MANIPULATION AND EXPLOITATION OF THE HOST CELL BY KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

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ABSTRACT

Louise Caroline Giffin: Manipulation and Exploitation of the Host Cell by Kaposi's Sarcoma-Associated Herpesvirus
(Under the direction of Blossom Damania)

Kaposi's sarcoma-associated herpesvirus (KSHV) is a human gamma-herpesvirus that is the causative agent of three human malignancies: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. KSHV causes a lifelong infection for which there is no known cure, and the cancers associated with KSHV typically have poor prognosis and no established treatment protocol. KSHV is a large DNA virus that encodes over eighty open reading frames that have diverse functions, including viral proteins that thwart the host immune system and that alter cellular growth pathways to promote viral persistence, with the incidental effect of inducing cellular transformation.

KSHV expresses several homologs of human proteins, including a homolog of interleukin 6 (hIL-6) called viral interleukin 6 (vIL-6). vIL-6 is a predominantly intracellular protein that localizes to the endoplasmic reticulum where it can signal through the gp130 subunit of the IL-6 receptor. We sought to examine how vIL-6 interacts with components of the host cell and alters host gene expression to promote vIL-6 function and induce pathogenesis. We identified hypoxia upregulated protein 1 (HYOU1) as a vIL-6-interacting partner, and found that HYOU1 supports vIL-6 protein expression and promotes vIL-6-mediated signaling, migration, and survival in

endothelial cells. We also found that vIL-6 significantly upregulates the expression of genes associated with cellular movement including the adhesion factor called carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). We determined that vIL-6 increases CEACAM1 expression via STAT3 signaling, and that CEACAM1 promotes vIL-6-mediated migration of endothelial cells. Both *de novo* and latent KSHV infection were also found to upregulate CEACAM1.

To further elucidate how KSHV modulates the cellular environment, we performed kinome profiling to identify cellular kinases that are differentially activated during latent and lytic KSHV infection in primary effusion lymphoma. Kinases regulate almost all cellular processes, and dysregulated kinase activation can drive tumorigenesis. This research may identify major pathogenic signaling pathways that are hyperactivated or shut off in KSHV-associated cancers. Further elucidating how KSHV and its encoded proteins alter the cellular environment to promote pathogenesis will identify targets for the development of novel therapeutics to treat KSHV-associated disease.

I dedicate this dissertation to my grandmother, Helen Louise Giffin,
for giving me my love of science and teaching me to always be curious.

And to my family, Neil, Mom, Dad, and Nik for their love and support.

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCBL	Body cavity-based lymphoma
BCR	B cell receptor
BEC	Blood endothelial cells
CAMK	Calcium/calmodulin-stimulated protein kinase
CCL2	Chemokine C-C motif ligand 2
CDK	Cyclin-dependent kinase
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
cGAS	Cyclic GMP/AMP synthase
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GTP	Guanosine triphosphate
HEK	Human embryonic kidney
hIL-6	Human interleukin 6
HAART	Highly active anti-retroviral therapy
HHV8	Human herpesvirus 8

HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
HUVEC	Human umbilical vein endothelial cell
HYOU1	Hypoxia upregulated protein 1
IFN	Interferon
IHC	Immunohistochemistry
IKK	IκB kinase
IL	Interleukin
IL-6R	Interleukin 6 receptor
IP	Immunoprecipitation
IPA	Ingenuity Pathway Analysis
IRF	Interferon regulatory factor
IRIS	Immune reconstitution inflammatory syndrome
ITAM	Immunoreceptor tyrosine activation motif
ITIM	Immunoreceptor tyrosine inhibitory motif
JAK	Janus kinase
KICS	KSHV inflammatory cytokine syndrome
KS	Kaposi’s sarcoma
KSHV	Kaposi’s sarcoma-associated herpesvirus
LANA	Latency-associated nuclear antigen
LEC	Lymphatic endothelial cells
MAPK	Mitogen-activated protein kinase
MCD	Multicentric Castleman’s disease
MHC	Major histocompatibility complex

MIB/MS	Multiplex inhibitor bead/mass spectrometry
miRNA	MicroRNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa B
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NLR	NOD (Nucleotide-binding and oligomerization, leucine right repeat)-like receptor
NTC	Non-targeting control
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
pDC	Plasmacytoid dendritic cell
PEL	Primary effusion lymphoma
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
qPCR	Quantitative Real time polymerase chain reaction
RCA	Regulator of complement activation
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RRV	Rhesus rhadinovirus
RTA	Replication and transcription activator
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	Src homology
shRNA	Short hairpin RNA

siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STAT3	Signal transducer and activator of transcription 3
STING	Stimulation of interferon-dependent genes
TCR	T cell receptor
TLK2	Tousled-like kinase2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	Tetradecanoyl phorbol acetate
TRAF	TNF receptor associated factor
Tyr	Tyrosine
UTR	Untranslated region
vFLIP	Viral FADD-like caspase-8 inhibitory protein
vGPCR	Viral G protein-coupled receptor
vIL-6	Viral interleukin 6
vIRF	Viral interferon regulatory factor
VKORC1v2	Vitamin K epoxide reductase complex subunit 1 variant 2
vPK	Viral protein kinase, ORF36
VSV	Vesicular stomatitis virus

CHAPTER 1: KSHV: PATHWAYS TO TUMORIGENESIS AND PERSISTENT INFECTION[1,2]

OVERVIEW

Kaposi's sarcoma associated herpesvirus (KSHV; also known as human herpesvirus 8) is the etiological agent of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. These cancers often occur in the context of immunosuppression, which has made KSHV-associated malignancies an increasing global health concern with the persistence of the AIDS epidemic. KSHV has also been linked to several acute inflammatory diseases. KSHV exists between a lytic and latent lifecycle which allow the virus to transition between active replication and quiescent infection. KSHV encodes a number of proteins and small RNAs that are thought to inadvertently transform host cells while performing their functions of helping the virus persist in the infected host. KSHV also has an arsenal of components that aid the virus in evading the host immune response, which help the virus establish a successful lifelong infection. In this comprehensive review, we will discuss the diseases associated with KSHV infection, the biology of latent and

[1] Louise Giffin and Blossom Damania. Manuscript was written by LG and edited by BD.

[2] Reprinted from Advances in Virus Research, Volume 88, Louise Giffin and Blossom Damania, "KSHV: Pathways to Tumorigenesis and Persistent Infection", Pages 111-159, 2014, with permission from Elsevier.

lytic infection, and individual proteins and microRNAs that are known to contribute to host cell transformation and immune evasion.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) was identified in 1994, and is the eighth and most recently discovered human herpesvirus. Shortly after, KSHV was found to be the etiological agent of three human malignancies including its namesake, Kaposi's sarcoma, as well as two B cell lymphoproliferative diseases called primary effusion lymphoma and multicentric Castleman's disease. Recently, KSHV has also been associated with several acute inflammatory diseases. KSHV infection typically occurs in the context of immunosuppression; thus KSHV-associated cancers have become a global public health concern alongside the acute inflammatory disease syndrome (AIDS) epidemic.

Like all herpesviruses, KSHV infection transitions between a quiescent latent and replicative lytic life cycles. KSHV encodes an arsenal of viral proteins and non-coding RNAs that are thought to manipulate the host cell environment to directly or indirectly drive pathogenesis and viral persistence. Unlike other human pathogens such as bacteria, fungi, and parasites, viruses must enter the host cell to replicate and rely on the function of many host factors to successfully propagate. Because of this intimate relationship between host and pathogen, viruses have evolved in close contact with cellular components and thus have developed unique ways of manipulating and exploiting the cellular environment to support viral infection. During co-evolution with the human host, herpesviruses also acquired the genetic material

to encode homologs of cellular proteins. Some of these homologs allow the virus to activate or inactivate key host pathways to the virus' advantage. Dissecting the function and role of individual viral components has helped create a picture of how KSHV induces disease. Understanding the mechanisms by which KSHV maintains a persistent infection and/or drives tumor development will help uncover potential therapeutic targets for the treatment of KSHV-associated malignancies.

MALIGNANCIES AND SYNDROMES LINKED WITH KSHV INFECTION

Kaposi's sarcoma: The classical form of Kaposi's sarcoma was first described in 1872 as a pigmented sarcoma of the skin by the Hungarian dermatologist, Moritz Kaposi (1). KS incidence rates started to increase dramatically with the onset of the AIDS epidemic in the 1980's (2). The correlation between HIV-infected individuals and KS suggested an infectious agent was involved. About a decade later, representational difference analysis used by Chang and Moore identified novel gammaherpesvirus DNA sequences in KS lesion biopsies (3). In the years following the discovery of KSHV, PEL and MCD were also found to be causally linked to this human herpesvirus (4-6) (Table 1).

There are four forms of KS that have been described (7-9). Classic KS, which was first identified by Moritz Kaposi, is found in elderly men of Mediterranean and eastern European descent (10). This form of KS is characterized by benign lesions on the upper and lower extremities, and rarely progresses to more aggressive disease. The second type of KS is African endemic KS, which occurs in eastern and central African countries (7, 11). The lymphadenopathic form of endemic KS is found

almost exclusively in young African children and cause significant mortality (12, 13). AIDS-associated, or epidemic KS has become the most common type of KS in the past three decades, and is the most aggressive form of the disease (2, 14, 15). AIDS-associated KS is considered an AIDS-defining illness (16). KS is currently the most common malignancy associated with HIV infection, and therefore is the most frequent cancer in many Sub-Saharan countries (17-21). The fourth type of KS is iatrogenic/post-transplant KS, which is associated with the use of immune suppressive therapy for the prevention of organ transplant rejection (22-24). Interestingly, it was found that this type of KS occurs more often in KSHV-infected recipients rather than KSHV-negative recipients that receive an organ from a KSHV-positive donor (25, 26).

KS lesions typically occur cutaneously on upper and lower extremities or on mucosal surfaces; however, lesions may also involve lymph nodes or may occur on visceral organs such as lung and spleen (22, 27). The immune status of the host and lymph node involvement are important factors in patient prognosis. KS disease progresses through six stages called the patch, plaque, nodular, lymphadenopathic, infiltrative, and florid stage (13, 28). KS lesions are highly angiogenic and as a result are usually red, purple, or brown in color. Additionally, the lesion vasculature is leaky, which allows for extravasation of erythrocytes and infiltration of inflammatory cells (29). KS tumor cells are of endothelial cell origin, and the primary KSHV-infected cells found in the lesion are highly proliferative spindle-shaped cells (30-32). Over 95% of KS lesions contain KSHV DNA (33), and most of the infected cells harbor the virus latently. Interestingly, KSHV infection of blood endothelial cells

(BEC) can induce expression of lymphatic endothelial cell (LEC) markers and vice versa. This transcriptional reprogramming results in poorly differentiated endothelial cells that express mixed lineage markers such as CXCR4, CD34, VEGFR3, LYVE1, and PROX1 (34-36). As opposed to a metastatic dissemination, KS lesions typically arise independently of one another; however this oligoclonality is not universal, and situations of monoclonal KS have also been reported (37, 38).

