL-6</td>
<td>pro-growth; pro-survival; pro-angiogenic; pro-virus replication</td>
<td>[67-69,115,152,169,175,183]</td>
</tr>
<tr>
<td>K3</td>
<td>K3 protein</td>
<td>downregulation of MHC&lt;sup&gt;b&lt;/sup&gt; class I; NK&lt;sup&gt;c&lt;/sup&gt; cell and CTL&lt;sup&gt;d&lt;/sup&gt; evasion</td>
<td>[94,184-186]</td>
</tr>
<tr>
<td>K4</td>
<td>vCCL-2</td>
<td>pro-angiogenic; pro-survival; pro-virus replication; Th2 polarization</td>
<td>[187-193]</td>
</tr>
<tr>
<td>K4.1</td>
<td>vCCL-3</td>
<td>pro-angiogenic; Th2 polarization</td>
<td>[193,194]</td>
</tr>
<tr>
<td>K5</td>
<td>K5 protein</td>
<td>downregulation of MHC class I; NK and CTL evasion</td>
<td>[94,184-186,195]</td>
</tr>
<tr>
<td>K6</td>
<td>vCCL-1</td>
<td>pro-angiogenic; pro-survival; pro-virus replication; Th2 polarization</td>
<td>[187,196]</td>
</tr>
<tr>
<td>K7</td>
<td>vIAP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>pro-survival</td>
<td>[197-199]</td>
</tr>
<tr>
<td>K8</td>
<td>K8.1A/K8.1B</td>
<td>target cell recognition; heparin binding</td>
<td>[200,201]</td>
</tr>
<tr>
<td>K9</td>
<td>vIRF1</td>
<td>pro-survival; inhibition of interferon signaling</td>
<td>[202-211]</td>
</tr>
<tr>
<td>K10</td>
<td>vIRF4</td>
<td>p53 destabilization</td>
<td>[202,212,213]</td>
</tr>
<tr>
<td>K10.5</td>
<td>vIRF3</td>
<td>pro-angiogenic; pro-survival; inhibition of IFN signaling</td>
<td>[117,202,214-217]</td>
</tr>
<tr>
<td>K11</td>
<td>vIRF2</td>
<td>inhibition of interferon signaling</td>
<td>[119,202,218]</td>
</tr>
<tr>
<td>K12</td>
<td>kaposin A, B, &amp; C</td>
<td>prevention of cytokine mRNA decay; activation of MAPK signaling; PROX1 stabilization</td>
<td>[219-223]</td>
</tr>
<tr>
<td>K13</td>
<td>vFLIP&lt;sup&gt;f&lt;/sup&gt;</td>
<td>anti-apoptotic via inhibiting caspase-8 activation; pro-survival via NFκB activation</td>
<td>[78,224-226]</td>
</tr>
<tr>
<td>K14</td>
<td>K14 cell surface protein</td>
<td>interacts with CD200 receptor; downregulation of TNFα and myeloid cell production</td>
<td>[227]</td>
</tr>
<tr>
<td>K15</td>
<td>K15 membrane protein</td>
<td>Pro-growth; pro-survival; pro-angiogenic</td>
<td>[228-231]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Variable immunoreceptor tyrosine-activation motif (ITAM) protein; <sup>b</sup> major histocompatibility complex; <sup>c</sup> natural killer; <sup>d</sup> cytotoxic T lymphocyte; <sup>e</sup> viral inhibitor of apoptosis; <sup>f</sup> viral FADD-like interleukin-1 beta-converting enzyme (FLICE) inhibitory protein.
Table 1.2. HHV-8 microRNAs.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Targets</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-K12-1</td>
<td>p21, caspase 3, THBS1, IkBα</td>
<td>inhibition of cell-cycle arrest; anti-apoptotic; pro-angiogenic; suppression of viral replication; induction of MAPK signaling</td>
<td>[232-235]</td>
</tr>
<tr>
<td>miR-K12-2</td>
<td>unknown</td>
<td>upregulated in KS lesions</td>
<td>[236]</td>
</tr>
<tr>
<td>miR-K12-3</td>
<td>caspase 3, NFIB, THBS1</td>
<td>anti-apoptotic; pro-latency; pro-angiogenic</td>
<td>[234,235,237]</td>
</tr>
<tr>
<td>miR-K12-4</td>
<td>caspase 3, Rbl2</td>
<td>anti-apoptotic; epigenetic modifications</td>
<td>[234,238]</td>
</tr>
<tr>
<td>miR-K12-5</td>
<td>BCLAF1, Rta, MyD88</td>
<td>anti-apoptotic; pro-latency; suppression of inflammatory cytokine production</td>
<td>[238-240]</td>
</tr>
<tr>
<td>miR-K12-6</td>
<td>MAF, THBS1</td>
<td>cell fate reprogramming; pro-angiogenic</td>
<td>[20,235]</td>
</tr>
<tr>
<td>miR-K12-7</td>
<td>Rta, MICB</td>
<td>pro-latency; NK evasion</td>
<td>[241,242]</td>
</tr>
<tr>
<td>miR-K12-8</td>
<td>unknown</td>
<td>upregulated in KS lesions</td>
<td>[236]</td>
</tr>
<tr>
<td>miR-K12-9</td>
<td>BCLAF1, Rta, IRAK1</td>
<td>anti-apoptotic; pro-latency; decreased inflammatory cytokine production</td>
<td>[239,240,243]</td>
</tr>
<tr>
<td>miR-K12-10</td>
<td>TWEAKR, BCLAF1</td>
<td>repression of caspase activation; anti-apoptotic</td>
<td>[239,244]</td>
</tr>
<tr>
<td>miR-K12-11</td>
<td>BACH1, SMAD5, MAF, THBS1</td>
<td>pro-survival; suppression of TGF-β signaling; cell fate reprogramming; pro-angiogenic; induction of MAPK signaling</td>
<td>[20,235,245-248]</td>
</tr>
<tr>
<td>miR-K12-12</td>
<td>unknown</td>
<td>upregulated during lytic reactivation</td>
<td>[249,250]</td>
</tr>
</tbody>
</table>
FIGURES

Figure 1.1. Genomic organization of HHV-8. Gammaherpesvirus-conserved “core” gene blocks are shown in blue. Divergent loci (DL-A through DL-D and IRF locus) outside of the conserved blocks are labelled. Signaling membrane receptors (pink), cytokines (blue), interferon regulatory factors (orange), and latency genes (green) are highlighted. The K1, vOX2/vCD200 (K14), and K15 receptors, the viral cytokines, and the viral IRFs have thus far been identified only in HHV-8 and closely-related macaque rhadinoviruses [251-253]. K7 (survivin) and K12 (kaposin) are unique to HHV-8. Latency genes encoding LANA, vCyclin, and vFLIP are present in and characteristic of other gamma-2 herpesviruses. . Abbreviations: see Table 1.1; modulator of immune recognition 2 (MIR2), thymidylate synthase (TS), modulator of immune recognition 1 (MIR1), viral B-cell lymphoma 2 (vBcl-2), viral chemokine ligand (vCCL), orexin receptor 2 (OX-2).
Figure 1.1
Figure 1.2. vIL-6 and hIL-6 three-dimensional structures. hIL-6 and vIL-6 are composed of four alpha helices (A-D) and fold into similar three-dimensional structures. This structural similarity allows both cytokines to initiate signaling through complexes containing the IL-6α receptor and gp130 signal transducer.

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Figure 1.3. Human and viral interleukin 6 signaling complexes. Human IL-6 (hIL-6) initiates signaling through hexameric signaling complexes containing two molecules each of hIL-6, gp80, and gp130; these complexes form exclusively at the plasma membrane. Viral IL-6 (vIL-6) is capable of signaling via identical hexameric complexes, though vIL-6 is unique in its ability to signal via tetrameric complexes (vIL-6$_2$-gp130$_2$) within the endoplasmic reticulum (ER). Following complex formation, tyrosine (Y) residues within the cytoplasmic tail of gp130 are phosphorylated. Depending on the specific tyrosine phosphorylation event, signal transducer and activator of transcription (STAT) 1 or 3 or mitogen-activated protein kinase (MAPK) signaling cascades are activated downstream of gp130.
Figure 1.3
Figure 1.4. Known and proposed functions of viral interleukin 6. Viral interleukin 6 (vIL-6) is a multifunctional viral protein and is active during both the latent and lytic phases of the virus life cycle. During latency in PEL cells, vIL-6 acts as an autocrine factor to promote cell growth and survival; it may perform a similar role in other cell types. During the lytic phase, vIL-6 is thought to promote pathogenically significant angiogenesis and inflammation through induction of cellular factors, including VEGF and hIL-6. vIL-6 inhibits neutrophil recruitment via downregulation of CCL-2. These activities are believed to promote virus replication in vivo. The hypotheses that vIL-6 autocrine signaling via gp130 can contribute to latently infected cell viability and to productive replication form the basis of the thesis project.
Figure 1.4
CHAPTER 2: METHODS
Summary

Many laboratory techniques were utilized to conduct the studies described in this dissertation. Detailed methods are provided in the chapters describing the studies in which they were used.
INTRODUCTION

In order to answer the research questions posed in this body of work, many techniques were employed. Several of the techniques have been well-described by others, but other protocols were specifically developed in the course of this project. Classic and novel techniques are described in general terms below; more detailed methods are provided in the relevant chapters that follow.

RNA EXTRACTION

HHV-8 and cellular mRNA were extracted from cell cultures in order to quantify gene expression levels. To extract RNA, cells were washed with phosphate-buffered saline (PBS) and suspended in TRizol (Thermo Fisher Scientific, Inc., Waltham, MA). Following a 3 minute (min) incubation in TRizol, chloroform was added to the mixture, and the samples were vortexed briefly and incubated for 10 min at room temperature. Samples were then centrifuged in a microcentrifuge (10 min at maximum speed at room temperature), and the top layer was removed and placed in a clean Eppendorf tube. Isopropanol was added to precipitate RNA, and samples were centrifuged in a microcentrifuge (10 min at maximum speed at 4°C) to pellet the RNA. Finally, RNA pellets were washed with 70% ethanol and resuspended in RNase- and DNase-free water. Concentrations of RNA samples were determined via spectrophotometer.

REVERSE TRANSCRIPTION

After viral and cellular RNA were extracted from cultured cells, the RNA was reverse-transcribed to complementary deoxyribonucleic acid (cDNA) to allow amplification of a gene of interest. The reverse-transcription reaction requires a pure RNA template devoid of contaminating DNA molecules. As a result, the extracted RNA was treated with DNase I (45 min incubation at room temperature) to destroy contaminating DNA.
molecules; ethylenediaminetetraacetic acid (EDTA) was subsequently used to inactivate the DNase I enzyme by chelating magnesium and calcium required for enzyme activity. Random hexamer primers and deoxynucleotide triphosphates (dNTPs) were added to DNase-treated samples. Samples were then heated (65°C for 5 min) and cooled on ice (5 min); this process facilitated hexamer primer annealing. Finally, dithiothreitol (DTT; Invitrogen, Carlsbad, CA), reverse transcriptase buffer (5X First Strand Buffer; Invitrogen), RNase inhibitor (RNaseOUT; Invitrogen), and reverse transcriptase (SuperScript II RT; Invitrogen) were added to the samples. RNA samples were then reverse-transcribed into cDNA by incubating at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. cDNA samples were stored at -20°C until further processing.

**POLYMERASE CHAIN REACTION**

Polymerase chain reaction (PCR) was used to amplify a DNA template in order to create vast numbers of copies of a given amplicon. PCR products are more stable than cDNA derived from reverse-transcription and can be further analyzed or manipulated. PCR reactions included a DNA template, dNTPs, forward and reverse primers, polymerase buffer, and DNA polymerase. PCR amplification times depended on the length of the PCR product and the polymerase used. Generally, PCR reactions included approximately 50 nanograms of DNA template, 4 μl of dNTPs (2.5 mM), 1 μl each of forward and reverse primers (10 μM each), 1X polymerase buffer, 1.5 μl of MgCl₂ (50 mM), and 1 unit (U) of Taq polymerase isolated from *Thermus aquaticus* (Invitrogen) in 1X polymerase buffer. For cloning purposes, 2.5 U of PFU Ultra High-Fidelity polymerase (Stratagene, La Jolla, CA) was used, and MgCl₂ was not added. PFU Ultra High-Fidelity polymerase is less error-prone than Taq polymerase and is therefore more suitable for cloning.
Amplification using Taq polymerase was conducted as follows: 94°C for 2 min (denaturing); 25-35 cycles of 94°C for 30 sec (denaturing), 55°C for 30 sec (annealing), and 72°C for 1 min per kb of DNA template (extension); and a final extension of 72°C for 10 min. Samples were then cooled to 4°C and stored at -20°C until they were used for further analysis. A similar protocol was employed for reactions containing PFU Ultra High-Fidelity enzyme: 95°C for 2 min; 25-35 cycles of 95°C for 30 sec, 50-55°C for 30 sec, 72°C for 1 min per kb of template DNA (for vector templates <10 kb); followed by a final extension of 72°C for 10 min. For vector templates greater than 10 kb, an extension time of 2 min per kb and an extension temperature of 68°C were used. PCR amplification was confirmed and visualized by agarose gel electrophoresis (described below).

**AGAROSE GEL ELECTROPHORESIS**

PCR amplification was verified by running PCR products on an agarose gel with an appropriate DNA size marker. PCR samples were mixed with DNA loading buffer (40% sucrose and 0.25% bromophenol blue) and loaded into agarose gel lanes. DNA size marker was loaded into a gel lane as a reference to determine the size of PCR products. As DNA is negatively charged, PCR products migrated from the negatively charged pole toward the positively charged pole through the gel matrix. Ethidium bromide was included in the agarose gel and intercalated into the DNA duplexes, allowing for the detection of labeled DNA fragments by ultraviolet (UV) light. Smaller DNA fragments migrated through the gel more rapidly than larger DNA fragments; thus, DNA was separated based on size.

While agarose gel electrophoresis was used to determine PCR product size, relative abundance was also assessed in some instances. For example, RT-PCR was often used to determine whether a specific shRNA adequately repressed its target gene expression. In this case, RT-PCR-derived DNA samples from control and shRNA-treated cells were compared on an agarose gel. Amplified target gene DNA should be less
abundant than amplified target gene DNA from control cells if the shRNA used was effective. Thus, target gene expression was semi-quantified by agarose gel electrophoresis.

**CLONING AND SHORT HAIRPIN RNA DESIGN**

Several plasmid constructions were prepared in order to address questions related to vIL-6 functions in cell growth, apoptosis, and virus replication. Some genes were cloned with tags (e.g. FLAG) to facilitate detection, while others were cloned with localization sequences (e.g. KDEL motif) to restrict the gene product to specific cellular locations. Gene segments were also cloned (e.g. soluble gp130 [sgp130]) and introduced into cells to inhibit the interaction between vIL-6 and gp130. Additionally, shRNAs were cloned into lentiviral vectors to deplete endogenous cellular or viral proteins in cultured cells. Finally, shRNA-resistant genes were cloned into lentiviral vectors for use in depletion-complementation experiments. Specific details regarding the primers, restriction enzyme sites, and vectors utilized for these constructions are provided in the relevant chapters.

In general, genes were amplified from various vector templates using PFU Ultra High-Fidelity polymerase and then purified. In brief, PCR products were precipitated from PCR reaction mixtures with sodium acetate and ethanol to remove primers, excess dNTPs, polymerase, and reaction buffer components. Purified PCR products were then digested with restriction enzymes to create appropriate 5’ and 3’ overhangs (or left undigested in the event of a blunt-ended ligation). Restriction enzyme-digested PCR products and vectors were then separated on an agarose gel, and the appropriate DNA bands were excised from the gel under the guidance of UV light. DNA was purified from the agarose gel using a DNA gel extraction kit (QIAquick Gel Extraction Kit; Qiagen, Valencia, CA). Gel-purified DNA vector and PCR product insert were then ligated using...
T4 DNA ligase (New England Biolabs; Ipswitch, MA) for 1 hr at room temperature or overnight at 16°C.

Ligated vector-insert constructions were transformed into competent DH5α E. coli cells via a chemical transformation technique. Briefly, ligated DNA constructions were incubated with competent bacterial cells on ice for 30 min prior to heat shocking (42°C) for 30-60 seconds. Ligation product-bacterial mixtures were then placed on ice briefly and plated on Luria broth (LB) agar plates with appropriate antibiotics for selection of transformed bacteria. Depending on the plasmid used in the transformation, agar plates were incubated at 30°C or 37°C overnight to allow for the growth of transformed bacteria. Individual bacterial colonies were picked from plates, amplified in liquid LB, and miniprepped to extract bacterial DNA. Colony-derived DNA was used for PCR-based or restriction enzyme-based techniques to validate cloning constructions. Bacterial-derived DNA was PCR amplified using construct-specific primers to verify the presence of the DNA insert, DNA signal sequence, or sequence tag. Restriction enzyme digestion was also used to verify the insertion of restriction enzyme-containing DNA inserts, signal sequences, or tags. Prior to use in experiments, all constructions were commercially sequenced to verify the modified DNA sequence.

Several experiments utilized shRNA-encoded lentiviral vectors to deplete endogenous viral or host proteins. Proteins were targeted by 19 nucleotide sequences embedded in a characteristic hairpin structure. Targeting sequences were designed with the aid of iRNAi (version 2.1; Nucleobytes, The Netherlands). Complementary oligonucleotides encoding candidate target sequences were annealed, phosphorylated, and ligated into the pYNC352 lentiviral vector between the BamHI and MluI restriction sites. shRNA-encoded lentiviral vectors were then sequenced. To assess depletion efficiency of specific shRNA-encoded lentiviral vectors, vectors were co-transfected into 293T cultured cells with overexpressed target protein. Twenty-four hrs post-transfection,
cell lysates were harvested and subjected to western blot analysis to determine protein levels in control and shRNA-overexpressing cells (described below). In some cases, target mRNA depletion was assessed via RT-PCR following shRNA-encoding lentiviral overexpression (described above).

**MINIPREP DNA EXTRACTIONS**

Transformed bacteria were amplified in liquid LB overnight at 30°C or 37°C depending on the transformed plasmid and harvested for the extraction of plasmid DNA. Bacterial cells were pelleted via centrifugation, and cell pellets were resuspended in STET buffer (8% sucrose, 5% Triton X-100, and 50 mM EDTA, Tris [pH 8.0]). Samples were vortexed prior to the addition of lysozyme (100 μg/ml) and then boiled for 1 min and centrifuged at maximum speed for 10 min at room temperature. Supernatants were transferred to clean tubes, and an equal volume of isopropanol was added to each tube to precipitate DNA. Tubes containing sample mixtures were chilled at -20°C for 10 min and then centrifuged at maximum speed for 10 min at 4°C to pellet DNA. DNA pellets were washed in 70% ethanol and finally resuspended in DNase-free water containing RNase A to eliminate contaminating RNA.

**CELL CULTURE**

HEK293T cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). BCBL-1, TRExBCBL1-RTA, JSC-1, BJAB, BC-1, and BC-3 cells were maintained in RPMI 1640 media supplemented with 10% FBS. Telomerase-immortalized microvascular endothelial (TIME) cells and TIME-TRE/RTA cells were grown in endothelial growth basal media (EBM-2) containing FBS and supplements (Lonza; Walkersville, MD). All cells were grown in a 5% CO₂ humidified chamber maintained at 37°C.
TRANSFECTION OF CULTURED CELLS

Transfection of cultured HEK293T cells was utilized for functional experiments assessing viral and cellular protein-mediated signaling, shRNA depletion efficiency, protein-protein interactions, and lentivirus production. Transfections of HEK293T cells were conducted using standard calcium-phosphate protocols. Briefly, HEK293T cells were plated such that they would achieve 50% confluence at the time of transfection. DNA plasmids were then mixed with calcium phosphate (2.5 M) and 2X HEPES-buffered saline (HBS; 12 mM dextrose, 50 mM HEPES, 10 mM KCl, 280 mM NaCl, and 1.5 mM Na₂HPO₄·2H₂O, pH 7.05), vortexed, incubated at room temperature for 5 min, and added to HEK293T cell culture media. Following 8 hr of incubation, culture medium was replaced with fresh DMEM containing 10% FBS. Transfection efficiency was determined for plasmids encoding green fluorescent protein (GFP) by visualizing GFP fluorescence. To assess the transfection efficiency of plasmids not encoding fluorescent markers, a subset of transfected cells were fixed and stained for transfected proteins via immunohistochemical methods (described below).

Transfected cells were harvested 24-48 hr post-transfection for either RNA or cell lysates. RNA was harvested using the TRizol method described above. For preparation of cell lysates, cells were washed with PBS, scraped from the surface of the well or flask, and incubated in lysis buffer (20 mM Tris [pH 8.0], 5 mM EDTA, 50 mM NaCl, 0.2% NP40, and 1X protease and phosphatase inhibitors [Sigma; St. Louis, MO]) for 30 min on ice. Samples were then centrifuged (maximum speed for 5 min at 4°C in a microcentrifuge) to pellet cellular debris, and supernatants (lysates) were collected and stored at -20°C until western blot analysis.
WESTERN BLOT ANALYSIS

Western blot analysis was conducted to observe signaling events in transfected HEK293T cells or transduced PEL cell cultures. Western blot analysis was also performed to determine the efficiency of shRNA-mediated depletion in HEK293T, PEL, and TIME cell cultures. Preparation of cell lysates used for western blot analysis was described above. Specific antibodies and product numbers are provided in the methods section of the relevant chapters.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate lysate proteins via electrophoresis. Similar to agarose gel electrophoresis, proteins were separated based on size. Smaller proteins migrated through the gel matrix more rapidly than larger proteins. Depending on the size of the target protein, 8-12% polyacrylamide gels were used. Smaller target proteins were more efficiently separated on higher percentage (e.g. 12%) polyacrylamide gels, whereas larger proteins were better separated on lower percentage (e.g. 8%) gels. SDS loading buffer (188 mM Tris-HCl [pH 6.8], 3% SDS, 30% glycerol, 0.01% bromophenol blue, and 15% β-mercaptoethanol) was added to each protein sample prior to denaturation via boiling (5 min), and a protein size marker was loaded onto the gel as a reference. Following gel electrophoresis, proteins were electrophoretically transferred onto nitrocellulose membranes using standard protocols. Membranes were then washed in TBS-T (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) and blocked with 5% nonfat milk in TBS-T for 1 hr at room temperature. After briefly washing blocked membranes in TBS-T, primary antibodies (diluted in SuperBlock Blocking Buffer in TBS; Thermo Scientific, Rockford, IL) were incubated with membranes for 1 hr at room temperature or overnight at 4°C. After primary antibody incubation, membranes were washed three times with TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in 5% non-fat milk in TBS-T) for 1 hr at room temperature. Membranes were
continuously rocked during both antibody incubations. Again, membranes were washed three times in TBS-T and then developed using standard chemiluminescence procedures with ECL reagents (GE Healthcare Bio-Sciences; Pittsburgh, PA). Images were captured digitally on a GeneGnomeXRQ machine (Syngene; Frederick, MD).

**IMMUNOFLOURESCENCE STAINING**

Immunofluorescence staining was performed to determine transfected or transduced protein expression, to visualize cellular localization of target proteins, and to calculate the percentage of apoptotic cells, proliferating cells, and LANA-positive cells. To prepare cells for immunofluorescence staining, cells were washed in PBS, plated on glass slides, and allowed to air dry. Once dry, slides were fixed in ice cold methanol for 10 min. Slides were then allowed to air dry completely.

Following fixation, primary antibody (diluted 1:100 in SuperBlock Blocking Buffer in PBS; Thermo Scientific) was added to slides and incubated in a humidified chamber at 37°C for 1 hr. Primary antibody was then removed, and the slide was washed three times with PBS. Secondary antibody (diluted 1:200 in SuperBlock Blocking Buffer in PBS) was then applied to the slide, and the slide was incubated for 1 hr as before. After washing three times with PBS, nuclei were stained with 4’,6’-diamidino-2-phenylindole (DAPI). Finally, coverslips were mounted on slides prior to visualization on a UV microscope.

**BRDU INCORPORATION**

To determine the percentage of dividing (S-phase) cells, 5-bromo-2-deoxyuridine (BrdU) incorporation was assessed. BrdU is a thymidine analog and is incorporated into replicating DNA; thus, BrdU incorporation occurs exclusively in actively replicating cells.

BrdU (0.01 M diluted in culture media, 1000X) was added to cell cultures, and the cultures were incubated for 2 hr at 37°C; cells were then washed in PBS, spotted on glass
slides, and allowed to air dry. Slides were fixed in methanol as described above and washed in PBS. Cells were then permeabilized with 2M hydrochloric acid (HCl) for 20 min at room temperature, and sodium borate (0.1 M) was then added to the slides for 2 min to neutralize HCl. Permeabilized cells were then washed with PBS containing 3% bovine serum albumin (BSA) and incubated with an Alexa Fluor 488-conjugated anti-BrdU antibody for 1 hr at 37°C. After washing the slide in PBS, nuclei were stained with DAPI prior to mounting of cover slips. Finally, slides were visualized on a fluorescence microscope. The percentage of BrdU-positive cells was calculated as the number of BrdU-positive cells in the DAPI-positive population. Multiple random fields were assessed to generate an average number of BrdU-positive cells in the sample.

**ANNEXIN V-CY3 STAINING**

Cells undergoing apoptosis express phosphatidyl serine (PS) on the outer leaflet of the plasma membrane lipid bilayer. In healthy cells, PS is localized to the inner leaflet of the lipid bilayer. Annexin A5 (or annexin V) binds with high affinity to PS, which is accessible only when PS is localized to the outer leaflet. Thus, annexin V can be used as a marker of apoptotic cells.

To calculate the percentage of cells undergoing apoptosis, cells were first washed with PBS and resuspended in annexin V binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl₂) containing stock Annexin V-Cy3 (BioVision; Mountain View, CA). Following a 5 min incubation at room temperature in the dark, cells were pelleted and washed with fresh annexin V binding buffer. Washed cell pellets were resuspended in 4% paraformaldehyde and incubated at room temperature for 20 min. Paraformaldehyde-fixed cells were spotted on glass slides, permeabilized with 0.25% Triton-X 100 in PBS, and stained with DAPI prior to visualization by fluorescence microscopy. The percentage of annexin V-Cy3-positive cells was calculated as the number of annexin V-Cy3-positive cells
in the total DAPI-positive population. Multiple random fields were assessed to obtain an average value of the percentage of annexin V-positive cells.

**LENTIVIRUS PRODUCTION**

After lentiviral vectors were cloned and assessed for efficiency via HEK293T transfection and western blot or RT-PCR analysis, lentivirus was produced for use in cell-based experiments. To produce lentivirus, HEK293T cells were plated in T_{75} flasks such that they achieved 50% confluence at the time of transfection. For each T_{75} flask, 12 μg of lentiviral vector, 9 μg of Pax2 gag/pol, and 3 μg of vesicular stomatitis virus G protein (VSV/G) were mixed with 1 ml CaCl\textsubscript{2} (2.5 M) and 1 ml 2X HBS. Transfections were conducted as described above. Two days post-transfection, culture medium was collected and passed through a 0.45 μm filter. Filtered medium was then centrifuged at 49,000 x g in a SW28 rotor (Beckman Coulter, Inc; Palo Alto, CA) for 2 hr at 4°C in an ultracentrifuge. Lentivirus pellets were resuspended in RPMI 1640 medium, aliquoted, and stored at -80°C. Titering of lentivirus was assessed by GFP fluorescence (for pYNC352-derived lentiviral vectors) or immunofluorescent staining of cells transduced with limiting dilutions of aliquoted lentivirus.

**LENTIVIRAL TRANSDUCTION OF CULTURED CELLS**

Transduction experiments were conducted following lentivirus titration. For adherent cells (e.g. HEK293T or TIME cells), culture medium was removed. Then, RPMI 1640 medium containing lentivirus and polybrene (1 μg/ml) was added to cells. After 8 hr of incubation at 37°C, medium containing lentivirus was removed and replaced with fresh cell-appropriate culture medium. For suspension cells (e.g. PEL cells), cells were pelleted, and culture medium was removed. Cell pellets were resuspended in lentivirus- and polybrene-containing RPMI 1640 medium and incubated for 8 hr as described above.
Following incubation with lentivirus, PEL cells were pelleted and resuspended in fresh RPMI 1640 medium containing 10% FBS and allowed to recover for 48 hr prior to initiation of cell growth assays or HHV-8 reactivation.

**CELL GROWTH ASSAY**

To determine the role of vIL-6 and cellular proteins on the growth of PEL cells in culture, PEL cells were first transduced with vIL-6- or cellular protein-specific shRNA-encoding lentivirus. Two days post-transduction, trypan blue-excluding (viable) cells were counted using a hemocytometer. Equivalent numbers (1 x 10^5 cells/well) of control (non-silencing shRNA-encoding lentivirus) and experimental (e.g. vIL-6-specific shRNA-encoding lentivirus) cells were plated in duplicate wells of a 24-well cell culture plate. Plated cells were then counted daily for three additional days to generate growth curves. At the end of the growth experiment, remaining cells were harvested for preparation of cell lysates for western blot analysis.

**HHV-8 R219 GENERATION AND INFECTION OF ENDOTHELIAL CELLS**

HHV-8 r219 virus was designed such that GFP expression is driven by the human elongation factor 1 alpha (EF-1α) promoter, and RFP expression is driven by the viral PAN RNA promoter [254]. GFP is expressed constitutively, while RFP is expressed exclusively in lytic cells as PAN RNA is expressed only during the viral lytic cycle. Therefore, GFP and RFP fluorescence can be used as markers to determine whether cells have been infected with HHV-8 r218 (GFP^+) or have been reactivated (RFP^+, lytic).

HHV-8 r219 virus was generated in Vero-r219 cells infected with baculovirus-encoded Rta (a gift from Prashant Dasai). Briefly, Vero-r219 cells were grown in DMEM supplemented with 10% FBS and puromycin (5 μg/ml). Medium was removed from Vero-r219 cells, and baculovirus-encoded Rta was added to the cell culture medium. The cells
and virus were incubated at 37°C for 2 hr with continuous rocking. Cells were then washed with fresh DMEM and incubated with DMEM containing 10% FBS and NaB (1 mM) for 24 hr. Medium was then replaced with DMEM supplemented with 10% FBS, and cells were incubated for an additional four days. Finally, culture medium was harvested and centrifuged briefly at 400 x g for 5 min to remove cellular debris. The supernatant was then centrifuged at 16,000 rpm (SW41 rotor, Beckman Coulter, Inc) for 2 hr at 4°C in an ultracentrifuge to pellet the virus. Viral pellets were resuspended in EBM-2 without serum or supplements, aliquoted, titered on naïve TIME-TRE/RTA cells, and frozen at -80°C until needed.