Disease	Presentation	Lineage and primary tumor cell	Clonality	KSHV genomes
Kaposi's sarcoma (KS)	Highly angiogenic. Lesions can be found on skin, visceral organs, or mucosal surfaces	Endothelial cell origin; tumor cells are spindle cells with mixed blood and lymphatic endothelial cell markers	Oligoclonal lesions	>99% of tumor cells contain KSHV genomes
Primary effusion lymphoma (PEL)	Non-Hodgkin lymphoma; B-cell expansion in body cavity	B cell; CD20-; markers resemble partially differentiated plasma cells	Monoclonal	Each tumor cell has 50-100 copies of the KSHV genome
Multicentric Castleman's disease (MCD)	Plasmablastic variant of MCD	B cell; IgM λ-restricted plasmablasts	Typically polyclonal	Unknown

Table 1.1: Characteristics of KSHV-associated malignancies

Primary Effusion Lymphoma: Shortly after its association with KS, KSHV was identified as the etiological agent of PEL (6). PEL is a non-Hodgkin lymphoma (NHL) comprised of malignant, latently-infected B cells that expand within the

pericardial, pleural, and peritoneal body cavities (39). Unlike KS, PEL is a monoclonal population of B cells as evidenced by clonal immunoglobin gene rearrangements, and each tumor cell has a high KSHV copy number ranging from 50-100 genomes per cell (40). Morphologically, PEL share features of both immunoblastic and anaplastic large-cell lymphomas (41). Most PEL express CD45 and activation markers including CD30, CD38, and CD7, and epithelial membrane antigen (EMA) (42). Interestingly, PEL expresses plasma cell markers such as CD138, VS38c, and MUM-1/IRF4, but has relatively low expression of B-cell associated antigens, suggesting that PEL resembles partially differentiated plasma cells rather than mature B cells (42-44). Unlike other NHLs, PEL typically does not exhibit c-myc rearrangements or mutations in the ras, bcl2, or p53 genes (39, 42). PEL is frequently coinfected with the Epstein Barr virus (EBV) (42).

Although PEL is characterized by a malignant serous effusion lacking a solid tumor mass, cases of solid PEL have also been reported (45). These tumors typically present as an extracavitary lymphoma in extranodal or lymph node tissue and are composed of immunoblastic-like cells. These solid PEL are also KSHV positive, and have similar morphology, immunophenotype, immunoglobulin gene rearrangements to classical PEL (41).

PEL is a very aggressive lymphoma, and the average survival time is about 6 months from diagnosis (46). The main prognostic factors that have been identified are the presence of highly active antiretroviral therapy (HAART) in HIV-positive patients before PEL diagnosis and the performance status of the patient prior to PEL diagnosis (46); however, it has also been suggested that the KSHV viral load may

also be an accurate predictor of clinical outcome of PEL patients (47). The level of immune suppression and the amount of circulating CD4+ lymphocytes may also contribute to the aggressiveness of PEL.

Multicentric Castleman's disease: Around the same time as PEL, the plasmablastic variant of MCD was also found to be associated with KSHV infection (4, 5). MCD also exists in a hyaline variant that is not associated with KSHV (48). MCD is an uncommon disseminated lymphadenopathy characterized by an abnormal proliferation of IgM λ-restricted plasmablasts within the mantle zone of B cell follicles (4, 49). MCD is considered non-neoplastic since the plasmablasts are typically polyclonal, however monoclonal B cell expansions have been observed (50). The plasmablasts are large with a vesicular nucleus containing one or more nucleoli (51). Systemic symptoms and inflammation as well as involvement of multiple organs often accompany MCD diagnosis (48, 52).

KSHV coinfection is observed in almost all HIV-positive MCD, although only a small proportion of cells in affected lymph nodes typically harbor the virus. Interestingly, KSHV infection in MCD is quite lytic as compared to KS and PEL (53, 54). KSHV is detected in less than 40% of HIV-negative MCD cases (4, 48, 52, 55); however, in patients coinfected with HIV and KSHV, MCD tends to be very aggressive with rapid disease progression. One cause of the high fatality among these cases is that other KSHV-associated malignancies including KS and PEL are frequently observed with HIV-associated MCD (56, 57). MCD progression is thought to be driven by dysregulated cytokine levels, including IL-6, IL-10, and vascular

endothelial growth factor (VEGF) (52, 58-60). In KSHV+ MCD, expression of the virally-encoded IL-6 (vIL-6) cytokine likely exacerbates inflammation and disease progression. vIL-6 can enhance cytokine signaling and further increase human IL-6 and VEGF expression (61-64). A cohort of plasmablastic MCD patients with detectable vIL-6 expression were found to have a rapidly fatal clinical course as compared to vIL-6-negative MCD patients, suggesting the importance of cytokine signaling in MCD progression (55).

KSHV-associated Inflammatory Cytokine Syndrome: In the past few years, several studies have reported patients that present with MCD-like inflammatory symptoms but lack lymphadenopathy or other pathological evidence of true MCD (65). These patients typically have elevated cellular and viral cytokine levels, including human IL-6, IL10, C-reactive protein, and the viral cytokine vIL-6 (65). As compared to KS patients, high KSHV viral loads are also observed, indicative of a lytic or reactivated KSHV infection (27, 65). Concurrent KS is frequently observed in these patients as well. Because of the systemic inflammatory symptoms, the proposed name for this disease is KSHV inflammatory cytokine syndrome or KICS. It differs from the chronic immune activation disease sometimes seen in HIV patients because two requirements for a KICS diagnosis include detection of high KSHV viral load and vIL-6 cytokine levels (54).

There has been some controversy as to whether KICS is truly a distinct syndrome, since its diagnosis is typically made by exclusion of an MCD diagnosis. It has been proposed that KICS is a heterogeneous condition or a “prodrome” that eventually evolves into KSHV+ MCD, although some patients never progress to this

point (54). Recently, a group investigated whether polymorphisms in the KSHV-encoded microRNAs (miRNA) could be correlated with the development of KICS (66). They found that a higher percentage KSHV+ MCD and KICS patients had single nucleotide polymorphisms (SNPs) in the KSHV miRNA loci than KS patients or KSHV+/KS-negative control patients. They also utilized classification tree analysis to determine combinations of SNPs that may predict development of KSHV+ MCD and KICS. Another recent case study identified a KICS patient with high viral loads of both KSHV and the ubiquitous human herpesvirus 6A, suggesting a possible role for other pathogens in development of KICS (27).

KSHV Immune Reconstitution Inflammatory Syndrome: A small percentage of patients that begin HAART to treat advanced HIV infection exhibit a rapid deterioration of their clinical status. This phenomenon is known as immune reconstitution inflammatory syndrome (IRIS). It is proposed that following immune reconstitution, the increase in functional CD4+ T cell populations causes an immune recognition and response to autoantigens or pathogens that were previously present but asymptomatic. Cases of IRIS have been reported against KSHV and other pathogens such as *Mycobacterium tuberculosis, Mycobacterium avium, Cryptococcus neoformans,* and human cytomegalovirus (CMV) (67). In many instances, treatment of the offending pathogen or use of anti-inflammatory drugs can improve prognosis. High morbidity is observed in patients experiencing KS flares following initiation of HAART (IRIS-KS), although administration of systemic chemotherapy can control flares and cause tumor regression (68, 69). Interestingly,

one study determined that IRIS-KS patients had a significantly higher CD4+ count at KS diagnosis following HAART initiation than patients who did not develop IRIS, and that the mean time to KS diagnosis following HAART was less than 2 months (70). They also found that patients receiving more potent HAART regimens were more prone to IRIS-KS development. Beginning HAART treatment prior to advanced HIV infection or diagnosis of AIDS-KS decreases the chance of IRIS-KS (69).

VIRAL LATENCY AND ASSOCIATED PROTEINS

Latency is the default lifecycle for KSHV following infection of a host cell. During latency, the latency associated nuclear antigen (LANA) circularizes and tethers the viral genome to the host chromosomes by simultaneously binding both the terminal repeats and host histones H2A and H2B (71-73). The viral genome is replicated by host machinery with each cell division, and therefore persists as it is passed to each daughter cell (74, 75). As mentioned, only a small portion of the viral genome is actively transcribed during latency, and this region is known as the latency locus. This locus includes the viral genes LANA, vFLIP, vCyclin, kaposin, and the viral microRNAs (76, 77). The LANA promoter controls expression of LANA, vCyclin, and vFLIP while the kaposin promoter drives expression of three kaposin transcripts, a bicistronic transcript for vCyclin and vFLIP, and the twelve viral pre-miRNAs (78, 79). Additionally, PEL express vIRF3 (LANA2) during latency (80). Transgenic mice expressing some or all of the KSHV latency locus have phenotypes characteristic of KSHV malignancies (76, 81). The viral latent genes and microRNAs

have been investigated in depth to understand the mechanism by which KSHV causes disease. In this section, we will discuss a few elements of the latency locus.

LANA: LANA is encoded by ORF73 and is KSHV's major latency protein. It is responsible for tethering the viral episome to the host genome via the terminal repeats and histone interactions (71, 73), which allows host machinery to replicate and distribute the latent genome to daughter cells (72, 74, 75). The phosphorylated DNA-damage response protein γH2AX and the cellular replication fork factors Timeless and Tipin are some of the many known cellular proteins that assist LANA in maintaining KSHV episomes (82-84). LANA has also been shown to positively and negatively affect the transcription of a number of host genes (85, 86). This is likely mediated through LANA's interaction with many transcription factors (84). LANA can also autoregulate its expression by inducing transcription from the LANA promoter (87). Furthermore, LANA and the LANA homologue in rhesus rhadinovirus (RRV) can repress transcription of the viral lytic transactivator, RTA (replication and transcription activator, ORF50) to help maintain latency (88-90).

LANA has several mechanisms by which it can promote host cell survival and proliferation. LANA can bind and inhibit p53 to reduce activation of p53-dependent reporter genes and cause chromosomal instability (91, 92). LANA can also bind and inactivate the tumor suppressor Rb leading to increased E2F-dependent reporter gene activation (93). Furthermore, LANA induces cytoplasmic β-catenin accumulation by binding and sequestering GSK-3β in the nucleus (94), thus allowing upregulation of the pro-growth proteins cyclin D and c-myc by the transcription factor LEF. LANA can also stabilize c-myc protein levels (95, 96). LANA has been shown

to increase telomerase expression, which increases the lifespan of infected cells (97). Finally, B cell-specific expression of LANA in a transgenic mouse model led to follicular hyperplasia, increased germinal center formation, and lymphomas, implicating LANA as a key player in KSHV-associated lymphomagenesis (81).

vCyclin: vCyclin is another latently-expressed protein and is encoded by ORF72. vCyclin shares sequence and functional homology with cellular cyclin D2 and can bind and activate the cyclin-dependent kinase CDK6 (98, 99). When in complex with cdk6, vCyclin can phosphorylate and inactivate the tumor suppressor Rb, the cdk inhibitor p27 (Kip), and the anti-apoptotic protein Bcl-2, collectively leading to cell cycle deregulation (100-102). vCyclin-cdk6 can also phosphorylate histone H1 and cdc25a (101). Interestingly, vCyclin transgenic mice develop lymphomas deficient in p53 (103). This is likely because vCyclin can also bind cdk9 which induces p53 phosphorylation and cell cycle arrest, so only cells which have lost p53 can continue to divide and expand into a lymphoma (104).

vFLIP: KSHV K13 encodes vFLIP, which is a viral homolog of cellular FLIP (FLICE [protein FADD-like interleukin-1 beta-converting enzyme, now called caspase-8] inhibitory protein). vFLIP is expressed during latency, and contains two death effector domains that can associate with FADD and prevent the CD95 death receptor from activating the apoptosis-inducing protease caspase 8 (FLICE) (105). It was subsequently shown that vFLIP can bind procaspase 8 directly to prevent its cleavage into active caspase 8 (106). Furthermore, vFLIP persistently activates nuclear factor kappa B (NFκB) signaling through binding to IKKα, IKKβ, RIP, and the NEMO complex (107, 108). This signaling contributes to the transforming potential of

vFLIP (109). *In vivo* studies demonstrate that vFLIP transgenic mice can develop lymphomas and B cell-derived tumors (110-112).