TIME-TRE/RTA endothelial cells were infected with HHV-8 r219 virus for use in virus reactivation experiments. Prior to infection, TIME-TRE/RTA cells were plated such that they would achieve approximately 90% confluence at the time of infection. Culture medium was removed, and HHV-8 r219 virus was added to cells. Cell cultures containing virus were centrifuged clockwise at 400 x g for 30 min at 37°C and then counterclockwise for an additional 30 min at the same speed and temperature. Following an additional 1 hr incubation at 37°C at 5% CO₂, medium containing virus was removed and replaced with fresh EBM-2 containing supplements and FBS. Two days post-infection, cells were visualized for GFP expression via fluorescence microscopy to determine infectious titers.

For reactivation experiments in endothelial cells, HHV-8 r219-infected TIME-TRE/RTA cells were then transduced with lentivirus targeting viral or host proteins and reactivated as described below.

**TITERING OF REACTIVATED HHV-8**

To assess the role of viral and host proteins in HHV-8 productive replication, protein-depleted TIME and PEL cells were reactivated, and viral titers were calculated. Transduced JSC-1 PEL cells were reactivated with NaB (0.5 μM) and 12-O-
tetradecanoylphorbol-13-acetate (TPA, 20 nM), and medium was harvested four days post-induction. Transduced TRExBCBL1-RTA and HHV-8 r219-infected TIME-TRE/RTA cells were induced with doxycycline (Dox, 1 μg/ml) and harvested at four (TRExBCBL1-RTA) or six (TIME-TRE/RTA) days post-induction. Harvested culture medium containing virus was passed through a 0.45 μm filter and centrifuged at 16,000 rpm (SW41 rotor, Beckman Coulter, Inc) in an ultracentrifuge for 2 hr at 4°C to pellet virus. Viral pellets were resuspended in EBM-2, and aliquots of harvested virus were applied to naïve TIME-TRE/RTA cells for 2 hrs. Cells and virus-containing medium were centrifuged clockwise at 400 x g for 30 min at 37°C and then counterclockwise using the same conditions. Following a 1 hr incubation at 37°C in a 5% CO₂ chamber, medium containing virus was removed, and fresh EBM-2 containing FBS and supplements was added. The following day, cells were trypsinized, washed with PBS, and plated on glass chamber slides. One day after the cells were plated, slides were fixed in methanol as described above and stained using an anti-LANA primary antibody and a Cy3-conjugated fluorescent secondary antibody. Nuclei were stained with DAPI, and slides were visualized via fluorescence microscopy. The percentage of LANA-positive cells was calculated as the number of LANA-positive cells in the total DAPI-positive cell population. Multiple random fields were assessed.

DEPLETION AND COMPLEMENTATION EXPERIMENTS

Depletion and complementation experiments were utilized to address several research questions, including whether an ER-localized vIL-6 signaling mutant could rescue PEL cell growth in vIL-6 depleted cells and whether ER-localized vIL-6/gp130-mediated STAT and MAPK signaling played a role in virus replication. These experiments required that cells be transduced with multiple lentiviruses.
For experiments assessing the rescue of vIL-6 depletion with an ER-localized vIL-6 signaling mutant, cells were first transduced with lentivirus encoding an ER-localized and shRNA-resistant vIL-6 signaling mutant. After allowing time for the transduced cells to recover, cultures were selected in puromycin (1 μg/ml) to eliminate untransduced cells. Then, puromycin-selected cells were transduced with lentivirus encoding vIL-6-specific or non-silencing shRNAs. Cells were allowed to recover for two days following the second lentiviral transduction. Then, cells were normalized and plated for cell growth assays as described above.

For depletion-complementation assays addressing virus replication, TRExBCBL1-RTA PEL cells were transduced with the first lentivirus (overexpressed vIL-6 variants or gp130-signaling mutants) and allowed to recover for two days prior to being transduced with the second (shRNA-encoding) lentivirus. GFP fluorescence was used as a marker of transduction efficiency of the shRNA-encoded lentivirus. A subset of doubly transduced TRExBCBL1-RTA cells were harvested in TRIzol for RT-PCR analysis to verify the expression of vIL-6 and gp130 signaling mutants. As before, cells were allowed to recover for two days prior to cell count normalization and reactivation with Dox. Virus was harvested from culture media and applied to naïve TIME-TRE/RTA cells for LANA staining as described above. Specific procedures are detailed in the methods sections of the appropriate chapters.
CHAPTER 3: INVOLVEMENT OF GP130 IN VIL-6-MEDIATED PEL CELL GROWTH AND SURVIVAL
Summary

Prior to initiating this dissertation work, vIL-6 had been implicated in the pathogenesis of HHV-8-associated diseases via pro-proliferative, anti-apoptotic, and pro-angiogenic activities. In PEL cells, vIL-6 is produced in functional amounts during viral latency and promotes the growth of these cells, mediating its activity from the ER, where it is predominantly localized. This vIL-6 activity is dependent, in part, on its interaction with a splice variant of VKORC1, termed VKORC1v2. However, the roles of gp130 and ER-localized vIL-6/gp130 interactions in PEL cells were unknown. Thus, this study sought to determine the influence of gp130 on PEL cell viability and growth, the role of ER-localized vIL-6/gp130 signaling in PEL cell maintenance, and the contributions of gp130-mediated MAPK and STAT signaling to PEL cell proliferation and survival.

Targeted depletion of gp130, vIL-6, ERKs 1 and 2, STAT1, and STAT3 was conducted to determine the role of these proteins in PEL cell growth and survival. Additionally, gp130-activated MAPK and STAT signaling was assessed following gp130 depletion to determine the contribution of gp130 to activation of these signaling pathways in PEL cells. Finally, two approaches were utilized to establish the significance of ER-localized vIL-6/gp130 signaling on the proliferation and viability of PEL cells.

This study found that the IL-6 signal transducer, gp130, is required for optimal PEL cell growth and viability. Levels of activated ERKs 1 and 2 and STATs 1 and 3, phosphorylated following gp130 stimulation, were reduced in gp130-depleted BCBL-1 and BC-1 PEL cells. Diminished STAT activation was also detected in two other PEL cell lines, JSC-1 and BC3. Growth effects of gp130 depletion could be mimicked by shRNA targeting of ERKs 1 and 2 or by depletion of STAT3. Finally, inhibition of vIL-6/gp130 interactions specifically within the ER compartment suppressed cell proliferation and viability, mirroring the effects of gp130 depletion. Combined, these data demonstrate that gp130, in addition to VKORC1v2, is essential for normal PEL cell growth and survival and that ER-localized
vIL-6/gp130 interactions are critical for these activities. Targeting of intracellular vIL-6/gp130 interactions could potentially provide a means of PEL therapy.
ROLE OF HUMAN HERPESVIRUS 8 INTERLEUKIN-6-ACTIVATED GP130 SIGNAL TRANSUDER IN PRIMARY EFFUSION LYMPHOMA CELL GROWTH AND VIABILITY

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INTRODUCTION

HHV-8 encodes several proteins that are believed to contribute to the onset and/or progression of endothelial KS and B-cell malignancies PEL and MCD [255-258]. vIL-6, like its cellular counterparts, is a growth factor for B-cells and other cell types and promotes inflammatory and angiogenic responses. These activities implicate the viral cytokine as a contributory factor in HHV-8 associated neoplasias [144,259]. In PEL cells, true latent expression of vIL-6 suggests that the viral protein can contribute to this disease in a direct, autocrine fashion by promoting PEL cell proliferation and survival, in addition to possibly maintaining viral latent reservoirs during normal (disease-free) infection [115,116].

While the three-dimensional structures of vIL-6 and hIL-6 are similar and both can bind to and induce dimerization of the gp130 signal transducer, vIL-6 is unique in that it is “pre-conformed” to mediate gp130 dimerization without first binding the non-signaling gp80 IL-6 receptor subunit [165,260,261]. However, vIL-6 can bind gp80 and form hexameric complexes (vIL-6<sub>2</sub>:gp130<sub>2</sub>:gp80<sub>2</sub>) in addition to tetrameric (gp80-devoid) complexes [159,165]. Hexameric and tetrameric complexes have distinguishable signaling properties [262], likely mediated in part by gp80 stabilization of vIL-6-induced gp130 dimerization [159,165]. Within the ER, vIL-6 induces the formation of tetrameric complexes exclusively [115,170]. ER-directed hIL-6 is unable to induce gp130 complexing
and signal transduction. vIL-6, hIL-6, and other cellular IL-6 proteins activate STATs 1 and 3 via gp130-associated Janus kinase (JAK)-mediated tyrosine phosphorylation of the transcription factors [156]. MAPK signaling is activated following SHP2 recruitment to gp130 and phosphorylation by JAK, which leads to downstream phosphorylation and activation of ERKs 1 and 2 [156]. In addition to differences in gp80-dependency of ligand-induced gp130 dimerization and the ability of vIL-6 to signal from the ER, inefficient secretion of vIL-6 distinguishes it from its cellular counterparts [170]. Thus, vIL-6 is found largely intracellularly, specifically within the ER, and its ability to signal from this compartment suggests that this may be functionally important for both virus biology and viral pathogenesis. Indeed, vIL-6 depletion-mediated inhibition of PEL cell growth in culture can be reversed by transduction of ER-retained (KDEL motif-tagged) vIL-6 [115]. Also, vIL-6 support of PEL cell growth can be inhibited by an ER-localized single-chain antibody specific to vIL-6 [150]. It is reasonable to hypothesize that vIL-6 may contribute to PEL pathogenesis via gp130 signaling. STAT3, a major target of such signaling and a transcription factor implicated in many human cancers [263-265], is activated in PEL cells and appears to be important for their viability, in part via the STAT3-induced pro-survival protein survivin [152].

However, demonstration of vIL-6-mediated signal transduction via gp130 in PEL cells and the role of gp130 in PEL cell biology have not been reported. Recently, vIL-6 was found to interact with the ER membrane protein VKORC1v2, a splice variant of warfarin target VKORC1 (variant 1) [174], and this interaction was shown to be important for the pro-growth and anti-apoptotic activities of vIL-6 in PEL cells [173]. Interaction of vIL-6 with VKORC1v2 occurs via a transmembrane-proximal region of the ER-lumenal domain; its precise mapping enabled the development of a vIL-6 refractory variant and an interaction-inhibitory peptide that helped reveal the functional relevance of the vIL-6-
VKORC1v2 interaction [173]. The mechanism of vIL-6 activity via VKORC1v2 appears to be independent of gp130.

In the present study, we have investigated directly the influence of gp130 on PEL cell proliferation and viability, the role of the vIL-6-gp130 interaction in gp130-mediated activities, and the contributions of gp130-activated MAPK and STAT signaling to PEL cell growth. Our data indicate that gp130 is essential for normal proliferation and viability of PEL cells and that ER-localized vIL-6/gp130-activated ERK and STAT3 signaling are critically important. These findings suggest that inhibitory targeting of vIL-6 interactions with the signal transducer within the ER may provide a useful strategy for PEL therapy or for targeting HHV-8 latency in normal B-cells should vIL-6 be expressed in this latent reservoir.

METHODS

Cell culture, transfections, and lentivirus production

Cell cultures were maintained as described in Chapter 2. HEK293T cells were transfected with plasmids using standard calcium-phosphate-DNA co-precipitation as explained in Chapter 2. Cells were harvested 24 to 48 hrs post-transfection. Cell lysates were prepared as outlined in Chapter 2, and lysates were analyzed directly or stored at -20°C prior to Western blot analysis. Lentivirus was produced by transfecting HEK293T cells with shRNA-, vIL-6-, or sgp130-encoding or empty lentivirus plasmids (pYNc352 and pDUET001) together with gag/pol and VSV/G expression vectors, as previously described and in Chapter 2 [173]. Infectious titers were established by using GFP-based or immunofluorescence microscopy to identify vector-transduced cells in PEL and other cultures prior to experimental utilization of lentiviral vectors (see Chapter 2). For vIL-6 “rescue” experiments, PEL cells were first transduced with pYNc419-vIL-6.KDEL or pYNc419-vIL-6.W167G.KDEL, allowed to recover for 5 days, selected in puromycin (1.0
μg/ml) for 2 weeks, and then transduced with either NS or vIL-6-specific shRNA encoded by pYNC352-derived lentviruses. For shRNA and sgp130-transduced cultures, cells were allowed to recover for 2 days prior to normalizing cell numbers for cell growth assays.

**Oligonucleotides and plasmids**

shRNAs were chosen or designed to selectively deplete gp130, STAT1, STAT3, ERK1, or ERK2. Complementary oligonucleotides encoding 19-nucleotide short hairpin regions were annealed, phosphorylated, and cloned into the BamHI and MluI sites of the pYNC352 lentivirus vector. The following, pre-validated shRNA sequences were used: STAT1 (5'-GCGTAATCTTCAGGATAAT-3' [266]), STAT3 (CATCTGCCTAGATCGGCTA [266,267] used independently or together with AGTCAGGTTGCTGGTCAAA [266]), ERK1 (GCCATGAGAGATGTCTACA [268]), and ERK2 (GAGGATTGAAGTAGAACAG [268]). Three shRNA sequences were designed and tested for gp130 depletion: GTGGGATCACCTATGAAGA (sh1), CTGTCCAAGACCTTAAACC (sh2), and TGCCCTTGGGAAGGTTACA (sh3); sh1 was used in subsequent functional analyses, alone or together with sh3. vIL-6 shRNA oligonucleotides and non-silencing (NS) control shRNAs were described previously [115]. For generation of a lentivirus vector expressing KDEL motif-tagged vIL-6 variant W167G [166], the corresponding vIL-6 coding sequences were first cloned between the BamHI and Smal sites of pSG5.KDEL [166] to generate contiguous vIL-6.W167G-KDEL coding sequences, and these sequences were then PCR amplified and inserted between the NotI and XhoI sites of pTYB6-derived lentivirus vector pYNC419 [166,269]. Lentiviral vector-cloned vIL-6 and vIL-6-KDEL and pSG5-cloned vIL-6, vIL-6-KDEL, vIL-6.W167G, hIL-6, hIL-6-KDEL, gp130-Fc, and chitin binding domain (CBD)-fused VKORC1v2 have been described previously [69,115,165,166,173]. The soluble gp130 (sgp130) expression vector was made by amplifying the first 972 nucleotides of gp130 (encoding domains 1-3) and inserting this sequence between the
EcoRI and BamHI sites within the pSG5.KDEL vector, generating pSG5-sgp130.KDEL. A sequence encoding the FLAG epitope was then inserted at the BamHI site using appropriate complementary oligonucleotides, generating pSG5-sgp130.FLAG.KDEL. The sgp130.FLAG.KDEL coding region was then amplified and ligated into the pDUET001 lentiviral vector [270] between the BamHI and XhoI sites. The integrities of all constructions were verified by sequencing.

**Immunoblotting and co-precipitation assays**

For western blotting, heat-denatured and reduced proteins in cell lysate samples were size fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes using standard procedures (see Chapter 2). Membranes were blocked, probed with primary antibodies, washed, incubated with appropriate HRP-conjugated secondary antibodies, and exposed as described in Chapter 2. For co-precipitation assays, cell lysates (prepared as described above) were added to either chitin beads (for precipitating VKORC1v2-CBD) or protein-A agarose beads (for precipitating gp130-Fc) and incubated with continuous rotation overnight at 4°C. Beads were then washed 3 times with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) prior to boiling and loading onto SDS-PAGE gels. Untreated lysate samples were also separated on SDS-PAGE gels to provide controls for protein expression, extraction, and transfer to immunoblotting membranes.