The Kaposins: ORF K12 encodes three transcripts that yield kaposin A, B, and C (113). Kaposin is highly abundant in PEL, and is transforming in cell culture-based assays (114). Kaposin A was shown to interact with the ARF guanine nucleotide exchange factor cytohesin-1 to mediate cellular transformation and activation of the ERK/MAPK pathway (115). Kaposin B plays a role in preventing the decay of cytokine mRNAs by binding and activating the p38/MAPK target kinase MK2 (116, 117). MK2 can inhibit the decay of mRNAs that contain AU-rich elements, which include cytokine mRNAs and the mRNA for PROX1. KSHV induces reprogramming of blood vascular endothelial cells towards a lymphatic lineage through upregulation of PROX1, and the ability of kaposin B to stabilize PROX1 mRNA is critical for this process (118).

KSHV microRNAs: Similar to other members of the herpesvirus family, KSHV encodes 12 viral pre-microRNAs (pre-miRNAs) that are processed by the host proteins Drosha and Dicer to generate mature miRNAs. The KSHV pre-miRNAs are transcribed from the latent Kaposin/K12 promoter. While 10 of the pre-miRNAs are located in a Kaposin intron, the remaining 2 are located in the Kaposin protein-coding region and the Kaposin 3' UTR (119-121) . The 12 viral pre-miRNAs generate 24 mature miRNAs that have all been detected in KSHV-infected cells. Furthermore, RNA editing of the 5' end of pre-miR-K12-10 can yield multiple mature miR-K12-10 species (122). Despite having a common promoter, the mature KSHV miRNAs each exist at high but variable levels in latent PEL cell lines, and miR-K12-

10 and miR-K12-12 levels are further increased during lytic replication (121-123). In PEL cell lines, over 90% of the expressed mature miRNAs are KSHV miRNAs. Several studies comparing clinical samples of KS biopsies and PEL to cultured PEL cell lines report that the KSHV pre-miRNAs are expressed at even higher levels *in vivo* and that their sequences are highly conserved between patients (124, 125). Functional KSHV miRNAs are also found in the virion, along with mRNAs, cellular miRNAs, and other small RNA species (126).

A number of validated host and viral mRNA targets of the KSHV miRNAs have been identified. These targets are involved in a variety of viral and cellular processes including maintenance of viral latency, immune evasion, cell cycle regulation, cell survival and proliferation, and apoptosis. miR-K9* has been shown to directly target the 3' UTR of the ORF50/RTA mRNA to prevent lytic reactivation (127), and miR-K12-5 may also indirectly suppress the RTA transcript (128). Furthermore, miR-K12-4 can target the DNA methyltransferase repressor Rbl2 to epigenetically maintain latency (128), and miR-K12-1 targets the 3' UTR of the NFκB repressor IκBα to enhance NFκB signaling and promote latency (129). Two components of the TLR/IL-1R signaling cascade, IRAK1 and MyD88, were identified as targets of miR-K12-9 and miR-K12-5, respectively, which results in reduced IL-6 and IL8 inflammatory cytokine production (130). Furthermore, miR-K12-7 can directly bind the 3' UTR of the mRNA of MICB, the stress-induced natural killer (NK) cell ligand, to repress MICB translation and promote viral immune evasion by diminishing NK cell function (131). KSHV miR-K12-1 is able to prevent cell cycle arrest by targeting the CDK inhibitor p21 to promote cell division (132). KSHV

infection may prime B cells for transformation by expression of miR-K12-11, which is an ortholog of cellular miR-155 (133-135). miR-K12-11 targets the host protein Jarid2 and both miR-155 and miR-K12-11 can induce expansion of splenic CD19+ B cells *in vivo* (133, 136). Additionally, miR-K12-11 and -1 can induce MAPK signaling, promigration factors, and cell invasiveness through indirectly suppressing the MAPK phosphatase DUSP1 (137).

The KSHV miRNAs also have several mechanisms of preventing apoptosis of host cells: miR-K12-10 variants inhibit TGF-β signaling by targeting the 3' UTR of the TGF-β type II receptor (TβRII) (138) and miR-K12-10a suppresses the tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) receptor (TWEAKER) to prevent TWEAK-mediated caspase activation, apoptosis, and proinflammatory cytokine production (139). Furthermore, it was demonstrated that miR-K12-1, -3, and 4-3p can target the 3' UTR of caspase 3 to downregulate this host protein and inhibit apoptosis (140). The KSHV miRNAs have a variety of cellular and viral targets, only a handful of which have been discussed here. Collectively, the miRNAs work to drive KSHV pathogenesis by promoting latency, providing favorable growth conditions, and preventing apoptosis of infected cells.

In addition to the genes described above, some reports have also shown that viral genes such as K1, vIL-6, and K15 are expressed at low levels during latency but are highly upregulated during lytic replication (141-143). These viral proteins are discussed in the section on lytic pro-growth proteins.

THE KSHV LYTIC CYCLE

The KSHV lytic cycle can ensue following primary infection or when a latently infected cell undergoes lytic reactivation. During the lytic cycle, a temporal transcriptional cascade begins that results in expression of viral immediate early, delayed early, and late genes followed by the subsequent assembly and egress of progeny virions (144). As discussed earlier, a variety of cell stresses can induce reactivation (145-149). Ultimately, expression of the lytic transactivator, ORF50/RTA, is required to initiate this complex stage of the viral lifecycle. RTA expression alone is sufficient to drive lytic replication, and suppression of RTA prevents reactivation (150-153). RTA is an immediate-early gene, and it is part of a polycistronic transcript that also encodes K8 and K8.1. Other immediate early genes include ORF45 and K4.2 (154). RTA has an activation domain and a DNA-binding domain on opposite ends of the protein. The DNA-binding domain allows RTA to directly bind and activate numerous viral promoters and the two KSHV origins of lytic replication, OriLyt-L and OriLyt-R (155, 156). The activation domain allows RTA to interact with cellular transcription factors and chromatin modification complexes to promote viral gene transcription (157).

Delayed early genes are sensitive to cyclohexamide treatment because in order to be transcribed they require the function of the proteins encoded by immediate early genes. The delayed early genes include the viral DNA polymerase and viral proteins required for viral DNA synthesis, as well as the viral thymidine kinase, nucleotide reductase, ORF57, the signal transduction proteins K1, K15, and vGPCR, and the immune evasion proteins K3 (MIR1) and K5 (MIR2) (144).

Following expression of the delayed early genes, viral DNA replication begins from OriLyt-L and OriLyt-R (158, 159). The viral replication machinery includes the viral DNA polymerase, helicase, polymerase processivity factor, primase, primase-associated factor, and single strand binding protein (160) and replication is thought to occur by a rolling circle mechanism similar to other herpesviruses. Viral DNA replication stimulates expression of the KSHV late genes, which mainly encode structural proteins such as the viral capsid and envelope proteins (161). Linear genomes are packaged into the newly forming capsids. KSHV ORF67 and ORF69 assist in nuclear egress (162, 163), and KSHV glycoprotein B is thought to play a role in viral maturation and egress from the host cell (164, 165).

LYTIC KSHV PROTEINS INVOLVED IN CELL GROWTH AND SURVIVAL

Lytic reactivation results in expression of all KSHV genes. As described earlier, several of the proteins encoded by the KSHV latency locus can drive cellular transformation. A number of proteins encoded by KSHV lytic genes also have pro-growth or transforming qualities, which are discussed in this section and summarized in Figure 1.1.

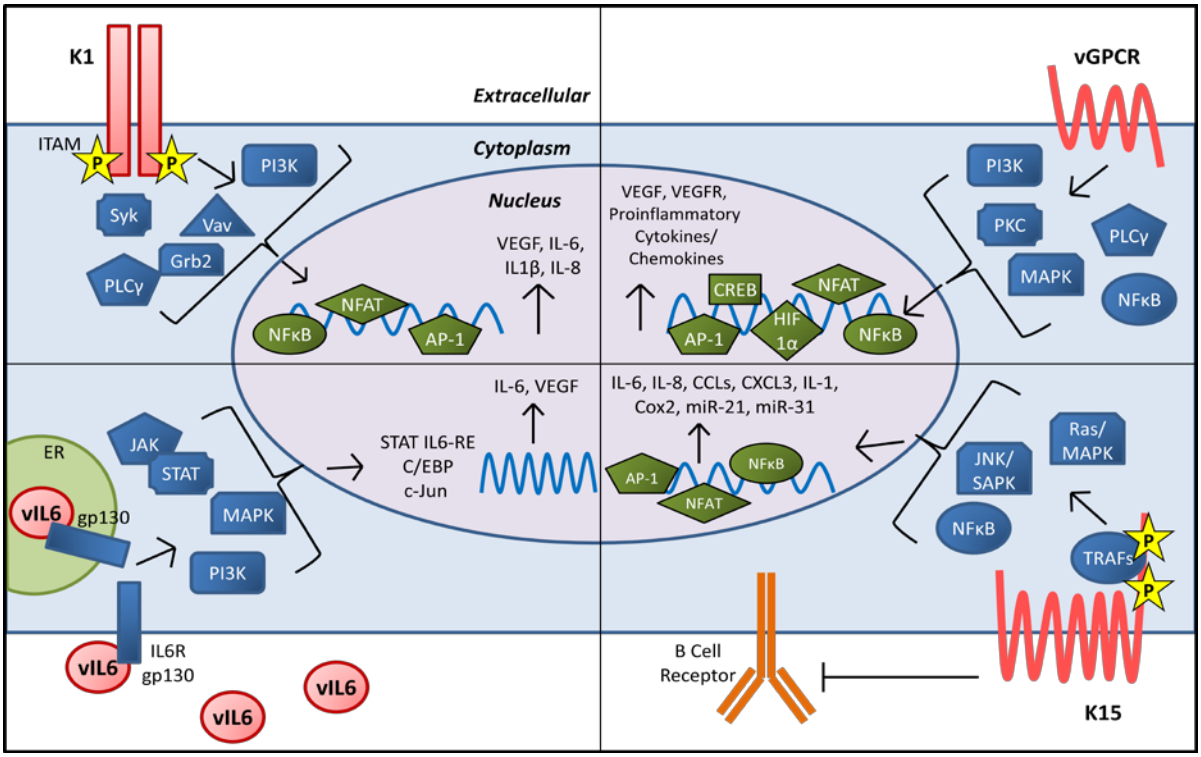


Figure 1.1: KSHV encodes a number of proteins that contribute to cell growth and transformation. A) K1 is a transmembrane protein with a constitutively active immunoreceptor tyrosine activation motif (ITAM) that activates signaling through SH2-containing proteins. K1 expression results in production of VEGF and pro-inflammatory cytokines. B) vGPCR is a constitutively active homolog of the IL8 receptor that results in activation of numerous cell signaling pathways and transcription factors to increase production of VEGF, VEGFR, and proinflammatory cytokines and chemokines. C) vIL-6 is a functional homolog of human IL-6 that can signal through shared IL-6 pathways including JAK/STAT, MAPK, and PI3K. This results in activation of multiple IL-6 response elements and production of human IL-6 and VEGF. D) K15 is a transmembrane protein with several tyrosine residues and SH2 and SH3 domains in its cytoplasmic tail that are critical for K15's interaction with cellular TRAFs and signaling through the MAPK and NFκB pathways. K15

signaling results in activation of numerous transcription factors and expression of pro-inflammatory cytokines and chemokines and several human miRNAs that are involved in cell motility.