**Antibodies**

The following commercially available antibodies were used for western blotting: BrdU (BD Biosciences, Rockville, MD; catalog number 558599); pSTAT3 and pSTAT1 (Cell Signaling Technology, Beverly, MA; catalog numbers 9131 and 9171); β-actin (Sigma, St. Louis, MO; catalog number A2228); pERK, ERK1, STAT3, p-tyrosine, and
gp130 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog numbers sc-7383, sc-94, sc-482, sc-508, and sc-655); hIL-6 (R&D Systems, Minneapolis, MN; catalog number MAB-206); and CBD (New England Biolabs, Ipswich, MA; catalog number E8034S). Rabbit antiserum to vIL-6 was generated in our laboratory and reported previously [166]. The gp130 (BD Biosciences, Rockville, MD; catalog number 555756) and FLAG (Sigma; catalog number F1804) primary antibodies and the Alexa Fluor 594 donkey anti-mouse IgG (H+L) secondary antibody (Invitrogen, Carlsbad, CA; catalog number A21203) were used for immunofluorescence staining.

**Apoptosis, proliferation, and cell growth assays**

Apoptosis was assessed via annexin V-Cy3 staining of cells as outlined in Chapter 2. Several fields were used to calculate the percentage of annexin V-Cy3-positive cells in the total DAPI-positive cell population. Cell proliferation rates were determined by BrdU-incorporation assay, which was detailed in Chapter 2. Slides were visualized under UV microscopy to assess the percentage of BrdU-positive cells in the total cell population (DAPI¹). Multiple fields were assessed to determine an average percentage of BrdU-positive cells. For cell growth assays, appropriate, high-efficiency infection with the GFP-positive lentiviral vectors was verified by >90% GFP fluorescence in the cultures, which was sustained over the course of the 3-day growth experiment. Cell growth assays were initiated following cell density normalization and seeding of cells (10⁵) in fresh media 48 hrs after lentiviral infection. Growth of the cultures was quantified by daily counting of trypan blue-excluding (viable) cells per ml using a hemocytometer. Cultures transduced with equivalent titers of lentiviral vector expressing NS shRNA were used as controls.
RESULTS

Contributions of gp130 to PEL cell growth. In previous studies, we found that depletion of vIL-6 in BCBL-1 and JSC-1 PEL cells led to autocrine-mediated reductions in cell proliferation and survival and that ER-localized vIL-6 activity was sufficient for these functions [115]. Subsequently, we determined that expression of VKORC1v2 and its interaction with vIL-6 were important gp130-independent contributors to the pro-growth and anti-apoptotic activities of vIL-6 in these cells [173]. However, the possibility remained that vIL-6 signaling through gp130, in the ER and/or at the cell surface, also contributes to vIL-6 effects on PEL cells. The potential contribution of gp130 to PEL cell growth was tested using lentiviral-delivered gp130-specific shRNA to deplete endogenous expression of the signal transducer. Three shRNAs for gp130 depletion were designed (Methods) and tested in lentivirus-shRNA vector-transfected HEK293T cells, and each significantly depleted overexpressed gp130 levels relative to vector-expressed gp130 observed in NS shRNA-transfected control cultures (data not shown). Two of these gp130 shRNA-expressing lentivirus vectors were used for single or dual transduction (see Methods) for subsequent experiments in PEL cells. The control NS shRNA-encoding lentiviral vector was used for comparison. Equivalent inoculating doses of pre-titered lentiviruses were used, sufficient to provide ~90% transduction efficiency, as determined by lentivirus-expressed GFP fluorescence. After a post-infection incubation period of two days, growth of cell density-normalized PEL cultures was monitored for an additional three days. For all PEL cell lines tested (BCBL-1, JSC-1, BC-1, and BC-3), gp130-targeted shRNAs led to significantly reduced cell growth relative to NS shRNA-transduced controls (Figure 3.1A). Potential off-target, generally cytotoxic effects of gp130-directed shRNA were controlled for by equivalent experiments in HHV-8-negative BJAB cells, which were essentially unaffected by gp130 shRNA transduction relative to NS shRNA-transduced controls.

The observed reductions in PEL cell growth following gp130 depletion could be
due to decreased cell survival and/or decreased rates of proliferation. This was investigated utilizing annexin V-Cy3 staining assays to detect apoptotic cells and BrdU incorporation analysis to identify S-phase cells in BCBL-1 and JSC-1 cultures transduced with either NS or gp130-specific shRNA. The data from these experiments revealed a correlation between gp130 depletion and both increased rates of apoptosis (Figure 3.1B) and decreased rates of DNA synthesis (Figure 3.1C). The combined results indicate that gp130 signaling supports PEL cell growth by promoting both cell proliferation and cell survival.

**Influence of gp130 on STAT and ERK signaling in PEL cells.** Signal transduction by gp130 involves activation of STAT and MAPK signaling. This proceeds via JAK-mediated tyrosine phosphorylation of gp130-recruited STATs 1 and 3 and tyrosine and threonine phosphorylation of ERKs 1 and 2 following the activation of the MAPK signaling cascade, which is triggered by JAK phosphorylation of gp130-associating SHP2 [156]. Active STAT3 has been reported previously to be expressed at constitutively high levels in PEL cells and to be important for PEL cell viability in culture [152]. However, the mechanism of constitutive STAT3 activation in PEL cells and the biological significance of ERK signaling in these cells are unknown. To address the role of gp130 in the activation of STAT3 and ERK in the context of PEL cells, we again utilized gp130 depletion via lentiviral vector-mediated shRNA transduction. Targeting of gp130 resulted in detectably reduced levels of phospho-STAT3 (pSTAT3, active) and pSTAT1 in JSC-1, BCBL-1, BC-1, and BC-3 cells (Figure 3.2A,B) and a marked decrease in phospho-ERK (pERK) levels in BCBL-1 and BC-1 cells but not in JSC-1 and BC-3 cells (Figure 3.2C). The data from these experiments identify, for the first time, the contribution of gp130 to constitutively high pSTAT3 and pSTAT1 levels in PEL cells and provide evidence that gp130 can significantly influence ERK signaling in at least some PEL cell types (shown here for BCBL-1 and BC-1).
Contributions of STATs to PEL cell growth. It has been reported by others that STAT3 is essential for the viability of PEL cells (BC-1, BCBL-1, and VG-1) and that this activity involves STAT3-mediated induction of caspase-inhibitory and anti-apoptotic protein survivin [152]. These studies utilized transduced dominant-negative STAT3 and the JAK pharmacological inhibitor AG490 to suppress levels of pSTAT3. Others have documented the importance of JAK-mediated, STAT-activating signaling in several PEL cell lines via the experimental use of JAK1-inhibitory curcumin; this was found to inhibit cell proliferation, promote apoptosis, and suppress IAP family proteins, including survivin [271]. To independently assess the role of STAT3 in PEL cell growth, the transcription factor was targeted for depletion by shRNA-encoding lentiviruses. Two previously reported STAT3-targeted shRNAs [266,267] were first tested in transfected HEK293T cells and verified as effective (data not shown). These shRNAs were each cloned into a lentiviral vector for introduction into PEL cells. Initial experiments indicated that single shRNA transduction was insufficient to achieve strong STAT3 depletion in PEL cultures, whereas co-infection of PEL cells with both lentiviral vectors was effective. For growth assays, BCBL-1 and JSC-1 cells were simultaneously infected with both STAT3-targeting lentiviral vectors or with an equivalent infectious dose of NS shRNA-encoding control lentivirus. Cell growth of post-transduction cultures was monitored by daily counting of trypan blue-excluding (viable) cells to determine cell densities (Figure 3.3A). Suppression of STAT3 relative to NS shRNA-transduced cultures was verified in terminal (day 3) cultures by western blotting of cell extracts (Figure 3.3A, right panels). The data revealed that STAT3 depletion substantially diminished growth of these PEL cells, consistent with previously published studies [152], and identified JSC-1 PEL cells (not previously examined) as dependent on STAT3 for normal growth.

Further analyses were undertaken to test the contributions of STAT3 to proliferation and survival of BCBL-1 and JSC-1 cells. These experiments involved
immunofluorescence detection of BrdU incorporation and annexin V-Cy3 staining, respectively, as outlined above and for the equivalent studies following gp130 depletion. Cells were harvested 5 days following lentiviral transduction (3 days post-normalization and culture) for BrdU and annexin V analyses. The results mirrored the effects of gp130 depletion, demonstrating that STAT3 depletion inhibited cell proliferation (proportion of cells in S phase) (Figure 3.3B) and increased the rate of apoptosis (Figure 3.3C). Thus, both of these components of cell biology are influenced by STAT3, which in turn is activated by gp130 in these PEL cell lines.

As gp130 signaling involves activation of STAT1 in addition to STAT3, we also explored the potential contribution of STAT1 activity to BCBL-1 and JSC-1 cell growth. Lentiviral-delivered shRNA [266] was used to deplete STAT1 in the respective cultures, and cell growth was compared to that of control (NS) shRNA-transduced cultures. The data revealed no significant effects of STAT1 depletion on BCBL-1 or JSC-1 cell growth, despite achieving robust depletion of pSTAT1 in BCBL-1 cells and a nearly 2-fold reduction of pSTAT1 in JSC-1 cells, as demonstrated by western blotting of terminal culture extracts (Figure 3.3D). Although STAT1 depletion was somewhat limited in JSC-1 cells, these data suggest that STAT1 activity is not critical for PEL cell growth.

**Contributions of ERKs 1 and 2 to PEL cell growth.** As gp130 signaling involves the activation (phosphorylation) of ERKs 1 and 2 in addition to STATs, we undertook experiments to investigate the influence of ERK signaling on PEL cell growth. ERKs 1 and 2 were simultaneously targeted for depletion in BCBL-1 and JSC-1 cells using techniques analogous to those employed for STAT depletion. The NS- or ERK-shRNA lentiviral vector-transduced cells were monitored for growth over a three-day period by daily calculation of viable (trypan blue-excluding) cell densities from triplicate cultures for each cell type. For both PEL cell lines, ERK depletion led to dramatic reductions in cell growth relative to control (NS) shRNA-transduced cultures (Figure 3.4A). Therefore, ERKs 1 and
2, in addition to STAT3, are essential for normal growth of these cells.

The shRNA-transduced JSC-1 and BCBL-1 cultures were further analyzed to assess the effects of ERK depletion on cell proliferation and survival, specifically. Rates of proliferation and apoptosis in these cells following ERK1 and ERK2 depletion were examined by BrdU incorporation and annexin V-Cy3 staining, respectively, as outlined above. These analyses revealed that both proliferation and cell survival were reduced appreciably (between two- and three-fold) by ERK depletion (Figure 3.4B,C).

Analysis of the effects of ERK depletion in two additional PEL cell lines, BC-1 and BC-3, indicated the general importance of ERKs 1 and 2 for PEL cell growth (Figure 3.4D). Effects were not as marked in these cell lines as they were for BCBL-1 and JSC-1 cells, but it is possible that this was due to reduced efficacy of ERK depletion (Figure 3.4D, bottom panels). In contrast, ERK depletion in the HHV-8-negative BJAB B-cell line, reported previously to be insensitive to inhibition of ERK kinase (MEK) by PD98059 [272], were unaffected by ERK shRNA transduction, verifying lack of general cytotoxic effects of lentiviral infection and ERK shRNA transduction (Figure 3.4D, right). Combined, the data from our ERK depletion studies reveal the importance of ERK signaling, sustained in part via gp130 signal transduction in at least some PEL cell lines (BCBL-1 and BC-1, shown here), for PEL cell growth and viability.

ER-localized gp130/vIL-6 interaction contributes to vIL-6 and gp130 activities. We have previously reported that ER-localized vIL-6 and its interaction with the ER protein VKORC1v2 are important for PEL cell proliferation and viability [173]. A previously characterized vIL-6 variant, vIL-6.W<sub>167</sub>G, was used to determine the contribution of ER-localized vIL-6/gp130 interactions to PEL cell growth and survival. This altered vIL-6 protein is mutated in a region (“site III”) that interacts with gp130 domain 1 and is unable to induce dimerization of gp130 absent gp80 co-expression and hexameric complexing [159,166]. As ER-localized vIL-6 signaling occurs exclusively through
tetrameric (vIL-6:gp130) complexes [171], vIL-6.W_{167}G should be unable to induce gp130 dimerization and signaling from the ER compartment. We first verified that ER-retained, KDEL motif-tagged vIL-6.W_{167}G was unable to induce gp130-mediated signaling in transfected HEK293T cells. Unlike wild-type vIL-6-KDEL, vIL-6.W_{167}G-KDEL was unable to induce tyrosine phosphorylation of gp130 or STAT3 (Figure 3.5A). Co-precipitation assays from transfected cell lysates confirmed that the W_{167}G mutation did not affect the interaction between vIL-6 and VKORC1v2, which was tagged with CBD to facilitate precipitation and enable immune-detection with a CBD-specific antibody (Figure 3.5B). KDEL-tagged, shRNA-resistant versions of wild-type and W_{167}G-mutated vIL-6 were then used to supplement vIL-6-depleted PEL cells to determine if the gp130 dimerization-defective but VKORC1v2 binding-competent variant could rescue cell growth. These experiments were analogous to those undertaken previously with vIL-6-KDEL, which demonstrated sufficiency of ER-localized vIL-6 for maintenance of BCBL-1 and JSC-1 cell growth and viability [171]. In contrast to vIL-6-KDEL, vIL-6.W_{167}G-KDEL was unable to complement endogenous vIL-6 depletion (Figure 3.5C), indicating that vIL-6-induced dimerization of gp130 and its downstream signaling is critically important for pro-proliferative activity of ER-localized vIL-6 in PEL cells.

The importance of intracellular gp130 signaling was further tested by utilizing lentiviral-transduced soluble gp130 (sgp130), which was KDEL motif-fused for ER retention. Initial experiments in transfected HEK293T cells were undertaken to verify the ability of this protein to inhibit ER-localized (vIL-6.KDEL), but not extracellular (hIL-6), signal transduction via endogenously expressed gp130 (Figure 3.6A). Additionally, the intracellular localization of sgp130.KDEL (Flag-tagged) was tested and compared to the distribution of full-length gp130 by α-Flag and α-gp130 antibody-based immunofluorescence assays in transfected cells; cells were co-transfected with GFP-KDEL, which provided an ER marker [115]. In contrast to native gp130, sgp130.KDEL was
present at high levels intracellularly with little, if any, surface fluorescence detectable (Figure 3.6B). Having confirmed the expression and appropriate ER localization of lentiviral vector-expressed sgp130.KDEL, this vector, or an empty vector control, was then used to transduce PEL cells to determine the effects of ER-localized gp130 inhibition on cell growth and apoptosis. The data revealed significant effects of sgp130.KDEL, relative to the empty vector, on the growth of both BCBL-1 and JSC-1 PEL cells (Figure 3.6C). BrdU incorporation assays using cells harvested at day 2 from the growth experiment confirmed inhibition of proliferation (i.e., percentage of cells in S-phase) by introduced sgp130.KDEL (Figure 3.6D). In a separate experiment, transduction of sgp130.KDEL into BCBL-1 and JSC-1 cells led to substantial increases in rates of apoptosis, as determined by annexin V-Cy3 staining (Figure 3.6E). As vIL-6 is the only known cytokine to signal through gp130 from the ER compartment (and ER-retained hIL-6 has been demonstrated to be unable to do so [115]), these data suggest strongly that vIL-6/gp130 signaling is of biological significance and contributes, wholly or in part, to the pro-growth and pro-survival effects of gp130 in these cells.