K1: K1 is a single-pass transmembrane glycoprotein encoded by the first open reading frame of KSHV (Figure 1.1 A). This protein is expressed on the cell and ER membranes and can be internalized to endosomes (166, 167). K1 is constitutively active and has a highly conserved intracellular immunoreceptor tyrosine-based activation motif (ITAM) on its C terminus (168-170). Upon K1 oligomerization, the ITAM becomes autophosphorylated and can activate various Src homology 2 (SH2)-containing signaling proteins including PI3K (p85)/Akt, PLCγ, Vav, Syk, Lyn, RasGAP, and Grb2 (171-173). Additionally, ITAM signaling results in activation of NFκB, nuclear factor of activated T cells (NFAT), Oct-2 and AP-1(173, 174).

Endothelial cells expressing K1 become immortalized in culture and primary marmoset T lymphocytes infected with a K1-expressing herpesvirus saimiri became immortalized to IL2-independent growth (175, 176). K1 can also induce focus formation in rat fibroblasts (176). *In vivo,* K1 transgenic mice display constitutively active NFκB and Src family tyrosine kinase signaling and a fraction of the mice develop tumors (174) There are several aspects of K1 signaling that likely contribute to its transforming function. K1's activation of Akt results in inactivation of the proapoptotic forkhead (FKHR) transcription factor family which protects cells from FKHR- and Fas-mediated apoptosis (171). Heat shock protein-90 and -40 (hsp90

and hsp40) were identified as K1 binding partners that are critical for both K1 expression and K1's anti-apoptotic function (177). K1 also induces angiogenesis and VEGF production in primary human endothelial cells and cells derived from K1 transgenic animals (173, 175, 178). Furthermore, K1 signaling can induce secretion of inflammatory cytokines that are implicated in KS lesion development, including IL-6, GM-CSF, IL-1β, IL-8, and IL-10 (172, 174). A unique mechanism that K1 utilizes to prolong the life of B cells is to downregulate surface expression of the B cell receptor (BCR) by binding the μ chain of the BCR to retain the complex in the ER (179). Overall, K1 is a multifunctional protein that can constitutively activate multiple pro-growth signaling pathways in KSHV infected cells.

Viral G protein Coupled Receptor (vGPCR): KSHV ORF 74 encodes the vGPCR, which is a seven-pass transmembrane protein that shares homology with the human IL8 receptor (Figure 1.1 B) (180). This lytic protein has been detected at low levels in cultured reactivated PEL and in KS, PEL, and MCD clinical specimens (181). Conflicting reports have demonstrated that vGPCR has the ability to both sustain (182) and repress (183) RTA expression and lytic replication. Although vGPCR can bind the CXC and CC families of chemokines, it is constitutively active even in the absence of ligand (184-186). vGPCR activates a number of important signaling pathways, including PLC, PKC, MAPK, PI3K/Akt/mTOR, and NFκB (187). Downstream signaling from these pathways activates the AP1, NFAT, NF-κB, HIF-1α, and CREB transcription factors which results in vGPCR-mediated production of VEGF, VEGF receptor (VEGFR), and proinflammatory cytokines and chemokines (187). Through these signaling pathways, vGPCR can immortalize and promote the

growth of endothelial cells in culture (188-190). It was also demonstrated that endothelial cell-specific expression of vGPCR can cause formation of KS-like angioproliferative lesions in mice (191). Similar to K1, vGPCR expression can also transform NIH3T3 fibroblasts, as well as rat kidney cells, which are then able to form tumors in nude mice (186). A line of transgenic mice expressing vGPCR in hematopoietic cells developed angioproliferative lesions resembling KS at multiple organ sites (192). However, another study with a line of transgenic mice with vGPCR expressed ubiquitously from an SV40 promoter found that lesions mainly occurred on the tail and/or legs and that only a small fraction of tumor cells actually expressed vGPCR (193). Collectively, this research suggests a model by which vGPCR drives transformation of cells by inducing paracrine secretion of proinflammatory cytokines and angiogenic growth factors which can then work in concert with KSHV latent proteins to promote tumorigenesis.

Viral Interleukin-6 (vIL-6): KSHV ORF K2 encodes the vIL-6 cytokine (Figure 1.1 C). vIL-6 is induced upon lytic replication, but it is also expressed at low levels during latency. Although vIL-6 has been detected in KSHV-associated malignancies (64, 194), levels are highest in MCD lesions and patient sera (55). This protein shares about 25% amino acid identity and 63% similarity with human IL-6 (hIL-6) (195-197). Additionally, vIL-6 shares many functional characteristics with hIL-6, and as a result the viral cytokine can activate the IL-6 receptor (IL6-R) and downstream signaling pathways, including the JAK/STAT, MAPK, and PI3K/Akt pathways (198, 199). These pathways induce a variety of transcription factors and response elements (RE) such as the STAT1/3 and STAT5 IL-6 RE, C/EBP, and c-

jun promoter IL-6 RE (JRE-IL-6) (62). Activation of these pathways leads to expression of hIL-6 (200) and VEGF (61). However, vIL-6 differs from the human cytokine in several regards. vIL-6 can signal intracellularly directly through the gp130 subunit of the IL6-R and does not require the extracellularly-located IL6-R gp80 subunit whereas hIL-6 requires both gp80 and gp130 (201-203); however, gp80 can still bind to vIL-6 and enhance signaling (63, 204, 205). Additionally, hIL-6 is secreted much more efficiently than vIL-6, and a large portion of expressed vIL-6 is actually retained in the endoplasmic reticulum (ER) (206, 207). In the ER, vIL-6 interacts with the ER chaperone calnexin which impacts vIL-6 localization and intracellular retention (142). Furthermore, vIL-6 undergoes N-linked glycosylation which is required for its signaling activities (208).

vIL-6 expression transforms NIH3T3 fibroblasts and these cells form tumors in nude mice (61). vIL-6 expression can also induce growth in mouse hybridoma (198), PEL (209, 210), BAF (205), and Hep3B hepatoma (195) cell lines. In endothelial cells, vIL-6 expression induces proliferation, tubule formation, and neoangiogenesis (211, 212). Additionally, vIL-6 can help cells escape interferon (IFN)-induced growth arrest (209). Furthermore, transgenic mice expressing vIL-6 under the MHC class I promoter develop plasmablastic MCD-like disease, which is abrogated in the absence of endogenous IL-6 (213).

K15: KSHV K15 is encoded by the rightmost open reading frame of the virus (Figure 1.1 D). K15 has two highly divergent alleles called the predominant (P) and minor (M) forms, and these are present in different strains of KSHV (214). K15 is expressed at low levels in latent PEL, but is robustly induced following lytic

reactivation. The transcript is spliced to yield multiple K15 proteins with 4-12 transmembrane domains that localize to lipid rafts (215, 216). The short K15 cytoplasmic tail contains SH3 and SH2 signaling motifs and binding sites for TRAFs 1, 2, and 3 (215, 217). Several critical tyrosine residues within these motifs are constitutively phosphorylated by cellular Src family tyrosine kinases, which mediate activation of downstream signaling pathways. Pathways activated by K15 signaling include the Ras/MAPK, JNK/SAPK, and NFκB pathways as well as the NFAT/AP1 transcription factors (217-219). This signaling activates transcription of a number of cellular cytokines and chemokines including IL-6, IL-8, CCL20, CCL2, CXCL3, IL-1α/β, and Cox2 (217, 220). K15 can also downregulate signal transduction and intracellular calcium mobilization induced by the BCR, which may help the virus maintain latency (216). A potential mechanism by which K15 accomplishes this may be through K15's interaction with the tyrosine kinase Lyn, which plays a role in the regulation of BCR signaling (219). Additionally, the K15 M allele induces cell motility, and this is dependent on K15-mediated upregulation of the human miRNAs miR-21 and -31 (221). K15 may contribute to KSHV-induced tumorigenesis through its ability to activate pro-growth signaling pathways, promote latency, and induce cell motility.

KSHV'S ACTIVATION AND EVASION OF THE HOST IMMUNE RESPONSE

The human immune system is designed to recognize invading pathogens in order to launch an innate and adaptive response to eliminate infection. KSHV utilizes a number of mechanisms to dampen the immune response so that it can persist for the lifetime of the host. In this section, we will discuss aspects of the innate and

adaptive immune response that are activated by KSHV infection as well as aspects that are suppressed by viral immune evasion techniques.

Immune Activation: Toll-like receptors (TLR) are innate pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) and induce NFκB signaling and production of type I IFN and proinflammatory cytokines (222). KSHV can activate TLR3 during infection of primary human monocytes, and this upregulates TLR3 expression and the production of IFNβ and CXCL10 (223, 224) which are then downregulated as latency is established (225). Although KSHV can reduce TLR4 activity in endothelial cells, TLR4 activation is still capable of inhibiting KSHV infection because cells lacking this receptor are more susceptible to infection (226). Thus, there is an initial TLR-mediated innate immune response to KSHV primary infection, but in many cases this response is subsequently downregulated by the virus. KSHV is also sensed by IFN gamma-inducible factor IFI-16, which triggers inflammasome formation and subsequent production of IL-1β (227, 228). Additionally, KSHV infection can activate plasmacytoid dendritic cells (pDCs) which results in TLR9-mediated production of IFNα (229).

KSHV-associated diseases typically occur in immune compromised patients, and it has been demonstrated that reconstitution of the immune system can result in KSHV-associated tumor regression (230). This suggests a role for the adaptive immune response, particularly the CD8+ T cell response, in controlling KSHV infection and pathogenesis (231-233). CD4+ T cells can weakly recognize KSHV latent antigens such as LANA (234). Additionally, it was found that CD4+ and CD8+ T cells from KSHV-seropositive patients frequently recognize select groups of early

lytic and late lytic KSHV genes (235). In a cohort of seven KSHV+/HIV+ KS patients on HAART, KSHV-specific immune responses were detected in six of the seven patients (236). Interestingly, 100% of the non-progressor patients had KSHV-specific CD8+ cytotoxic T lymphocytes (CTLs) that simultaneously secreted IFNγ and TNFα in response to KSHV antigen whereas only 60% of the patients with progressive disease had a CD8+ CTL response (236). Although most studies of the adaptive immune response to KSHV have investigated the T cell response, KSHV infection also generates a humoral response to a variety of viral antigens (237).