DISCUSSION

The work reported here addresses the contribution of gp130 signaling to the constitutively high levels of active STAT3 reported in PEL cells, the influence of gp130 activity on STAT1 and ERK activation in these cells, and the functional relevance of vIL-6-mediated gp130 activity to PEL cell growth. To our knowledge, our data are the first to document a mechanism for the constitutively high levels of STAT3 in PEL cells, to establish a critical role of the gp130 signal transducer in supporting PEL cell proliferation and viability, and to identify the relevance of gp130 targeting by vIL-6 to these activities. Thus, in addition to the important association of vIL-6 with VKORC1v2 for PEL cell growth and survival [115], activity of the viral cytokine through its well-established signal
transducer is also critical. This activity is mediated from the ER, where vIL-6 is predominantly localized. A VKORC1v2 binding-competent, gp130 dimerization-defective vIL-6 variant (W_{167}G) that was restricted to the ER was unable to rescue the effects of vIL-6 depletion, in contrast to KDEL motif-tagged wild-type vIL-6. Furthermore, ER-localized sgp130, used as a competitive inhibitor of gp130/vIL-6 interactions in the ER compartment, enabled identification of the specific role of vIL-6 as a mediator of gp130 function in the context of PEL. Thus, gp130 and ER-localized vIL-6 signaling via gp130 have been shown for the first time to support PEL cell proliferation and viability. Therefore, gp130 and its interaction with vIL-6 have been identified as potential targets for therapeutic intervention.

Another important finding from this study is that ERK activation contributes significantly to the growth and survival of PEL cells. Depletion of ERK led to severe abrogation of cell proliferation (3-fold reductions in BrdU incorporation) and to greatly increased rates of apoptosis (2- to 3-fold) for BCBL-1 and JSC-1 cultures, effectively halting culture growth. Significant effects of ERK depletion on the growth of BC-1 and BC-3 cells were also identified. At least for BCBL-1 and BC-1 cells (included in this study), the high levels of active, tyrosine-phosphorylated ERK (pERK) are dependent on gp130. However, gp130 depletion did not diminish the levels of pERK in JSC-1 and BC-3 cells; pERK was actually increased in both cell lines. These patterns were observed in multiple repeat experiments in these PEL cell lines. In contrast to the effects of ERK depletion on PEL cell growth, ERK depletion in HHV-8-negative BJAB cells did not affect cell growth at all. The role of ERK signaling in HHV-8 biology has previously been noted only in connection with de novo infection, establishment of latency, and lytic reactivation. Thus, pharmacological inhibition of ERK activation suppresses TPA-induced lytic gene expression and replication in PEL cultures [273-276]. Additionally, activation of ERK-targeting signaling pathways and their requirement for de novo infection, post-entry lytic
gene expression, and latency establishment have been reported in endothelial cell systems [275,277,278]. Our results now add to this body of knowledge regarding the role of ERK signaling in HHV-8 biology by identifying ERK as an important factor for maintenance of PEL cell viability and by establishing gp130 signaling as a mechanism contributing to high levels of active ERK in at least some PEL cell types.

Previous studies by Aoki and colleagues [152] demonstrated that the JAK inhibitor AG490 and ectopic expression of a dominant-negative STAT3 protein (STAT3D, DNA binding-refractory) led to dramatically reduced PEL cell viability. The PEL cell lines investigated were BCBL-1, BC-1 and VG-1. Our experiments investigating the role of STAT3 utilized BCBL-1 and JSC-1 cells, characterized in depth previously with respect to latent expression and pro-growth and pro-survival activities of ER-localized vIL-6 [115]. A caveat of the experiments utilizing AG490 is that this pharmacological inhibitor can affect JAK-activated receptors other than gp130 and receptor-mediated activation of STATs other than STAT3; AG490 may also have non-specific effects on other pathways. Of additional note is that experimental use of the STAT3D protein, while very effective at blocking endogenous STAT3 activity, also has the potential to inhibit STAT1 and other STAT3-hetrodimerizing proteins; thus it may affect STAT3-independent mechanisms. However, the previously reported data from Aoki et al. [152] combined with our independently-derived and concordant results from STAT3 depletion experiments provide very strong evidence that STAT3 signaling is critical to PEL cell biology. STAT3 signaling was shown to induce the pro-survival protein survivin, which was found to be an important factor in STAT3-mediated pro-survival effects in PEL cells [152]. However, it is noteworthy that the JAK-targeting STAT signaling inhibitor curcumin, while demonstrated in an independent study to induce cell death with concomitant suppression of survivin in most of the PEL cell lines tested, did not lead to a significant loss of survivin expression in BCBL-1 cells (used in the present study and that of Aoki et al. [152]) [271]. Thus, survivin
may not be the only STAT3-regulated factor critical to PEL cell viability. Nonetheless, our data reveal that gp130 and ER-localized vIL-6 activity through the signal transducer can contribute to high constitutive levels of active pSTAT3, a factor that has been linked to cell growth and survival in PEL and other neoplasias as well as under normal physiological conditions [264,279,280], and to promotion of cell proliferation and viability. It is noteworthy that gp130-dependent constitutively high levels of active STAT3 have also been detected in endothelial cells latently infected with HHV-8, although via (paracrine) mechanisms independent of vIL-6 [281]. Furthermore, de novo infection of blood endothelial cells leads to STAT3 induction and gp130-dependent, but vIL-6-independent, lymphatic endothelial cell reprogramming [18]. Thus, STAT3 signaling, in part mediated via gp130, may be generally important for maintenance of HHV-8 latency and viability of latently infected cells and may also contribute to virus-associated neoplasia.

In conclusion, the data presented here document for the first time the significance of gp130-mediated signaling for proliferation and survival of PEL cells, the importance of gp130 as a contributor to constitutively high levels of functionally significant STAT and/or ERK signaling in at least some PEL cell lines, and the contribution of ER-localized vIL-6/gp130 interactions for PEL cell growth and viability. These data broaden our understanding of the role and mechanisms of action of latently expressed vIL-6 in PEL cell biology and provide further support for the idea that drug targeting of the viral cytokine and its signal transducer, within the ER compartment, may represent a useful therapeutic strategy.
FIGURES

Figure 3.1. Effects of gp130 depletion on PEL cell growth and viability. (A) Triplicate cultures of BCBL-1, JSC-1, BC-1, and BC-3 PEL cells and HHV-8-negative BJAB B-cells were transduced either singly (BCBL-1, JSC-1, and BJAB) or dually (BC-1 and BC-3) with gp130-directed shRNA(s) by lentiviral infection, and cells were counted for three days following normalization of cell numbers (see Chapter 2 and Methods above). Error bars represent divergence from mean values determined for the triplicate cultures. Western blotting of cell lysates derived from pooled cultures at the end of the growth experiment (on day 3) was carried out to verify gp130 depletion (shown below the associated growth curves). For BC-3 cells, complete cytostatic effects of gp130 shRNA transduction resulted in insufficient cell numbers to process for immunoblotting. Levels of gp130 in BJAB cell lysates were below the limit of detection; the growth data show lack of general cytostatic effects of lentiviral-mediated gp130 shRNA transduction. (B) Samples of gp130 and NS shRNA-transduced BCBL-1 and JSC-1 cultures harvested on day 2 from the growth curve experiments shown in panel A were analyzed for proliferation. Rates of proliferation were determined by detection of BrdU incorporation using Alexa Fluor 488-conjugated BrdU-specific antibody for immunofluorescence visualization of S-phase cells and counterstaining with DAPI to detect all nuclei (see Chapter 2 and Methods above). Relative proliferation rates are shown as the percentage of cells in S-phase (BrdU-positive) for the respective cultures. Data were derived from two random fields (>100 cells/field) for each slide-spotted culture sample. Examples of BrdU and DAPI co-staining (sections of a single field, BCBL-1 cells) are shown below the quantified data. (C) Rates of apoptosis in these same PEL cultures were determined by annexin V-Cy3 binding. DAPI staining was again used to visualize nuclei, enabling calculation of the percentage of cells undergoing apoptosis. Examples of annexin V-Cy3 and DAPI co-staining (of JSC-1 cells) are shown below the graphs. Cells were harvested on day 3 for the apoptosis assays.
Figure 3.1
Figure 3.2. Contribution of gp130 signaling to levels of active STATs and ERKs in PEL cells. Phosphotyrosine-specific antibodies were utilized in immunoblotting to detect and determine the levels of phosphorylated (active) gp130-targeted STAT3 (panel A), STAT1 (panel B), and ERKs 1 and 2 (panel C) (pSTAT3, pSTAT1, and pERK) present in lysates of PEL cells depleted of gp130 relative to levels of these proteins in NS shRNA-transduced controls. Antibodies detecting total (phosphorylated and unphosphorylated) STAT3 and ERK1/2 and β-actin were used to verify equivalent protein loading on SDS-PAGE gels and transfer to blotting membranes. For each set of lysates (used for single or multiple phospho-protein detection), effective depletion of endogenous gp130 in the lentiviral-shRNA infected PEL cultures was verified by immunoblotting for gp130. Monitoring of growth of NS-shRNA versus gp130-shRNA cultures for BCBL-1, JSC-1, and BC-1 cells over three days (as in Figure 3.1) confirmed the growth-inhibitory effects of gp130 depletion (data not shown); cells were harvested on the third day for western analyses. For BC-3 cultures, all cells were harvested 48 hrs after lentiviral infection (“day 0”) to ensure sufficient material for immunoblotting.
Figure 3.2
Figure 3.3. Contributions of gp130-activated STATs to PEL cell growth. (A) Appropriate lentiviral-shRNA vectors (see Methods above) were used in combination to transduce BCBL-1 and JSC-1 PEL cells to deplete STAT3. Growth curves were derived from triplicate cultures transduced separately with the NS and STAT3-targeting lentiviral-shRNA vectors and monitored in parallel (see Chapter 2). Error bars represent deviations from mean cell density values at each 24-hr timepoint over the three-day experiment. Pooled cultures were harvested at the end of the experiment (day 3) for western analysis of cell lysates to check for depletion of STAT3. (B) Proliferation of STAT3-depleted versus NS shRNA-transduced BCBL-1 and JSC-1 cells were examined by BrdU incorporation assay, as outlined in Figure 3.1, applied to cells harvested on day 3 (5 days after shRNA transduction). (C) Rates of apoptosis in these same culture samples were determined by annexin V-Cy3 binding assays and co-staining of cell nuclei with DAPI (see above, Figure 3.1 legend, Chapter 2). (D) STAT1 depletion experiments were performed to examine the potential contributions of STAT1 to BCBL-1 and JSC-1 cell growth. STAT1 depletion was mediated by transduction of PEL cultures with a lentiviral vector encoding a STAT1 mRNA-targeting shRNA (see Methods above), and NS shRNA-encoding lentivirus was used as a control as before. While substantial depletion of STAT1 was apparent for each cell line (immunoblots, right), growth of neither was altered significantly as a result. Chemiluminescence signals from western blots were digitally captured, quantified using imager-associated software, and normalized to β-actin signal. Levels of pSTAT1 in JSC-1 cultures was 1.9-fold reduced relative to STAT1 levels in the corresponding NS shRNA-transduced controls; no pSTAT1-specific signal was detected in BCBL-1 cell lysates from STAT1 shRNA-transduced cultures.
Figure 3.3
Figure 3.4. Analysis of culture growth, cell proliferation, and apoptosis rates as a function of ERK depletion. (A) Potential contributions of ERK to the growth of BCBL-1 and JSC-1 cultures were examined by monitoring growth of cells transduced by lentiviral vectors encoding either ERK-targeting or NS (control) shRNAs. Cell growth assays were conducted as described in Chapter 2 and the Methods above. Error bars indicate deviations from average values derived from triplicate cultures. Effective ERK depletion was verified by immunoblotting of lysates from BCBL-1 and JSC-1 cells pooled from the respective triplicate cultures and harvested at the end of the experiment. β-actin was used as a control for equivalent protein loading and membrane transfer. (B) Relative proliferation rates of BCBL-1 and JSC-1 cultures transduced with NS or ERK1/2-directed shRNAs were determined by BrdU incorporation assay (see Chapter 2 and Methods above). Cultures were harvested 4 days after shRNA transduction and 2 days post-normalization prior to growth monitoring (panel A). The proportions of BrdU-positive, S-phase cells are expressed as a percentage of the total cell population (DAPI⁺) for BCBL-1 and JSC-1 cultures (dark and light bars, respectively). Data, expressed as averages, were derived from multiple random microscopic fields (>100 cells/field) for each. (C) Apoptosis in the same cultures (harvested 5 days post-transduction and on day 3 of counting) was identified by annexin V-Cy3 staining; data are expressed as a percentage of the total DAPI⁺ population. Average values from multiple random microscopic fields are shown. (D) Growth experiments equivalent to those shown in panel A were undertaken to determine the effects of ERK depletion in two additional PEL cell lines, BC-1 and BC-3, in addition to HHV-8-negative BJAB cells, previously shown to be refractory to ERK kinase (MEK) inhibition [272] and used here as a control for potential non-specific cytotoxic effects of ERK shRNA transduction. The PEL cell lines, but not BJAB cultures, were growth-inhibited by ERK shRNA transduction relative to NS shRNA-expressing controls. Effective ERK depletion in each of the three cell lines was verified by immunoblotting for
ERKs 1 and 2; β-actin antibody was used to check for equal protein loading and membrane transfer. Blots for each of the cell lines are shown below the respective growth curves.
Figure 3.4
Figure 3.5. ER-localized vIL-6 signal transduction via gp130 supports PEL cell growth. (A) Previously described vIL-6 variant W167G, which cannot form tetrameric (vIL-6;gp1302, ER-localized) complexes [159,166], was tested in transfected HEK293T cells to verify its predicted inability to mediate signal transduction when restricted to the ER compartment. KDEL-tagged, ER-directed forms of vIL-6 and vIL-6.W167G were expressed together with Fc-tagged gp130 from appropriate expression plasmids. After 24 hrs, cell lysates were analyzed by immunoblotting for pSTAT3, an indicator of gp130 signaling, and protein A-agarose-precipitated gp130-Fc was probed with phosphotyrosine-specific antibody for detection of activated gp130. Only wild-type vIL-6 (vIL-6.K) was able to signal via gp130 in the ER compartment, inducing gp130 and STAT3 phosphorylation, in contrast to vIL-6.W167G-KDEL (W167G.K) and empty vector (vec). (B) Testing of VKORC1v2 binding by vIL-6.W167G was undertaken by co-immunoprecipitation assay, which was used previously for the detection of the vIL-6/VKORC1v2 interaction [173]. CBD-tagged VKORC1v2 was precipitated with chitin beads from lysates of transfected cells, and precipitated material was analyzed by immunoblotting for the presence of vIL-6.W167G, wild-type vIL-6 (positive control), or KDEL motif-tagged hIL-6 (hIL-6.K, negative control), expressed in separately transfected cultures. Both vIL-6 proteins were precipitated and therefore competent for interacting with VKORC1v2; binding of VKORC1v2 by vIL-6.W167G verified that the “site III” mutation affected only gp130 binding. (C) Experiments utilizing shRNA-mediated vIL-6 depletion and complementation with KDEL-tagged, ER-restricted vIL-6 (vIL-6.K) and vIL-6.W167G (W167G.K) were undertaken to determine whether vIL-6 signaling through ER-localized gp130 was important for PEL cell growth. BCBL-1 and JSC-1 cells were first transduced with lentiviral vectors expressing either vIL-6.K or vIL-6.W167G.K (designed to be refractory to vIL-6 shRNA) and then transduced with lentiviral vectors expressing non-silencing (NS) or vIL-6-directed shRNAs. Triplicate cultures were monitored for growth as outlined in Chapter 2 and in the Methods above; error bars indicate standard deviations.
from mean values of cell density. Below the plots, ethidium bromide-stained agarose gels are shown containing RT-PCR-amplified vIL-6 mRNA sequences extracted from lysates of PEL cell samples derived from the growth assay cultures. The data provide evidence of appropriate and equivalent expression of each of the vIL-6 constructions. RT-PCR products amplified with PCR primer pairs for vIL-6.K (v) and β-actin (a), used as a normalization control, are indicated. Omission of reverse transcriptase (-RT) provided a control for RNA-derived vIL-6.K PCR products (i.e., lack of DNA contamination).
Figure 3.5
Figure 3.6. Inhibitory targeting of vIL-6/gp130 signaling diminishes PEL cell proliferation and survival. (A) Functional testing of KDEL motif-fused (ER-directed and retained) and Flag epitope-tagged soluble gp130 (sgp130.K) as an inhibitor of ER-localized vIL-6 signaling in transfected HEK293T cells. Cultures were co-transfected with expression vectors for sgp130.K (or empty vector control, vec) and either vIL-6, vIL-6-KDEL (vIL-6.K), or hIL-6. Cell lysates were harvested 48 hrs post-transfection for preparation of cell lysates. These were analyzed by western blotting for detection of pSTAT3, an indicator of gp130 signaling, and β-actin (protein loading control). Both vIL-6 (mainly ER-localized but also secreted) and KDEL-tagged vIL-6 (completely ER-retained [115]) signaling were inhibited by sgp130.K, whereas signaling by hIL-6 (efficiently secreted) was unaffected. The data verified the ER-restricted, vIL-6/gp130-inhibitory activity of sgp130.K. (B) Confirmation of intracellular localization of KDEL motif-fused and Flag-tagged sgp130 and comparison to (surface-expressed) full-length gp130 via Flag- and gp130-based immunofluorescence staining of transfected cells. Alexa Fluor 594-conjugated secondary antibody was used for detection of (red) fluorescence signal. Each protein was expressed together with KDEL-tagged GFP (GFP.K, used previously as an ER marker [115]) in vector-co-transfected HEK293T cells. As expected, Flag-specific staining for sgp130.Flag.KDEL [sgp130(Flag).K] was evident intracellularly and was coincident with ER-localized GFP signal, whereas gp130 staining was localized mainly to the cell surface. Stacked (“total”) and representative individual Z-sections (“slice”) are shown for two examples (1 and 2) taken from each of the transfected cultures. (C) BCBL-1 and JSC-1 cells were transduced with equivalent titers of “empty” (vec) or sgp130-KDEL (sgp130.K) expressing lentiviral vectors, and growth of cell density-normalized cultures was monitored after a two-day recovery period as described in Chapter 2 and in the Methods above. Error bars represent standard deviations from derived average values. (D) Relative proliferation rates of PEL cells at “day 2” (from growth assays) were determined by BrdU incorporation.
assays (see Methods and Chapter 2). (E) In a separate experiment, transduction of sgp130.K into BCBL-1 and JSC-1 cultures led to induction of apoptosis, as determined by annexin V-Cy3 staining. Cells were harvested and analyzed 3 days post-transduction with test (sgp130.K) or control (vec) lentiviral vectors.
Figure 3.6
CHAPTER 4: ROLE OF GP130 IN VIL-6-MEDIATED VIRUS REPLICATION
Summary