Evasion of the Adaptive Immune Response: KSHV employs a variety of mechanisms to evade KSHV-specific adaptive immune responses (reviewed in (238)). These techniques mainly involve repressing viral antigen presentation, T cell activation, B cell receptor (BCR)-mediated B cell activation, and B cell differentiation.

KSHV infection of B cells, dendritic cells (DCs), macrophages, and endothelial cells results in decreased expression of the major histocompatibility complex class I (MHC-I) (239, 240). MHC-I is critical for the presentation of viral antigens to the T cell receptor (TCR) of CD8+ T cells. KSHV K3 and K5 (also called modulator of immune recognition (MIR) 1 and 2, respectively) are capable of ubiquitinating the MHC-I cytoplasmic tail to trigger endocytosis and proteasomal degradation of the complex (241, 242). vIRF1, vFLIP, and the virally-encoded shutoff and exonuclease protein (KSHV SOX) can also cause downregulation of MHC-I (243, 244). vIRF3 and cellular suppressor of cytokine signaling 3 (SOCS3) were recently found to inhibit antigen presentation by the MHC-II complex by reducing the level of MHC-II transcripts (245, 246). Additionally, vIRF3 expression renders PEL

resistant to recognition by KSHV-specific CD4+ T cells. LANA, which is expressed in all KSHV-infected cells, has an acidic central repeat domain that prevents its antigenic processing to further hinder this process (247, 248). In addition to repressing antigen presentation, KSHV infection also causes downregulation of the costimulatory molecules CD80, CD86, CD1a, and CD83 on antigen presenting cells (APCs) (249). K5 likely plays a role in this, because it has been shown to downregulate CD86 and ICAM-1 (250). These costimulatory molecules are required for TCR-mediated activation of CTLs, so the downregulation of these proteins is a mechanism by which KSHV infection inhibits the adaptive T cell immune response.

As discussed previously, B cells are one of the main target cells of KSHV infection. B cells are a critical part of the adaptive immune response, and following binding of antigen to the B cell receptor (BCR), these cells proliferate and differentiate into antibody-producing plasma cells or memory B cells (251). Antibodies eliminate infection by binding to antigen that is either in the extracellular space or presented on the surface of infected cells. Antibody binding generally results in neutralization or phagocytosis of the pathogen or infected cell. If a B cell is unable to be activated through its BCR or unable to differentiate into a plasma cell, antibody production will not occur. One hypothesis is that KSHV targets these two aspects of B cell biology as a mechanism of adaptive immune evasion. The KSHV K5 protein can utilize its ubiquitin ligase activity to downregulate bone marrow stromal antigen 2 (BST-2, also called tetherin) which is an IFN-inducible protein that plays a role in B cell differentiation (252). As mentioned earlier, the KSHV K1 signaling protein plays a role in downregulation of the BCR on the cell surface (179).

Furthermore, KSHV K15 is capable of disrupting signaling from the BCR and possibly accelerating BCR internalization to further reduce BCR-mediated B cell activation (216, 253). Collectively, this inhibition of both B cell differentiation and BCR signaling may help KSHV evade the B cell immune response.

Evasion of the Innate Immune Response: A large portion of the KSHV genome is devoted to evading the innate immune response of the host. The innate immune functions targeted by viral proteins include interferon production, interferon regulatory factor (IRF) activation, natural killer (NK) cell activity, complement activation, inflammasome activation, and chemokine activity. In this section we will discuss the strategies utilized by KSHV to hinder the innate immune response to allow the virus to persist for the lifetime of the host (Figure 1.2).

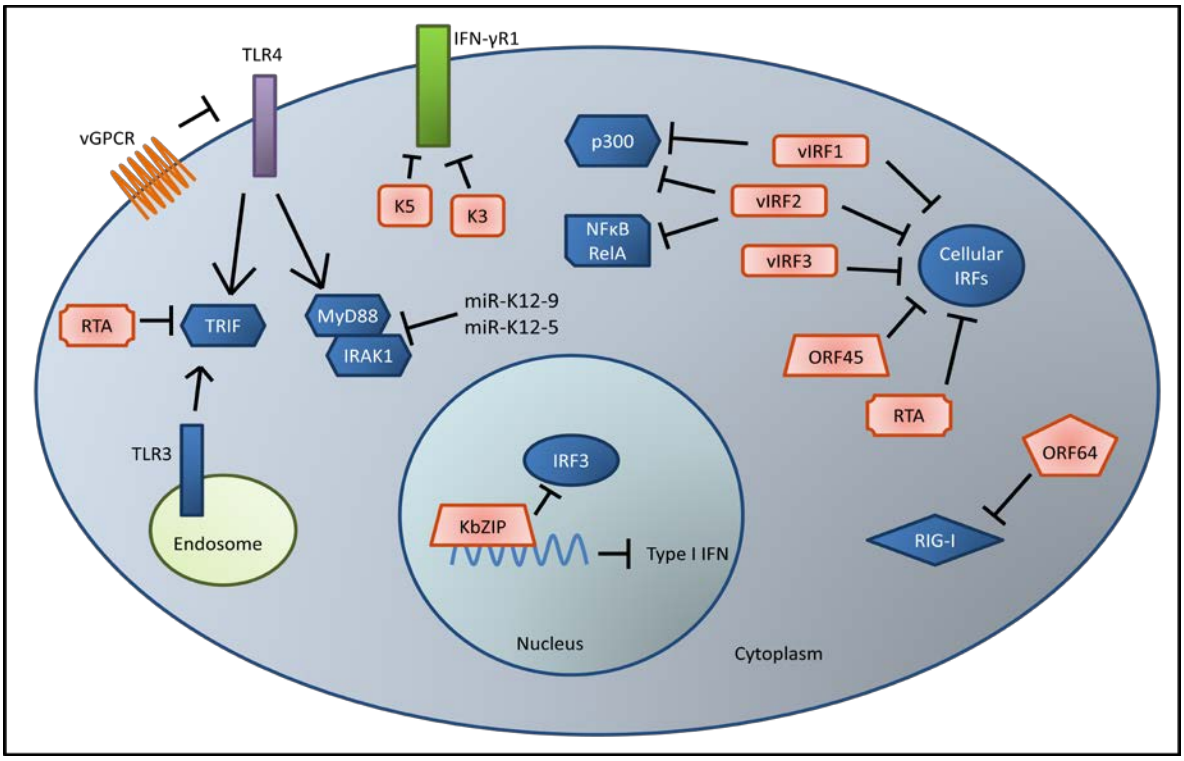


Figure 1.2: KSHV evasion of the host interferon response. KSHV encodes viral interferon regulatory factors (vIRF 1-3) that antagonize the function of cellular IRFs, p300, and NFκB to suppress production of type I IFN. ORF45, RTA, and KbZIP have also been shown to interfere with IRF signaling. K3 and K5 are able to degrade the IFN3γR1 to reduce antiviral IFNγ signaling through the JAK/STAT pathway. Viral infection and expression of vGPCR reduces TLR4 expression, and RTA can induce degradation of the TLR3 and TLR4 mediator TRIF. miR-K12-9 and 5 downregulate IRAK1 and MYD88 which are also components of TLR signaling pathways. Reduction of TLR signaling results in reduced expression of type I IFN. Finally, ORF64 is able to deubiquitinate RIG-I which suppresses RIG-I mediated production of IFNβ.

KSHV employs multiple mechanisms of inhibiting both IFN production and signaling since IFN is a potent antiviral defense that is detrimental to KSHV

persistence (Figure 1.2) (254). The virus encodes four homologs of the cellular IRFs called vIRF 1-4 (255). The cellular IRFs are a large family of transcription factors that drive expression of type I IFN (IFNα and β) and a variety of cytokines and chemokines. Of the four KSHV-encoded vIRFs, only vIRFs 1, 2, and 3 have been shown to impact IFN signaling. vIRF1 can bind to and inhibit the transcriptional activities of IRF1, IRF3, and IRF7 (256, 257). Additionally, vIRF1 can bind and sequester the transcriptional coactivator p300 that is required for IRF1- and IRF3-mediated transcription of type I IFN (256, 257). vIRF2 is able to bind to cellular IRF1, 2, and 8 as well as NFκB RelA and p300 (258). vIRF2 is able to block type I IFN signaling and IFNα-, IFNλ-, and IRF1-dependent transactivation of the IFN stimulated response element (ISRE) promoter (259). More recently, IRF3 was identified as a binding partner of vIRF2, and it was shown that this interaction both suppresses IRF3-mediated transcription of IFNβ and enhances caspase-3-dependent degradation of IRF3 (260). vIRF3 can interact with cellular IRFs 3 and 7 which diminishes the DNA-binding abilities of IRF7 (261). vIRF3 can also interact with IRF5 to inhibit IRF5-mediated IFN promoter activation and production of type I IFN (262, 263). Recently, it was shown that vIRFs 1 and 2, but not vIRF3, are capable of suppressing endogenous IFNβ message and protein expression following activation of TLR3 (225). Because KSHV can activate and upregulate the TLR3 pathway (223), this suggests that the vIRFs have a crucial function in evading the innate type I IFN response to KSHV infection. The vIRFs also have the ability to promote cell growth and prevent apoptosis (reviewed in (255)). Therefore, the vIRFs may have a twofold function in infected cells: first, to inhibit IFN to create a safe

environment for KSHV, and second, to promote cell survival to allow for persistence of the virus in the host.

KSHV ORF45 also influences the IFN response. It was demonstrated that KSHV ORF45 can interact with the inhibitory domain of cellular IRF7 (264). This interaction prevents IRF7's phosphorylation and nuclear accumulation, which are both necessary for IRF7-mediated transcription of type I IFN (265). ORF45 also competes with IRF7 for phosphorylation by IKKε and TBK1 which reduces overall levels of IRF7 phosphorylation (266). Infection of cells with an ORF45-null virus triggered a strong IRF7-dependent type I IFN response that rendered them resistant to subsequent vesicular stomatitis virus (VSV) infection (267). Interestingly, ORF45 is contained within the KSHV virion, which allows the virus to dampen the IFN response immediately upon infection (268). KSHV RTA can also act as an E3 ubiquitin ligase that induces the ubiquitination and degradation of IRF7 to reduce transcription of type I IFN genes (269). KSHV ORFK8 encodes a transcription factor KbZIP that can bind to the positive regulatory domain (PRD) I/III region of the IFNβ promoter to block IRF3-mediated IFNβ transcription (270).

In addition to inhibition of type I IFN, KSHV can also repress signaling by IFNγ. The K3 and K5 proteins are able to induce degradation of the IFN-γ receptor 1 (IFN-γR1) which normally triggers IFNγ-mediated activation of the JAK/STAT pathway (271). Signaling through this pathway induces expression of a wide variety of antiviral genes, which is suppressed following reduction of IFN-γR1 expression by K3 and K5 (271). Between the vIRFs, ORF45, RTA, KbZIP, K3, and K5, KSHV

utilizes a variety of mechanisms to evade IFN activation, suggesting the importance of avoiding this antiviral response in order for KSHV to persist in the host.