HHV-8 vIL-6 has been implicated in the pathogenesis of HHV-8-associated disease and plays a role in maintaining PEL cell growth and viability in addition to contributing to elevated levels of phosphorylated STAT3 via its interaction with gp130. Furthermore, STAT3 and ERK1/2 are required for optimal PEL cell growth and survival. Additionally, vIL-6 interactions with VKORC1v2 contribute to the pro-growth and survival activities of vIL-6. While the previous study (Chapter 3) investigated the role of vIL-6 in PEL cell latency, the effects of vIL-6/gp130 signaling on virus replication are unknown. Thus, the goal of this study was to determine the role of vIL-6 and gp130 in HHV-8 productive replication in both PEL and endothelial cells.

To assess the effects of vIL-6 and gp130 signaling on HHV-8 replication, vIL-6, gp130, and gp130-induced STAT3 were depleted in both PEL and endothelial cultures. Furthermore, depletion-complementation experiments were utilized to determine the relevance of the ER-localized vIL-6/gp130 interaction as it relates to virus replication. Finally, ER-localized gp130 signaling mutants were employed to evaluate the specific contributions of vIL-6/gp130-mediated STAT and ERK signaling to virus replication.

Depletion and depletion-complementation experiments revealed that ER-localized vIL-6 activity via gp130 and gp130-activated STAT signaling, but not ERK activation, were critical for vIL-6 pro-replication activity. Additionally, vIL-6 and gp130 depletion did not affect de novo infection or the establishment of latency. These data significantly extend current understanding of vIL-6 function and associated mechanisms in HHV-8 biology.
HUMAN HERPESVIRUS 8 VIRAL INTERLEUKIN-6 SIGNALING THROUGH GP130 PROMOTES VIRUS REPLICATION IN PRIMARY EFFUSION LYMPHOMA AND ENDOTHELIAL CELLS

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INTRODUCTION

The previous chapter described the role of vIL-6 in PEL cell growth and viability and determined that the ER-localized vIL-6/gp130 interaction is critical for these pro-growth and survival activities of the viral cytokine. Additionally, the elevated levels of phosphorylated STAT3 commonly observed in PEL could be attributed, in part, to vIL-6/gp130 signaling. This chapter aims to expand on these findings and to characterize the role of vIL-6 and its gp130-mediated downstream signaling components in the reactivation and replication of HHV-8 in both PEL and endothelial cells.

As has been stated previously, HHV-8 is associated with PEL in addition to MCD and KS [256-258,282]. HHV-8-encoded vIL-6 promotes cell growth and survival, inflammation, and angiogenesis and is implicated in pathogenesis and promotes disease progression [115,144,147,169,259]. However, its role in normal virus biology remains unclear. vIL-6 and hIL-6 share ~25% sequence identity, fold into similar three-dimensional structures, and initiate signaling through similar complexes [157-159,165,260,261]. While both hIL-6 and vIL-6 form hexameric signaling complexes comprised of two gp80 receptor subunits, two gp130 signal transducers, and two IL-6 molecules (gp802-gp1302-IL-62), vIL-6 is unique in that it can also signal from the ER via tetrameric complexes (gp1302-vIL-62) [159,165,170]. vIL-6 can be expressed during PEL cell latency in addition to being
maximally expressed during the lytic stage of the viral life cycle [115,116]. Furthermore, latently infected PEL cell viability is maintained in part via vIL-6/gp130 signaling from the ER and also through its interaction with VKORC1v2 [169,173]. The vIL-6/VKORC1v2 interaction has also been shown to contribute to productive replication [175].

Upon vIL-6-induced gp130 dimerization, phosphorylation of specific gp130 tyrosine residues initiates either STAT1/3 (Y767, Y804, Y905, and Y915) or MAPK (Y759) signaling [156,161-163]. Activated STAT3 and ERK1/2 have been shown to promote growth and survival of PEL cells, and vIL-6/gp130 signaling contributes [40,152,169]. However, the role of vIL-6 in HHV-8 lytic replication is unclear. One study utilized a vIL-6-knockout recombinant virus to assess the role of the viral cytokine in virus replication in HHV-8 negative BJAB cells, but no effect was observed [283]. A second study found that vIL-6 depletion in BCBL-1 and JSC-1 PEL cells resulted in less HHV-8 virus production following chemically-induced reactivation in culture. No other studies have been conducted to assess whether vIL-6 plays a role in HHV-8 productive replication; thus, we sought to determine the effects of vIL-6/gp130 signaling on HHV-8 lytic replication in both PEL and endothelial cells.

METHODS

Cell culture, transfections, and lentivirus production

HEK293T, Vero-r219, TRexBCBL1 RTA, JSC-1, and TIME-TRE/RTA cells were maintained as described in Chapter 2. For all transfections, HEK293T cells were transfected using standard calcium-phosphate-DNA co-precipitation methods as detailed in Chapter 2. Cells were harvested 24 to 48 hrs post-transfection, and lysates were prepared as outlined previously (Chapter 2). Lysates were analyzed immediately via western blot analysis or stored at -20°C for later use.
To produce lentivirus, HEK293T cells were transfected with pYNC352-encoded shRNAs (specific for vIL-6, gp130, NS, or STAT3) or pDUET001-encoded plasmids (empty vector, wild-type [WT] gp130-Flag, gp130.STAT-Ys, gp130.ERK-Y, and gp130.ΔYs) or pYNC419-encoded plasmids (mvIL-6.KDEL, mvIL-6-W_{167}G-KDEL, or luciferase) with Pax2 gag/pol and VSV/G expression vectors as described previously and in Chapter 2 [173]. To harvest lentivirus, medium was collected 2 days post-transfection, filtered, and centrifuged as detailed in Chapter 2. Viral pellets were resuspended in RPMI 1640 medium and stored at -80°C until needed. Prior to experimental use, lentiviruses were titered on HEK293T cells to ensure equivalent titers as described previously and in Chapter 2 [169].

HHV-8 r219 virus was produced as described in Chapter 2 for the infection of naïve TIME-TRE/RTA cells. Harvested r219 virus was titered on naïve TIME-TRE/RTA cells via virus-encoded GFP expression prior to infection of TIME-TRE/RTA cells (Chapter 2). After allowing cultures to recover for two days, r219-infected TIME-TRE/RTA cells were transduced with lentiviral vectors to assess HHV-8 reactivation (Chapter 2).

Plasmids and Primers

The pYNC352 shRNA-encoded lentiviral vectors (NS, vIL-6, gp130, and STAT3) have been described [115,169]. The mvIL-6.KDEL, mvIL-6-W_{167}G.KDEL, and luciferase lentiviral pYNC419 vectors have been reported [115,169]. pSG5-derived vIL-6 and hIL-6 and the pDUET001 vector have also been reported [166,168,270]. To generate the pDUET001-encoded gp130 WT and signaling mutants that are resistant to depletion by gp130-specific shRNA, wild-type gp130, gp130.STAT-Ys (Y759F-mutated), gp130.ERK-Y (Y759 only, all other Ys mutated to F), and gp130.ΔYs (pan Y-to-F-mutated) (described in [168]) were PCR-amplified from the pEF-BOS vector using gp130-BamHI-FWD (5' TTGGATCCATGTTGACGTTGCAGACTTGG 3’) and gp130-1205res-REV (5’
AGATGGTCTGTCCCTCGTACGTAATTCCGCTTGCTTCTTCTTCACTCCAGT 3’) primers to amplify the 5’ end of the gene. To add a C-terminal FLAG epitope and to amplify the 3’ end of WT gp130 and gp130.STAT-Ys, gp130-1205res-FWD (5’ GAAGAAGCAAGCGGAATTACGTACGAGGACAGACACCATTCTAAAAGCACC 3’) and gp130-WT-Flag-REV (5’ TTCTCGAGTCACTTGTCATCGTCGTCCTTTGTAATCTCTGAGG CATGTAAGCGCCTTG 3’) primers were used for PCR amplification. Similarly, gp130-1205res-FWD and gp130-deltaY-Flag-REV (5’ TTCTCGAGTCACTTGTCATCGTCGTCCTTTGTAATCTCTGAGG CATGTAAGCGCCTTG 3’) primers were used to amplify the 3’ end of gp130.ERK-Y and gp130.ΔYs and to add a FLAG epitope to the C-terminus. The 5’ and 3’ amplicons for each gp130 gene (WT or signaling mutant) were then used as templates to amplify across the entire length of gp130-Flag; the resulting PCR products were resistant to the gp130-specific shRNA and contained C-terminal Flag epitope tags. Finally, the gp130-shRNA-resistant and Flag epitope-tagged amplicons were ligated into pDUET001 between the BamHI and XhoI restriction sites, replacing the GFP ORF. All of the constructions were verified by sequencing.

RT-PCR primers were designed to amplify gp130-Flag sequences in transduced TRExBCBL1-RTA cells in order to verify the expression of wild-type and gp130 signaling mutant plasmids. The following primers were used: gp130-Flag-FWD (5’ TAGATGGCGGTGATGGTATTT 3’) and gp130-Flag-REV (5’ CTTGTCATCGTCGTCCTTGTA 3’). Previously validated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to amplify GAPDH as a positive control: GAPDH-FWD (5’ TTGCCATCAATGACCCCTTCA 3’) and GAPDH-REV (5’ CGCCCCACTTGATTTTGGAGA 3’); these primers were designed at the RRC Core Genomics Facility at the University of Illinois at Chicago.
**Antibodies and Western blotting**

The following commercially available antibodies were used: β-actin (Sigma, St. Louis, MO; catalog number A2228), pSTAT3 (Cell Signaling Technology, Beverly, MA; catalog number 9131), STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog number sc-482), pERK (Santa Cruz Biotechnology; catalog number sc-7383), LANA (Advanced Biotechnologies, Columbia, MD; catalog number 13-210-100), and gp130 (Santa Cruz Biotechnology; catalog number sc-655). Rabbit anti-serum to vIL-6 was generated previously in our laboratory [166]. Cy3-goat anti-rat IgG fluorescent antibody (Life Technologies, Carlsbad, CA; catalog number A10522) was used to detect LANA by immunofluorescence assay.

Western blotting was conducted by separating heat-denatured and reduced lysate samples via SDS-PAGE and transferring samples to nitrocellulose membranes using standard protocols described in Chapter 2. Following antibody incubation, immuno-targeted proteins were visualized via standard chemiluminescence assay.

**Quantification of HHV-8 titers following reactivation**

For HHV-8 reactivation experiments in TRExBCBL1-RTA and JSC-1 PEL cells, cells were transduced with pYNC352-encoded shRNAs as described previously [169] and in Chapter 2, allowed to recover for 2 days, normalized for cell number, and then induced with Dox (TREx-BCBL1/RTA cells; 1 μg/μl) or TPA (20 nM) and NaB (0.5 μM) for JSC-1 cells. NaB was removed from JSC-1 cultures after 12 hrs of incubation. Transduced PEL cells were maintained in Dox or TPA for 4 days, and HHV-8 virus was harvested from culture media as detailed in Chapter 2. Cells were counted at the time of virus harvest to ensure no drastic differences in cytotoxicity between cultures. Aliquots of harvested virus were added to naïve TIME-TRE/RTA cells plated at ~90% confluency in 12-well plates,
centrifuged, and incubated for an additional hr as outlined in Chapter 2. One day post-infection, cells were trypsinized and added to chamber slides for LANA immunostaining.

For reactivation of HHV-8 in TIME-TRE/RTA endothelial cells, cells were seeded at ~90% confluency in 12-well plates and then infected with HHV-8 r219 virus (described above and in Chapter 2). After recovering for 48 hrs, TIME-TRE/RTA GFP fluorescence was assessed to verify a high viral titer (~90% of cells infected). Cells were then infected with pYNC352-encoded shRNA-expressing lentiviruses as described above and in Chapter 2. Two days post-shRNA lentiviral infection, cells were induced with Dox (1 μg/μl). Medium was removed from infected and induced cells every 48 hrs for 6 days, and fresh medium containing Dox was added to cell cultures. Harvested media containing virus were stored at -80°C. Reactivated TIME-TRE/RTA cells were also visualized daily for RFP fluorescence to assess rates of reactivation. Medium collected from each sample was pooled and centrifuged at 16,000 rpm for 2 hr at 4°C; viral pellets were resuspended in EMB-2 without serum or supplements. Naïve TIME-TRE/RTA cells were incubated with an aliquot of collected virus and then transferred to chamber slides for immunostaining as described above.