As mentioned earlier, the TLRs are pattern recognition receptors (PRR) that can be activated by invading pathogens. TLR activation triggers the production of antimicrobial cytokines and chemokines such as IFN, CCLs, and CXCLs through a variety of signaling proteins including NFκB, IRFs, and TRAFs. KSHV infection is able to downregulate TLR4 expression partly through the actions of vGPCR and vIRF1, and this subsequently suppresses expression of TNF-α, IL1-β, IL-6, and IFNβ (226). Furthermore, it was recently discovered that the ubiquitin ligase activity of KSHV RTA may cause the degradation of TRIF (Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing β-IFN), which is a critical mediator of TLR3- and TLR4-induced type I IFN production (272). As mentioned previously, the KSHV-encoded miRNAs miR-K12-9 and miR-K12-5 target IRAK1 and MYD88, which are both essential components of TLR and IL-1 receptor signaling pathways (130) .

In addition to the TLRs, which are membrane-bound PRRs, host cells also express cytosolic receptors. These cytosolic PRRs include the RNA helicases RIG-I and MDA5, the NLR (nucleotide-binding and oligomerization, leucine-rich repeat containing) protein family, and the cytosolic DNA sensing pathway cGAS/STING (cyclic GMP AMP synthase/stimulation of IFN-dependent genes). RIG-I detects viral RNA and becomes ubiquitinated by TRIM25, which allows it to interact with the downstream signaling complex MAVS/IPS-1 (273, 274). Activation of this complex leads to the induction of type I IFN, which can limit KSHV infection (275) KSHV encodes a deubiquitinase (DUB), ORF64, which is capable of deubiquitinating RIG-I

to suppress RIG-I-mediated activation of the IFNβ promoter during reactivation (276).

NLRs sense a variety of microbial ligands, and their activation results in the assembly of an inflammasome complex which activates caspase-1 to generate mature IL-1β and IL18 (277). Production of IL-1β and IL18 in response to infection can lead to hyperinflammatory caspase 1-mediated cell death, called pyroptosis. KSHV ORF63 has homology to parts of cellular NLRP1, but lacks the effector caspase activation and recruitment (CARD) domain that is critical for inflammasome formation and function. ORF63 is able to interact with NLRP1 to prevent formation of both the NLRP1 and NLRP3 inflammasome and subsequent activation of caspase 1 (278). The function of ORF63 appears to be important for supporting viral gene expression and genome replication as well as suppressing IL-1β production.

A new cytosolic DNA sensor called cGAS was recently identified. cGAS activates the effector STING to induce IFN production and a subsequent antiviral response (279, 280). It was recently found that KSHV infection activates the cGAS/STING pathway, and employs multiple mechanisms to dampen this activation (281). One of these mechanisms is expression of vIRF1, which blocks the cGAS/STING-induced IFN response by disrupting the interaction between STING and the effector molecule TBK1.

KSHV encodes three homologs of cellular chemokines: viral CC-chemokine ligand 1 (vCCL1, also called vMIP1), vCCL2 (vMIP2), and vCCL3 (vMIP3) (195). vCCL1 is a ligand and agonist of CCR8 (282), whereas vCCL2 is a ligand that actually blocks signaling through multiple chemokine receptors including CCR-1, -2,

-5, and -8 and CXCR-1, -2, and -4 (283). vCCL3 is an agonist for CCR4 (284). Collectively, binding of the viral chemokines to their respective cellular chemokine receptors is able to elicit a Th2-polarized response that is less cytotoxic to KSHV-infected cells than a Th1-polarized response (284, 285).

The complement pathway acts as a bridge between the innate and adaptive immune system, since activation of complement can occur in an antibody-dependent or independent mechanism. Furthermore, phagocytosis of complement-bound pathogens or infected cells (opsonization) generates pathogen-derived antigens required to prime the adaptive immune system. Complement activation can occur through the classical, lectin, or alternative pathways which all result in the cleavage of complement component C3 into C3a and C3b by the C3 convertase (286). C3b can then be deposited onto the surface of pathogens or infected cells to facilitate lysis, neutralization, or phagocytosis. Since complement activation occurs through an amplifying cascade of proteolytic events, cellular regulators of complement activation (RCA) proteins keep this pathway in check to avoid hyperinflammatory responses (286). KSHV ORF4 encodes a structural and functional homolog to cellular RCA proteins called the KSHV complement control protein (KCP) (287, 288). KCP is able to prevent cleavage of C3 through accelerating the decay of the C3 convertase, by acting as an inhibitory cofactor to inactivate C3b and downstream complement molecules, and by preventing deposition of C3b onto target surfaces (289, 290). By evading the complement pathway, the virus is able to avoid neutralization of extracellular virions by complement deposition, decrease the

elimination of infected cells, and reduce the acquisition of viral antigens by phagocytes and APCs to inhibit the adaptive immune response.

As discussed in the adaptive immune evasion section, KSHV downregulates MHC-I expression on APCs. NK cells are designed to sense and kill cells displaying abnormal MHC-I levels through their leukocyte Ig-like receptor 1 (LIR1) and killer inhibitory receptor (KIR), which recognize endogenous MHC-I molecules on cells. To prevent the elimination of infected cells with reduced MHC-I, KSHV utilizes multiple mechanisms to inhibit NK cell function. In addition to downregulating MHC-I, KSHV K5 also downregulates surface expression of ICAM-1 and B7-2 (CD86) to avoid NK-mediated cell cytotoxicity (240, 242, 250). NK cell killing requires activation of the NKG2D and NKp80 receptors. As mentioned earlier, the KSHV miRNA miR-K12-7 targets the NKG2D ligand MHC class I-related chain B (MICB) 3'UTR. This results in decreased expression of this NKG2D ligand and effectively reduces NK cell killing ability (131). K5 also decreases the surface expression MICB and another NKG2D ligand, MICA, as well as the NKp80 ligand activation-induced C type lectin (AICL) (291). In these ways, KSHV has cleverly devised mechanisms to not only reduce activation of the adaptive immune system by downregulating MHC-I, but also to avoid the detrimental side effects of abnormal MHC-I levels on infected cells.

CONCLUSIONS

KSHV expresses a diverse repertoire of proteins and small RNAs that aid the virus in establishing a lifelong infection in the host. Many of these viral components inadvertently cause transformation of host cells, linking KSHV with the development

of several human malignancies. These cancers pose a large threat to global public health, particularly in areas that are still struggling with limited treatment options for HIV infection. Two decades of KSHV research has elucidated many of the mechanisms by which KSHV is able to establish and maintain infection in the host and initiate tumorigenesis; however, despite this extensive research, there are still aspects of viral infection and transformation that are not well understood. Further elucidating the unique mechanisms that KSHV uses to persist so successfully in the host will hopefully uncover novel therapeutic targets for the treatment of KSHV disease.

CHAPTER 2: MODULATION OF KSHV VIL-6 FUNCTION BY HYPOXIA UPREGULATED PROTEIN 1[3]

OVERVIEW

Kaposi's sarcoma-associated herpesvirus (KSHV, also called human herpesvirus 8) is linked to the development of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). KSHV expresses several proteins that modulate host cell signaling and deregulate cell growth. One of these proteins is viral interleukin-6 (vIL-6) which is a homolog of human interleukin-6. vIL-6 is able to prevent apoptosis and promote pro-inflammatory signaling, angiogenesis, and cell proliferation. Although it can be secreted, vIL-6 is mainly an intracellular protein that is retained in the endoplasmic reticulum (ER). We performed affinity purification and mass spectrometry to identify novel vIL-6 binding partners and found that the cellular ER chaperone hypoxia upregulated protein 1 (HYOU1) interacts with vIL-6. Immunohistochemical staining revealed that both PEL and KS tumor tissues express significant amounts of HYOU1. We also show that HYOU1 increases endogenous vIL-6 protein levels and that HYOU1 facilitates vIL-6-induced JAK/STAT signaling, migration, and survival in

[3] Louise Giffin, Feng Yan, M. Ben Major, and Blossom Damania. Copyright © Journal of Virology, Aug 2014, 88(16):9429-41. BD and LG designed all experiments. LG performed all experiments except for the mass spectrometry run and analysis, which was performed by FY and MBM. Manuscript was written by LG and edited by BD. Grants to BD provided funding for all experiments.

endothelial cells. Furthermore, our data suggest that HYOU1 also modulates vIL-6's ability to induce CCL2, a chemokine involved in cell migration. Finally, we investigated the impact of HYOU1 on cellular human IL-6 (hIL-6) signaling. Collectively, our data indicate that HYOU1 is important for vIL-6 function and may play a role in the pathogenesis of KSHV-associated cancers.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV; Human Herpesvirus 8) is the causative agent of several human malignancies including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (3-6). These malignancies often occur in the context of immunosuppression, and as a result KSHV-associated malignancies have increased in incidence since the onset of the AIDS epidemic (2). KSHV is a member of the gamma-herpesvirus subfamily and has a double-stranded DNA genome that expresses over eighty open reading frames (ORFs) (40). KSHV infection usually exists in a latent state in which a small subset of the viral genome is expressed. When the virus undergoes lytic reactivation, all viral genes are expressed and progeny virions are produced.

It is thought that several latent and lytic genes contribute to modulation of host cell signaling to induce tumorigenesis. One of these genes is ORF K2 which encodes a viral homolog of human interleukin-6 (hIL-6) called viral interleukin-6 (vIL-6) (195-197). vIL-6 shares 25% identity and 63% similarity to hIL-6 at the amino acid level. vIL-6 is expressed at low levels in latently infected PEL and is highly upregulated upon lytic reactivation (143, 144, 207). All KSHV-associated

malignancies have detectable vIL-6 levels (55, 64, 194). vIL-6 expression transforms NIH3T3 cells, and vIL-6-expressing cells injected into mice form larger tumors as compared to control cells (61). Additionally, transgenic mice engineered to express vIL-6 under the MHC Class I promoter display a phenotype reminiscent of KSHV-associated plasmablastic MCD that is also dependent on mouse IL-6 expression (213). vIL-6 drives production of hIL-6 (200) and vascular endothelial growth factor (VEGF) (61) and can promote angiogenesis (211). Importantly, vIL-6 activates similar signaling pathways to the human cytokine, including the JAK/STAT, MAPK, and PI3K pathways (62, 198, 199).

vIL-6 differs from hIL-6 in several ways: hIL-6 must bind the IL-6 receptor (IL-6R, gp80) before activation of the gp130 signal transducer subunit, whereas vIL-6 can directly bind gp130 to induce signaling (201-203); however, involvement of gp80 can enhance vIL-6 signaling (205). Another difference is that hIL-6 is rapidly secreted from cells whereas vIL-6 is primarily retained within the endoplasmic reticulum (ER) (206, 207). In this compartment, vIL-6 binds gp130 in a tetrameric complex to induce intracellular signaling (207). The cellular ER protein, calnexin, has been shown to interact with vIL-6 to stabilize vIL-6 folding and maintain its intracellular distribution (142). The ER transmembrane protein vitamin K epoxide reductase complex subunit 1 variant 2 (VKORC1v2) was recently identified as an additional intracellular binding partner of vIL-6 (292, 293). vIL-6 binds to VKORC1v2's C terminus which is present in the ER lumen, but data suggest that this binding domain is not responsible for retention of vIL-6 in the ER. Overexpression of VKORC1v2's vIL-6 binding domain or depletion of VKORC1v2

abrogates vIL-6's pro-growth phenotype in PEL cells independently of gp130 signaling (292). Furthermore, it was found that vIL-6 promotes PEL cell survival by suppressing the pro-apoptotic properties of the VKORC1v2 binding partner, cathepsin D (294). This suggests that VKORC1v2 uses a mechanism independent of gp130 signaling to promote vIL-6 function and PEL cell survival.

We performed affinity purification and mass spectrometry to identify novel binding partners of intracellular vIL-6. We found that a protein called hypoxia upregulated protein 1 (HYOU1; also called oxygen regulated protein 150 or ORP150) is able to bind vIL-6. HYOU1 is an ER resident chaperone protein that is a member of the heat shock and ER stress protein families (295). HYOU1 is expressed in many different cell types and can be upregulated by various cellular conditions including hypoxia and ER stress (295, 296). Furthermore, HYOU1 is upregulated in some human cancers including head and neck and breast cancer (297, 298). The HYOU1 transcript was originally cloned from astrocytes under hypoxic conditions (299), which makes it a relevant protein in KSHV biology since hypoxia plays a role in the KSHV lifecycle (300). Previous work has indicated that HYOU1 can suppress hypoxia-induced cell death (301) and induce angiogenesis by facilitating VEGF processing (302).

We found that HYOU1's interaction with vIL-6 is important for vIL-6-induced intracellular STAT3 signaling and vIL-6 expression in PEL cells. Furthermore, we show that HYOU1 is required for several vIL-6 biological functions including promotion of endothelial cell survival and migration. We found that vIL-6 increases extracellular levels of chemokine (C-C motif) ligand 2 (CCL2, also called monocyte

chemoattractant protein 1 or MCP1) in a HYOU1-dependent manner. CCL2 is implicated in the migration and metastasis of tumor cells and the extravasation of immune cells (303, 304). Finally, we investigated the impact of HYOU1 on cellular human IL-6 (hIL-6) signaling. Our results suggest that by modulating vIL-6 function HYOU1 may contribute to KSHV-associated tumorigenesis, making HYOU1 an attractive target for the treatment of KSHV-associated malignancies.

METHODS

Cell Culture and Generation of Stable Cell Lines

Human embryonic kidney (HEK) 293 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Corning). BCBL1 PEL cells were cultured in RPMI 1640 medium (Corning) containing 0.05 mM β-mercaptoethanol. TREx BCBL1 and TREx BCBL1-RTA PEL cells (305) were cultured in RPMI 1640 medium (Corning) containing Tet System Approved FBS (Clontech) and 20 μg/mL hygromycin B (Roche). hTERT-immortalized human umbilical vein endothelial cells (hTERT-HUVEC) were cultured in EBM-2 (Lonza) with the EBM-2 bullet supplement (Lonza) as described (178). All media were additionally supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin (PS), and 1% L-glutamine. Charcoal filtered FBS was obtained from Life Technologies. Cells were transfected with XtremeGene HP transfection reagent (Roche) at a ratio of 2 μL XtremeGene: 1μg plasmid DNA as per the manufacturer's protocol. Cells were transfected with 50-100 nM siRNA utilizing Lipofectamine RNAimax (Invitrogen) as per the manufacturer's protocol. For lentiviral transductions, adherent cells were

grown to 70% confluence and inoculated with lentivirus in the presence of 8 μg/mL polybrene. Spinoculation was used for PEL cell transductions as previously described (223). All transfections and transductions were incubated for 48-72 hours to allow for protein expression or knockdown. hTERT-HUVEC cells and HEK293 cells stably expressing empty vector or vIL-6 were generated by lentiviral transduction. HEK293 cells stably expressing a non-targeting shRNA or a HYOU1 targeting shRNA plasmid (described below) were also generated by lentiviral transduction. For all stable cells, media were changed 24 hours post-transduction and the puromycin concentration was increased from 0.1 μg/mL to a final concentration of 0.5 μg/mLfor hTERT-HUVEC and 1.0 μg/mL for HEK293 cells over 2 weeks.

Plasmids, Lentiviral vectors, shRNAs, and siRNAs

The pcDNA3.1-vIL-6-His clone was a kind gift from Yuan Chang and Patrick Moore. A C-terminal FLAG tag was added to vIL-6 and cloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen). pSG5-based eukaryotic expression vectors for untagged hIL-6 and hIL-6 with an ER-targeting motif containing KDEL and additional sequences (306) were a kind gift from John Nicholas and were previously described (207). The non-targeting control (NTC) siRNA duplex was purchased from Dharmacon (D001810-01) and the HYOU1-targeting siRNA duplex was designed and purchased from Invitrogen (NM_001130991_stealth_455) utilizing the Block-iT RNAi Designer as previously described (307). Plasmids for the pLKO.1 non-targeting control (NTC) shRNA and a HYOU1-targeting shRNA (TRCN0000029220) were purchased from Sigma and used to generate lentivirus.

FLAG-tagged vIL-6 was cloned into the lentiviral vector pSuper-CMV puro (Invitrogen). All lentiviruses were produced using the ViraPower Lentiviral Expression System (Invitrogen) as per the manufacturer's instructions.

Mass Spectrometry, Immunoprecipitations, and Western Blots

Twenty million 293T cells were transfected with pcDNA3 or vIL-6 expression vectors for 48 hours. Cells were harvested on ice in NP-40 lysis buffer (0.1% NP-40, 150 mM NaCl, 50 mM Tris HCl pH 8.0, 30 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 Roche protease inhibitor tablet per 50 mL) followed by one freeze-thaw cycle. Samples were clarified by centrifugation at 16 000 x *g* for 10 minutes and protein content was determined by Bradford assay (Bio-Rad). Equal amounts of protein were loaded on FLAG antibody-conjugated beads (EZview Red ANTI-FLAG M2 Affinity Gel; Sigma) and rocked at 4^{o}C overnight. Beads were washed twice with lysis buffer followed by 2 washes with 50 mM NH_4HCO_3. Samples were eluted with 3x FLAG peptide (Sigma) diluted in 50 mM NH_4HCO_3 and 0.1% PPS Silent Surfactant (Protein Discovery). Samples were treated with 5 mM dithiothreitol at 60^{o}C for 15 minutes. Proteins were trypsinized using the FASP protein digestion protocol (Protein Discovery) and tryptic peptides were separated by a nanoAquity UPLC system (Waters Corp.) with a 2-cm trapping column and a self-packed 25-cm analytical column (75-µm ID, Michrom Magic C18 beads of 5.0-µm particle size, 100-Å pore size) at room temperature. The flow rate was 350 nl/min over a gradient of 1% buffer B (0.1% formic acid in acetonitrile) to 35% buffer B in 200 min. Full mass spectrum scan [300 to 2000 mass/charge ratio (*m*/*z*)] was acquired in an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) at 60 000 resolution setting;

data-dependent MS^2 spectra were acquired by collision-induced dissociation with the 15 most intense ions. All raw data were searched with Sorcerer-SEQUEST server (Sage-N Research) against the human UniProtKB/Swiss-Prot sequence database. Search parameters used were a precursor mass between 400 and 4500 atomic mass units (amu), up to 2 missed cleavages, a precursor-ion tolerance of 3 amu, semi-tryptic digestion, a static carbamidomethyl cysteine modification, and variable methionine oxidation. False discovery rates (FDRs) were determined by ProteinProphet, and minimum protein probability cutoffs resulting in a 1% FDR were used.

For HYOU1 immunoprecipitations, cell lysates containing equal amounts of protein were precleared by incubation with Protein A beads and normal rabbit IgG. Beads were pelleted and supernatants were incubated with 8 μL HYOU1 antibody or rabbit IgG for 5 hours prior to the addition of 50 μL Protein A beads overnight. Beads were pelleted and washed twice with lysis buffer and twice with cold TBS. Bound proteins were eluted by boiling in Laemmeli buffer for 5 minutes. For FLAG-vIL-6 immunoprecipitation, HEK293 cells stably expressing empty vector or vIL-6 were transfected with 75 nM NTC or HYOU1-targeting siRNA for 72 hours. Equal amounts of cell lysates were precleared by Protein A beads and normal rabbit IgG then incubated with 50 μL FLAG antibody-conjugated beads overnight. Bound proteins were eluted with 3x FLAG peptide. All immunoprecipitation inputs and eluates were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in 1x TBS/0.1% Tween (TBST) followed by overnight incubation with primary antibody at $4^{o}C$. Blots were then

incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. Blots were developed with PicoWest chemiluminescent reagent (Thermo). Primary antibodies used were Flag (Bethyl, A190-101P), Actin (Santa Cruz, sc-1615), Tubulin (Cell Signaling, 9099), phospho-STAT3 Tyr705 (Cell Signaling, 9131), Total STAT3 (Cell Signaling, 4904), HYOU1 (Abcam, ab134944), human IL-6 (Abcam, ab32530), gp130 (Santa Cruz, sc-655), LANA (Advanced Biotechnologies (13-210-100), RTA (a gift from Ren Sun), K8.1 (Advanced Biotechnologies, 13-212-100), and vIL-6 (Advanced Biotechnologies, 13-214-050). Anti-rabbit, anti-mouse, and anti-rat (Cell Signaling) and anti-goat (Santa Cruz) horseradish peroxidase-conjugated secondary antibodies were used. Normal rabbit IgG (Santa Cruz, sc-2027) and Protein A beads (Santa Cruz sc-2003) were used for preclearing immunoprecipitation samples.

Immunohistochemistry

Immunohistochemical staining was carried out as described previously (308). PEL xenograft tumors were obtained from a previous study (308). Prepared sections of confirmed KSHV-positive KS lesions from specimen IDs 30035001 and 30035006 were obtained from the AIDS and Cancer Specimen Resource (ACSR). For HYOU1 staining, slides were deparaffinized and rehydrated then incubated in 3% H_2O_2 in 10% methanol. Antigen retrieval was accomplished by boiling sections in 1 mM ethylenediaminetetraacetic acid (pH 8.0) for 10 minutes. Slides were incubated with blocking buffer (10% normal horse serum, 5% BSA, 0.3% Triton X-100) for 1 hour followed by overnight incubation with HYOU1 antibody (1:100) or blocking buffer as a negative control. Sections were washed and incubated with biotinylated goat anti-

rabbit secondary antibody for 1 hour followed by signal amplification with the Vectastain ABC rabbit IgG kit (Vector labs). Sections were stained with 3, 3'-diaminobenzidine (DAB) peroxide substrate according to the manufacturer's protocol (Vector Labs). For LANA staining, antigen retrieval was accomplished by boiling sections in Retrievagen A pH 6.0 (BD Pharmingen). Slides were incubated in blocking buffer (1.5% rabbit serum, 1% BSA, 0.1% Triton X100, 0.05% Tween-20, 0.1% gelatin from cold water fish skin in 1x PBS) for 1 hour followed by overnight incubation with LANA antibody diluted 1:100 in antibody buffer (1% BSA, 0.1% Triton X-100, 0.1% gelatin from cold water fish skin in 1x PBS) or plain antibody buffer for the negative control. Sections were washed and incubated with biotinylated rabbit anti-rat secondary antibody for 30 minutes followed by signal amplification with the Vectastain ABC rat IgG kit. Slides were stained with NovaRed substrate according to the manufacturer's protocol (Vector Labs). All slides were counterstained with hematoxylin . Slides were imaged using a Leica DM LA histology microscope with 20x/0.25 numeric aperture (NA) and 40×/0.75 NA objectives.