For depletion-complementation experiments in TRExBCBL1-RTA cells, cells were first transduced with pYNC419-expressing mviL-6.KDEL, mviL-6.W167G.KDEL, or luciferase to assess vIL-6 effects on virus replication or were transduced with pDUET001-expressing empty vector, WT gp130-Flag, gp130.STAT-Ys, gp130.ERK-Y, or gp130.ΔYs to determine the contribution of STAT and ERK signaling to virus replication. Cells were allowed to recover for 2 days prior to being transduced with pYNC352-encoded shRNAs as described in Chapter 2. Dually-transduced cells were induced with Dox; medium was collected, and virus was harvested as outlined in Chapter 2. Naïve TIME-TRE/RTA cells were infected with aliquots of harvested virus as described above, and cells were transferred to chamber slides prior to immunostaining.
Chamber slides containing infected TIME-TRE/RTA cells were stained for LANA expression to quantify HHV-8 infection, as outlined in Chapter 2. Chamber slides were washed, fixed in ice cold methanol, and incubated with anti-LANA antibody (1:100 dilution in PBS blocking buffer [Thermo Fisher Scientific, Waltham, MA]) as outlined in Chapter 2. After thoroughly washing, slides were incubated with Cy3-goat anti-rat secondary antibody (1:200 dilution in PBS blocking buffer), washed, and then stained with DAPI to detect nuclei. The percentage of LANA* cells in the total DAPI* cell population was determined from 6 random fields (>50 cells/field) in each duplicate culture.

*De Novo* Infection

TIME-TRE/RTA cells were transduced with lentivirus-encoding shRNAs specific for NS, gp130, or vIL-6. Two days post-transduction, cells were infected with a subsaturating dose of HHV-8 r219 virus. One day post-HHV-8 r219 infection (and 3 days post-lentiviral transduction), infected and transduced TIME-TRE/RTA cells were washed, fixed, and stained for LANA and DAPI expression as described in Chapter 2. Multiple (>6) fields were visualized for each duplicate culture, and the percentage of LANA* cells in the total DAPI* cell population was calculated as outlined above.

*Annexin Staining*

TIME-TRE/RTA cells were plated on chamber slides at a confluency of ~80%. The following day, TIME-TRE/RTA cells were transduced with NS-specific or STAT3-specific lentivirus-encoded shRNAs. One day post-transduction, cells were fixed and stained for annexin V-Cy3 as described in Chapter 2. Multiple fields were visualized, and mean values were reported.
RESULTS

\textbf{gp130 and vIL-6 promote HHV-8 replication in PEL and endothelial cells.} The gp130 signal transducer was depleted in TRExBCBL1-RTA and JSC-1 PEL cells [100,111] and in TIME-TRE/RTA endothelial cells [203] latently infected with HHV-8 r219 [254] using previously established techniques [115,169]. Following depletion, lytic replication was induced with Dox or TPA and NaB. HHV-8 was collected from PEL and endothelial culture media, and infectious viral titers were determined as undertaken previously [175]. Viable (trypan blue-excluding) TRExBCBL1-RTA cell densities at the time of media harvest were not negatively affected by gp130 transduction (1.31x10^6 and 1.41x10^6 cells per ml for NS shRNA-transduced duplicate cultures versus 1.75x10^8 and 1.83x10^6 cells per ml for gp130 shRNA-transduced cells). Depletion of gp130 led to decreased HHV-8 titers in PEL (~3-fold) and endothelial cultures (>8-fold) (Figure 4.1A, B, C). Similarly, vIL-6 depletion in endothelial cells reduced viral titers by ~3-fold (Figure 4.1D). TIME-TRE/RTA cells were visibly unaffected by gp130 or vIL-6 depletion, and rates of apoptosis (% annexin V-Cy3^+ cells) were equivalent in gp130- and vIL-6-depleted cultures relative to NS shRNA-transduced controls (data not shown). Rates of reactivation, measured by ORF59-antigen immunofluorescence (PEL cells, data not shown) and RFP imaging (Figure 4.1C,D, bottom panels), 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, reported previously [175]. In contrast to reactivated productive replication, neither gp130 nor vIL-6 depletion had any substantial effect on latency titers (measured 3 days post-infection) following \textit{de novo} infection of endothelial cells (Figure 4.1E), a process known to involve a burst of expression of a subset of lytic genes, including vIL-6, prior to rapid latency establishment [284,285].
**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 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 [115]) but not vIL-6.W167G.KDEL (ER-retained and gp130-dimerization incompetent in the ER [166,169]) (Figure 4.2). 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-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 [156,161-163]. Lentiviral vector-cloned gp130 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.ΔYs (inactive). Functional testing in transfected HEK293T 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 pERK levels (Figure 4.3A). 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-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 (Figure 4.3B), 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 [152,169], we examined its role in productive replication. STAT3 depletion led to reduced HHV-8 titers induced from both PEL and endothelial cultures, although effects in TPA/NaB-induced JSC-1 cultures were modest despite robust STAT3 depletion (Figure 4.4). 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 between NS and STAT3-directed shRNA-transduced cultures (Figure 4.4 panels A and B, bottom), indicating that pro-proliferative and anti-apoptotic activities of STAT3 operating in latently infected PEL cells [152,169] 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 (Figure 4.4D). Our data show that STAT3 supports HHV-8 replication, and together with the vIL-6 and gp130 depletion-rescue data (Figures 4.2 and 4.3) indicate that vIL-6/gp130 signaling via STAT3 contributes significantly to the pro-replication activities of vIL-6 and gp130.

**DISCUSSION**

The likely role of vIL-6 in pathogenesis has been described in numerous reports [115,144,147,169,258,259]. 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 [283], and our own preliminary vIL-6 depletion experiments in PEL cells [175]. Previously, vIL-6 was shown to promote HHV-8 replication in BCBL-1 and JSC-1 PEL cells, but its role in endothelial cells and additional PEL cells was not evaluated. 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, but not gp130-activated MAPK signaling, is likely critical for vIL-6/gp130-enhanced replication.

While it possible that gp130-activated STAT1 signaling could also contribute, lack of detectable effects of STAT1 depletion on PEL cell growth [169] coupled with the well-established role of STAT1 in promotion of anti-viral interferon and apoptotic signaling [286] indicate that this is unlikely. It is noteworthy that previous studies have reported the importance of STAT3 signaling for replication of viruses, including VZV and hepatitis C virus [287,288]. For VZV, STAT3-activated survivin was found to be involved in pro-replication activity, reflecting the pro-survival activities of STAT3 via survivin reported in PEL cells [152]. However, HCMV blocks STAT3 phosphorylation, though requires it for optimal replication, and mouse cytomegalovirus induces phosphorylation of STAT3 but not STAT3-responsive cellular genes, suggesting novel, virus-redirected activities of the transcription factor [289,290]. 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, in which productive replication contributes significantly.
FIGURES

Figure 4.1. Involvement of gp130 and vIL-6 in promotion of HHV-8 replication. (A) HHV-8 titers following gp130 depletion in TRExBCBL1-RTA cells. The gp130 signal transducer was depleted in TRExBCBL1-RTA cells using lentiviral-encoded shRNAs (NS, control or gp130-specific) described previously [169]. Cell numbers were normalized, and cultures were reactivated with Dox. Aliquots of harvested virus were applied to naïve TIME-TRE/RTA cells to determine infectious virus titers by staining for LANA, as described previously [175]. Multiple random fields were assessed to quantify the number of LANA-positive cells in the total DAPI-positive population. (B) Importance of gp130 in HHV-8 replication in JSC-1 cells. JSC-1 cells were depleted of gp130 (as described in 4.1A) and induced with NaB and TPA. 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 [254], then transduced with shRNA-encoding lentiviral vectors (NS, control; gp130-specific; or vIL-6-specific), and finally induced with Dox. 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 Dox treatment (examples shown). Media containing virus were collected for six days and pooled prior to virus concentration and titration. (E) NS, gp130, and vIL-6 shRNA-transduced TIME-TRE/RTA cells were infected with HHV-8 r219 two 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 three days post-HHV-8 r219 infection. For all panels, data were derived by counting of LANA+ cells in multiple fields 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.
Figure 4.1.
**Figure 4.2. Importance of ER-localized vIL-6/gp130 interactions 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.W_{167}G (W_{167}G.K) [115,169] or with lentivirus encoding luciferase (negative control). Two days post-transduction, cells were transduced with control (NS) shRNA-encoding lentivirus or lentivirus expressing vIL-6-specific shRNA. Dually-transduced cells were then normalized for cell density and induced the following day with Dox. Virus was collected and titered as before (see Figure 4.1 legend). RNA was harvested from a subset of cells using TRIzol, and cDNA was amplified for vIL-6.KDEL [169] and GAPDH (normalization and positive control) mRNA to verify expression of the introduced vIL-6 genes (v, vIL-6 RT-PCR; g, GAPDH RT-PCR; -RT, no reverse transcriptase). Viable cell densities at the time of Dox treatment (day 0, normalized) and at virus harvest (day 4) are shown (bottom panel). v, vIL-6-KDEL; v_{167}.K, vIL-6.W_{167}G-KDEL; luc, luciferase.
Figure 4.2
Figure 4.3. Contributions of gp130-mediated STAT and ERK signaling to HHV-8 productive replication. (A) Functional assessment of gp130 signaling-tyrosine variants. HEK293T cells were co-transfected 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 post-transduction, cells were lysed for western blot analysis to detect 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 [270]) or vectors expressing Flag-tagged wild-type gp130, gp130.STAT-Ys, gp130.ERK-Y, or gp130.ΔYs. Two days post-transduction, cells were transduced with either NS (control) or a gp130-specific shRNA-encoding lentiviral vector. Following Dox-induced HHV-8 reactivation for four days, virus was collected and titered as described previously. To verify appropriate expression of wild-type gp130 and the gp130 signaling mutants, a subset of cells was lysed in TRIZol for preparation of RNA samples. RNA was reverse transcribed and amplified using 3’ Flag- and 5’ gp130-directed primers 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, GADPH RT-PCR; -RT, no reverse transcriptase.
Figure 4.3
Figure 4.4. Role of STAT3 in HHV-8 replication in PEL and endothelial cells. (A) TRExBCBL1-RTA cells were transduced with control (NS) and STAT3-specific shRNA-encoding lentiviral vectors. After two days, lytic reactivation was induced with Dox for four 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 Figure 4.1. 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 four days for virus titration. A subset of cells was lysed for western blotting at the time of induction to verify STAT3 depletion. Viable cell densities at the start and end of the experiment are shown (bottom). (C) Dox-induced TIME-TRE/RTA cultures were analyzed in the same way except that virus was harvested following six days of Dox treatment; parallel cultures were harvested at the time of induction for western blot analysis. (D) Rates of apoptosis (% annexin V-Cy3+/DAPI+ cells) in similarly transduced TIME-TRE/RTA cultures were unaffected by STAT3 depletion.
Figure 4.4
DISCUSSION OF MAJOR FINDINGS

The goal of this project was to further characterize the function of vIL-6 as it relates to normal HHV-8 biology and pathogenesis. Many previous studies have sought to determine the role of vIL-6 in viral pathogenesis and tumorigenesis. While pathogenesis studies are important, virus-induced tumorigenesis is an unintentional byproduct of HHV-8 infection and occurs in rare cases. In most immunocompetent individuals, host immune responses are able to control the virus, and HHV-8-associated malignancies are not observed. When the immune system is incapable of controlling virus replication and spread, the resulting increase in infection events provides many more opportunities for the virus to cause malignant transformation. Thus, determining how vIL-6 facilitates viral infection under normal physiological conditions is crucial for understanding how the virus maintains its fitness and expands within the host. The development of therapeutics to combat viral infection and HHV-8-mediated neoplasia will require a detailed understanding of virus-host interactions under normal, non-transformative conditions.

Studies presented here indicate that vIL-6 promotes latently infected PEL cell growth and survival, and they identify an important and therapeutically-targetable mechanism of PEL cell maintenance. Additionally, these studies demonstrate a role of vIL-6 signaling in normal virus latency in untransformed B-cells and possibly other cell types. Thus, disruption of vIL-6 signaling through specific abrogation of its interaction with gp130 could be a promising means not only to target PEL therapeutically but also to attack latently infected cells more generally and limit viral load. Furthermore, this project has identified intracellular signaling by vIL-6 specifically within the ER as leading to high levels of activated (phosphorylated) STATs 1 and 3 and ERKs 1 and 2. Moreover, STAT3 and ERK1/2 expression are important for PEL cell growth and survival, and these pathways could be targeted pharmacologically. These findings are in agreement with a previous study that determined that STAT3 promotes PEL cell growth and survival via the
upregulation of survivin though data described in this dissertation project were generated using an alternative method to assess the contribution of STAT3 to PEL cell proliferation and viability. This current work (described in Chapter 3) utilized shRNA-encoding lentiviral vectors to effectively and specifically deplete STAT3 in multiple PEL cell lines in order to confirm previous findings and to establish that vIL-6/gp130 signaling from the ER compartment contributes significantly to the increased levels of phosphorylated STAT3. Furthermore, a similar approach was undertaken to assess the role of ERK1/2 signaling as it relates to PEL cell growth and survival. For the first time, ERK1/2 was found to play a major role in the proliferation and viability of PEL cells in culture. This finding, along with data demonstrating that depletion of ERK1/2 in HHV-8 negative BJAB cells had no effect on cell growth, suggests that ERK1/2 activity may be specifically required by HHV-8-infected PEL cells. The effects of STAT3 and ERK1/2 on PEL cell growth were assessed by measuring rates of both cell proliferation and apoptosis; depletion of both signaling molecules led to decreased rates of cell growth and increased rates of apoptosis. Moreover, vIL-6-mediated signaling through gp130 was found to be responsible, at least in part, for high levels of both phosphorylated STAT3 (in all PEL cell lines tested) and ERK1/2 (in some PEL cell lines). These data also suggest that the constitutively high levels of phosphorylated STAT3 observed in PEL cells can be attributed largely to vIL-6/gp130 signaling. Finally, additional experiments were performed to evaluate the importance of ER-localized vIL-6 signaling; data from these depletion-complementation experiments indicate that vIL-6/gp130 ER-localized signaling is responsible for promoting PEL cell growth and survival in culture, as a vIL-6 mutant (W167G, incapable of dimerizing gp130 in the ER) was unable to rescue PEL cell growth following vIL-6 depletion. Thus, the study described in Chapter 3 adds to the body of work regarding vIL-6 function and intracellular signaling in the context of PEL cell proliferation and viability and has implications not only for PEL therapy but also for normal HHV-8 latency.
In addition to functioning as a pro-growth and pro-survival viral protein in PEL cells, we hypothesized that vIL-6 might also play a positive role in productive virus replication due to its role in prolonging cell survival. Thus, vIL-6 function in HHV-8 lytic replication was assessed in both PEL and endothelial cells. Using shRNA-encoding lentiviral vectors, vIL-6 and gp130 were depleted in both cell types. Data from these experiments indicate that vIL-6 and gp130 are required for efficient HHV-8 replication. Prior to these experiments, little was known regarding the function of vIL-6 in HHV-8 productive replication. One study utilized a vIL-6 knock-out recombinant virus to assess replication in HHV-8 negative BJAB cells, and in this setting, vIL-6 appeared to have no effect on virus replication [283]. In a second study, the function of vIL-6 in HHV-8 replication was evaluated in two PEL cell lines (BCBL-1 and JSC-1), and decreased viral titers were observed following vIL-6 depletion via vIL-6-specific lentivirus-encoded shRNA [175]. These studies also identified vIL-6-targeted VKORC1v2 and associated suppression of pro-apoptotic CatD were involved in promoting HHV-8 replication. The findings described in Chapter 4 are novel in that they demonstrate that vIL-6 promotes virus replication in both endothelial cells and in an additional PEL cell line (TREx-BCBL1/RTA). Additionally, the contributions of gp130 to HHV-8 productive replication were evaluated using similar methods, and gp130 depletion led to decreased viral titers in both cell types. Furthermore, vIL-6 depletion-complementation experiments were conducted using both wild-type and mutant vIL-6 (W167G) to determine the contribution of vIL-6/gp130 intracellular signaling on productive replication in PEL cells. This experiment demonstrated that vIL-6/gp130 ER-localized signaling is required for effective virus replication, as the ER-localized vIL-6 mutant (which was unaffected with respect to VKORC1v2 interaction) was incapable of rescuing HHV-8 replication following vIL-6 depletion. As expected, ER-directed wild-type vIL-6 was capable of rescuing replicative titers following lentivirus-mediated shRNA depletion of vIL-6. STAT and MAPK signaling are activated by vIL-6/gp130 signaling, and
these downstream signaling molecules were evaluated to determine their contributions to HHV-8 productive replication. A set of gp130 signaling mutants were over-expressed following gp130 depletion in a depletion-complementation assay, and data from this experiment indicate that vIL-6/gp130-mediated STAT3 signaling is required for optimal HHV-8 replication and that MAPK signaling is not required for HHV-8 replication. Thus, vIL-6, gp130, and STAT3 have been identified for the first time as positive regulators of HHV-8 replication.

The studies described here and in other reports suggest that STAT signaling is critically important for virus replication and host cell proliferation and survival. STATs are known to be upregulated following viral infection and the activation of type 1 interferons. While STATs 1 and 2 facilitate and promote the host IFN response, STAT3 negatively regulates type 1 interferons [291]. Depletion and/or knockout of STAT3 in cell lines leads to an enhanced type 1 IFN response, and the addition of STAT3 following depletion dampens the type 1 IFN response [291]. Additionally, STAT3 knockout mice are embryonic lethal; conversely, STAT1 knockout mice are viable but are unresponsive to IFNα and IFNγ, and STAT2 knockout mice also display greater susceptibility to viral infections [292-294]. Therefore, the activation of STAT3 following virus infection is beneficial to the virus because STAT3 attenuates the host antiviral response by suppressing the pro-type 1 IFN response promoted by STATs 1 and 2.

Many viruses, including HHV-8, modulate STAT3 activity to promote virus replication and to evade host immune responses. For example, VZV induces STAT3 phosphorylation and upregulates survivin both in cell culture and in virus-infected SCID mice; inhibition of STAT3 and/or survivin expression limits VZV replication and spread in cell-based models [287]. VZV also inhibits STAT1, which ultimately attenuates the host type 1 IFN response [287]. HCMV inhibits STAT3 phosphorylation and sequesters the transcription factor (in its unphosphorylated form) in the nucleus; consequently, IL-6-
mediated gene expression is inhibited [289]. However, STAT3 activity is necessary for maximal HCMV replication, and inhibition of STAT3 by various chemical inhibitors resulted in drastically reduced viral titers [289]. Murine CMV (MCMV) inhibits the transcription of STAT3-responsive genes (including suppressor of cytokine signaling 3 [SOC3], a negative regulator of STAT3) while simultaneously upregulating STAT3 phosphorylation [290]. HSV1 latency is maintained by STAT3 expression, and the inactivation of STAT3 via inhibitors or a dominant negative STAT3 induces HSV1 productive replication [295]. In addition to herpesviruses, the rabies virus P protein interacts with STAT3, inhibiting the accumulation of the transcription factor in the nucleus [296]. Furthermore, targeting of STAT3 by the rabies virus abrogates gp130-mediated signaling [296]. The mumps virus, a member of the paramyxovirus family, also interacts with STAT3 via its V protein; this interaction leads to the proteasomal degradation of the transcription factor and the inhibition of IL-6-induced signaling [297]. HIV-1-encoded Nef activates STAT3 by inducing the expression of soluble factors, including Mip-1α, in monocyte-derived macrophages [298]. Hepatitis C virus (HCV) also modulates STAT3 activity via interactions with the HCV core protein [288]. Addition of constitutively active STAT3 leads to increased HCV replication, and the inhibition of STAT3 via siRNA or chemicals (AG490, STA-21, and S31-201) results in lower HCV RNA levels [288]. Thus, the targeting and modulation of STAT3 activity is conserved by many viruses across multiple families, suggesting that STAT3 function is crucial to virus biology. Whether STAT3 acts to enhance or inhibit virus replication and whether a particular virus induces or suppresses STAT3 activation presumably is dependent on the particular biological requirements of the viruses, their specific abilities to modify STAT3 activity, and the cell types infected.

STAT3 not only plays a role in regulating virus infection, but it is also involved in oncogenesis. STAT3 interacts with RelA, a nuclear factor κB (NFκB) family member, sequestering it within the nucleus; ultimately, this interaction leads to constitutive NFκB
signaling in tumor cells [299]. Furthermore, STAT3 and NFκB are linked via IL-6. NFκB regulates IL-6 expression at the transcriptional level, and IL-6 signaling promotes STAT3 phosphorylation and activation [300]. Thus, NFκB signaling leads to elevated levels of IL-6, and in turn, high levels of phosphorylated STAT3; this positive feedback loop between NFκB and STAT3 results in increased inflammation via the upregulation of pro-inflammatory cytokines [301]. Inflammation is known to contribute to tumorigenesis, and several cancers have been linked to inflammation caused by virus infection. Virus-induced inflammatory cancers include HHV-8-associated KS, liver cancer (associated with hepatitis B and/or C virus), cervical cancer (linked to human papillomavirus [HPV]), and T cell leukemias and lymphomas (associated with human T lymphotrophic virus [HTLV-1]) [255,302-304]. Viral and cellular cytokines, VEGF, host immune cell extravasation, and repetitive DNA damage promote inflammation. Therefore, STAT3 activation can lead to inflammation and a microenvironment that is hospitable for tumor development and growth.

In immunocompetent individuals, HHV-8 infection does not generally result in oncogenesis. However, virus infection promotes key pathways, such as angiogenesis and inflammation, which are often involved in tumor development. HHV-8-encoded vIL-6 increases the expression of VEGF in cell culture and mouse models [147]. VEGF promotes angiogenesis and causes increased permeability of existing vasculature, which enhances the infiltration of immune cells into the lesion [305]. HHV-8-encoded vIL-6 also inhibits neutrophil recruitment during acute inflammation in a murine peritoneal inflammation model [148]. Data from this mouse model indicate that vIL-6 promotes STAT3 phosphorylation, CCL-2 production, and inhibition of IL-1β-mediated release of CXCL8 (IL-8) [148]. CCL-2 recruits monocytes and basophils to the lesion and directs the immune response away from the anti-viral Th1 response and toward the antibody-mediated Th2 response [148,306]. HHV-8-encoded viral chemokines (vCCL-1, vCCL-2,
and vCCL-3) also promote Th2 polarization, which weakens the host anti-viral immune response and promotes maintenance of the virus (Table 1.1). IL-8 is a chemotactic factor for neutrophils and promotes angiogenesis. HHV-8-encoded vFLIP induces IL-8 production that counteracts CCL-2-mediated suppression of IL-8 release, and vFLIP-regulated induction of IL-8 is dependent on functional NFκB [226,307]. Thus, STAT3 is a pleiotropic factor that promotes the growth and viability of virus-infected cells, enhances inflammation and IL-8 expression via interactions with NFκB, and drives Th2 polarization. HHV-8 vIL-6 induces STAT3 phosphorylation; therefore, the viral cytokine mediates pro-inflammatory, pro-angiogenic, and pro-survival signaling in virally infected cells.

**IMPLICATIONS FOR CLINICAL RESEARCH**

Treatment of viral infections and virus-associated neoplasias has been difficult due to the fact that some viruses, such as herpesviruses and HIV, have both latent and lytic phases. Traditional antiviral drugs were designed to target virus-encoded proteins such as TK, DNA polymerase, reverse transcriptase, and protease. Unfortunately, these drugs require that the virus be actively replicating. Because the virus is not productively replicating during latency, many proteins are not expressed and cannot be effectively targeted during this phase of the viral life cycle. A minority of viral proteins are expressed during latency. These proteins interact with host proteins to promote latency and genome maintenance. Therapeutic targeting of intracellular interactions between viral and host proteins during viral latency may provide a means to target the latent virus population.

Data presented in this body of work suggest means of novel therapeutic interventions for the treatment of HHV-8-associated disease. Specifically, the establishment that interactions between vIL-6 and gp130 are critical for viral latency and viral replication supports the targeting of this protein-protein interaction in future drug
design efforts. By preventing this interaction with small molecules or peptides, critical viral processes might be inhibited. Many experimental approaches were undertaken to evaluate the vIL-6/gp130 interaction. Specific findings are stated below.

1) Expression of both gp130 and vIL-6 in HHV-8-positive PEL cells is required for cell growth and survival in culture.

2) Gp130 contributes to elevated levels of active (phosphorylated) STAT1 and STAT3 in PEL cells.

3) Gp130 contributes to levels of active (phosphorylated) ERK1/2 in a subset of PEL cell lines.

4) STAT3 and ERK1/2 signaling are required for PEL cell proliferation and survival, but STAT1 is not.

5) ER-localized vIL-6/gp130-mediated signaling promotes PEL cell growth and survival.

6) Optimal HHV-8 replication requires expression of vIL-6, gp130, and STAT3 in both PEL and endothelial cells; these proteins do not play a substantial role in de novo infection (of endothelial cells) or the establishment of latency.

7) The ER-localized vIL-6/gp130 interaction promotes HHV-8 productive replication.

8) Gp130-mediated STAT3 signaling promotes virus replication.

Taken together, these data suggest that the ER-localized vIL-6/gp130 interaction is critical for multiple viral processes, including growth of latently-infected cells, cell viability, and productive viral replication. Therapeutic interventions targeting the vIL-6/gp130 interaction or downstream signaling could theoretically inhibit the growth of latently infected cells while also limiting the production of virus in lytically infected cells.
FUTURE DIRECTIONS

While these studies have shown that vIL-6-mediated signaling through gp130 helps maintain viral latency and is required for optimal lytic replication, many questions remain. Data indicate that STAT3 and ERK1/2 participate in vIL-6 signaling downstream of gp130, but the effects of STAT3 and ERK1/2 signaling on cellular gene expression in virally-infected cells are not known. Future studies should investigate virus-induced transcriptional changes downstream of STAT3 and ERK1/2 signaling. Results from these studies may provide additional insight into the ways in which the virus harnesses host signaling cascades to enhance its own fitness.

Due to the importance of vIL-6/gp130 interactions within the ER, screens should be conducted to identify small molecule or peptide inhibitors of this interaction within the ER compartment. Should an in vitro screen identify robust inhibitors of this interaction, potential inhibitors should be utilized in functional assays to assess their effects on the growth, survival, and replication potential of virally infected cells. Validation of potential inhibitors in cell-based assays is the first step in the lengthy process of developing FDA-approved therapeutics for the treatment of human disease.
FIGURES

Figure 5.1. ER-localized vIL-6 and gp130 signaling in PEL cell growth and survival. vIL-6 interacts with both gp130 and VKORC1v2 within the ER. A previous study indicated that the vIL-6/VKORC1v2 interaction was important for the growth and viability of PEL cells in culture [173]. The study outlined in Chapter 3 demonstrated that vIL-6 signaling through gp130 in the ER was critical for the proliferation and survival of PEL cells. Depletion of gp130 led to reduced cell growth and increased apoptosis in all four PEL cell lines tested (BCBL-1, JSC-1, BC-1, and BC-3). Furthermore, gp130 was found to contribute to levels of phosphorylated STATs 1 and 3 (in all PEL cell lines tested) and phosphorylated ERKs 1 and 2 in a subset of PEL cells (BCBL-1 and BC-1). STAT3 had been previously shown to promote PEL cell growth/viability in culture via the upregulation of survivin [152], but the role of ERK1/2 in the growth and viability of PEL cells had not been established previously. Data described in Chapter 3 indicate that ERK1/2 expression is critical for PEL cell proliferation and survival in all four PEL cell lines tested. The asterisks (*) denote that STAT3 was required for PEL cell growth and survival in the two cell lines tested (BCBL-1 and JSC-1). To establish that ER-localized vIL-6/gp130 signaling is responsible for vIL-6-mediated pro-growth and pro-survival functions, two approaches were utilized. First, vIL-6 depletion-complementation experiments were conducted, and ER-directed and mutated vIL-6 (W167G mutation, incapable of dimerizing gp130 in the ER though capable of binding to VKORC1v2) was unable to rescue the growth of vIL-6-depleted PEL cells; ER-directed and wild-type vIL-6 was able to rescue the vIL-6-depletion phenotype. Secondly, introduction of an ER-directed soluble gp130 molecule (sgp130.K) into PEL cells inhibited the vIL-6/gp130 interaction in the ER, leading to decreased PEL cell growth and increased apoptosis. In summary, ER-localized vIL-6/gp130 signaling promotes cell growth and survival via the activation of STAT3 and ERK1/2.
Figure 5.1
**Figure 5.2.** Role of vIL-6 and gp130 in HHV-8 replication in PEL and endothelial cells. vIL-6 and gp130 are required for maximal HHV-8 productive replication in both PEL and endothelial cells. Depletion of vIL-6 or gp130 results in lower HHV-8 titers following reactivation. Additionally, STAT3, which is activated by vIL-6/gp130 signaling, promotes HHV-8 replication; depletion of STAT3 leads to decreased viral titers in both PEL and endothelial cells. To assess the role of ERK signaling in HHV-8 replication, a series of gp130 mutants were employed as described in Chapter 4. The gp130.ERK.Y mutant was capable of initiating ERK signaling but not STAT3 signaling, and gp130.STAT.Ys could activate STAT3 (and STAT1) signaling but not ERK signaling. In a depletion-complementation experiment, gp130.STAT.Ys, but not gp130.ERK.Y, was capable of rescuing HHV-8 titers following depletion of endogenous gp130. These results indicate that vIL-6/gp130-mediated STAT signaling, but not ERK signaling, is necessary for maximal HHV-8 replication. Finally, a vIL-6 depletion and complementation experiment was conducted to determine the contribution of ER-localized vIL-6/gp130 signaling to productive replication. ER-directed wild-type vIL-6 was capable of rescuing HHV-8 titers following vIL-6 depletion, but ER-directed and mutated vIL-6 (W167G; incapable of dimerizing gp130 in the ER) was unable to rescue the vIL-6-depletion phenotype. In summary, ER-localized vIL-6/gp130-mediated STAT3, but not ERK1/2, signaling is required for maximal HHV-8 replicative titers.
Figure 5.2.
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RESEARCH EXPERIENCE

2009–2014  Graduate Student, Cellular and Molecular Medicine
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            Research: Viral Oncology. Described the role of endoplasmic reticulum-
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            signaling, and virus production. Developed an assay to determine
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            viral and/or host proteins. Research findings will facilitate the search for
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2008  Summer Intern, Pediatric Oncology Education
       St. Jude Children’s Research Hospital, Memphis, TN
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       Research: Molecular Pharmacology research. Mutagenized human
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2007–2008  Undergraduate Research
            Clemson University, Clemson, SC
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