Endothelial Cell Assays

Scratch assay: Stable empty vector- or vIL-6-expressing hTERT-HUVEC were plated at $1.5x10^5$ cells/well of a 6 well dish. The following day, cells were transduced with lentivirus expressing NTC or HYOU1-targeting shRNA as described. Media were changed after 24 hours, and 48 hours post-transduction cells were serum-starved overnight with plain EBM-2. The monolayer was scratched with a P10 pipet tip, and a Nikon Eclipse Ti inverted microscope was used to obtain brightfield

images of the cells at 40x or 100x magnification at the indicated timepoints. ImageJ software (NIH) was used to quantify the area of each scratch over time.

CCL2 ELISA: Stable empty vector- or vIL-6-expressing hTERT-HUVEC were plated at $1.2x10^5$ cells/well of a 6 well dish. The following day, cells were transfected with 100 nM NTC or HYOU1-targeting siRNA as described. Seventy-two hours post-transfection, media were replaced with serum-free EBM-2 without supplements for 48 hours. Following harvest, the media were cleared of debris by centrifugation and a CCL2 ELISA was carried out as per the manufacturer's protocol (Life Technologies). Samples were all run in triplicate. Absorbance was read at 450 nm on a VersaMax tunable microplate reader (Molecular Devices) and a standard curve was generated using a best fit power trendline in Microsoft Excel. Error bars represent the standard deviation and CCL2 concentrations were compared using a two-tailed Student's t test with $p < 0.05$ considered significant. Results are representative of at least 3 experiments.

Survival Assay: Stable empty vector- or vIL-6-expressing hTERT-HUVEC were plated at $1.2x10^5$ cells/well of a 6 well dish. The following day, cells were transfected with 100 nM NTC or HYOU1-targeting siRNA as described. Seventy-two hours post-transfection, media were replaced with serum-free EBM-2 without supplements and a Nikon Eclipse Ti inverted microscope was used to obtain brightfield images of the cells at 40x or 100x magnification at the indicated timepoints. Media were replaced with fresh serum-free media before each timepoint to remove dead cell debris.

Cytotoxicity Assay: Three thousand cells/well of stable empty vector- or vIL-6-expressing hTERT-HUVEC were plated and transfected in two white walled 96 well plates using RNAimax and 100 nM NTC or HYOU1-targeting siRNA according to the manufacturer's protocol for reverse transfection (Invitrogen). Samples were transfected in triplicate. Three days post-transfection, one plate was used to measure baseline cytotoxicity at day 0 using the CytoTox Glo kit (Promega) as per the manufacturer's instructions with a FLUOstar Optima luminometer (BMG Labtech). The media of the second plate was replaced with serum-free EBM-2 without supplements for 6 days followed by a second cytotoxicity assay. The percentage of dead cells at each timepoint was calculated from the raw data. Data is shown as a fold change in cell death which was calculated by dividing the percentage of dead cells at day 6 by the percentage at day 0. Error bars represent the standard deviation of the mean, and a one way ANOVA with Tukey's post-hoc test was used to compare all samples with a p value < 0.05 considered significant.

RESULTS

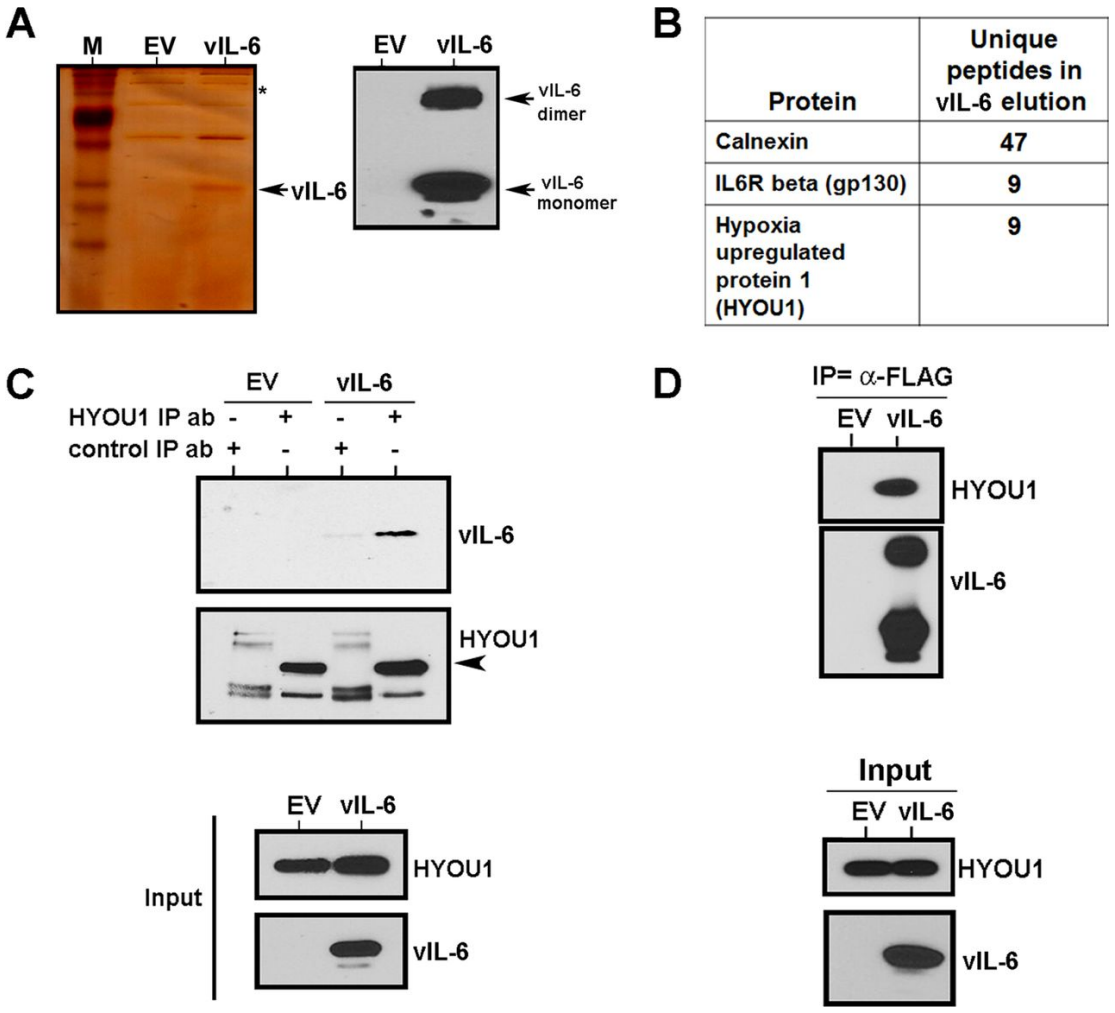
Identification of HYOU1 as a vIL-6 Binding Partner

The biological effects of extracellular vIL-6 have been extensively studied (61, 62, 195, 198-200) but less is known about vIL-6's interactions with intracellular proteins. We sought to identify additional cellular proteins that bind to vIL-6 and impact its function. We cloned vIL-6 with an N-terminal FLAG tag into the pcDNA3.1 vector (pcDNA3.1-vIL-6), and transfected pcDNA3.1 empty vector or pcDNA3.1-vIL-6 into HEK293T cells. Cells were harvested and subjected to an immunoprecipitation

with FLAG antibody-conjugated beads. vIL-6-bound proteins were eluted with 3x FLAG peptide. A fraction of the elution was subjected to SDS-PAGE and subsequent silver staining or Western blotting for vIL-6 to ensure successful vIL-6 expression and pull down (Figure 2.1 A). The vIL-6 concentration in the lysate was high enough that dimerization of the vIL-6 protein was evident by Western blot (Figure 2.1 A, right panel). The remainder of the eluted samples were digested with trypsin and analyzed by shotgun mass spectrometry (309). Proteins with peptide counts that were higher in the vIL-6-FLAG sample than the empty vector sample were identified as potential vIL-6 binding partners (Figure 2.1 B). Consistent with previous findings (142), over 40 unique calnexin peptides and 9 unique gp130 peptides were identified in the vIL-6 sample. The vIL-6 sample also had 9 unique peptides identified for the protein hypoxia upregulated protein 1 (HYOU1; ORP150) while no HYOU1 peptides were identified in the empty vector control.

To confirm that HYOU1 interacts with vIL-6, we again transfected HE293T cells with empty vector or FLAG-tagged vIL-6 constructs. Lysates from these cells were immunoprecipitated with HYOU1 antibody or rabbit IgG as a control, and bound proteins were eluted by boiling in Laemmli buffer. Eluates and input samples were resolved on an SDS-PAGE gel and analyzed by Western blotting. As seen in Figure 2.1 C, vIL-6 coimmunoprecipitated with HYOU1. To determine if vIL-6 interacts with HYOU1 in a more relevant cell type, BCBL1 PEL cells were transduced with lentivirus expressing empty vector or FLAG-tagged vIL-6 and lysates were harvested 48 hours later. The FLAG-tagged vIL-6 was immunoprecipitated by FLAG antibody-conjugated beads and bound proteins were

eluted with FLAG peptide. Our data again showed that HYOU1 coimmunoprecipitated with vIL-6, suggesting that there is a bona fide interaction between HYOU1 and vIL-6 in multiple cell types.



Protein	Unique peptides in vIL-6 elution
Calnexin	47
IL6R beta (gp130)	9
Hypoxia upregulated protein 1 (HYOU1)	9

Figure 2.1: vIL-6 binds the ER chaperone protein hypoxia upregulated protein 1 (HYOU1). (A) HEK293T cells were transfected with empty vector (EV) or FLAG-tagged vIL-6 and lysates were harvested for a FLAG bead immunoprecipitation. Portions of the bead eluates were analyzed by SDS-PAGE and subsequent silver staining (left panel; M is the size marker) or Western blot for vIL-6 (right panel). An asterisk denotes the expected location of HYOU1. (B) HYOU1, Calnexin, and gp130 were identified as binding partners of

vIL-6 by mass spectrometry. (C) HEK293T cells were transfected with empty vector or vIL-6 constructs and lysates were harvested and immunoprecipitated with HYOU1 or rabbit IgG control antibody. Bound proteins were eluted with Laemmli buffer. Samples were analyzed by SDS-PAGE and Western blotting for the indicated proteins. (D) BCBL1 PEL cells were transduced with lentivirus expressing empty vector or FLAG-tagged vIL-6. Lysates were immunoprecipitated with FLAG beads and eluates and inputs were analyzed by SDS-PAGE and Western blotting for the indicated proteins.

To determine if HYOU1 is expressed in tumors associated with KSHV infection, sections of BC-1 PEL tumors grown in immunodeficient mice (308) and human KS sections were stained for HYOU1 using DAB peroxide substrate. Matched tumor sections were also stained for LANA to confirm KSHV infection. HYOU1 and LANA staining were observed in both PEL xenografts and in KS lesions (Figures 2.2 A and 2.2 B). In the PEL xenografts in particular, HYOU1 displayed distinct perinuclear staining which is consistent with its ER localization (Figure 2.2A).

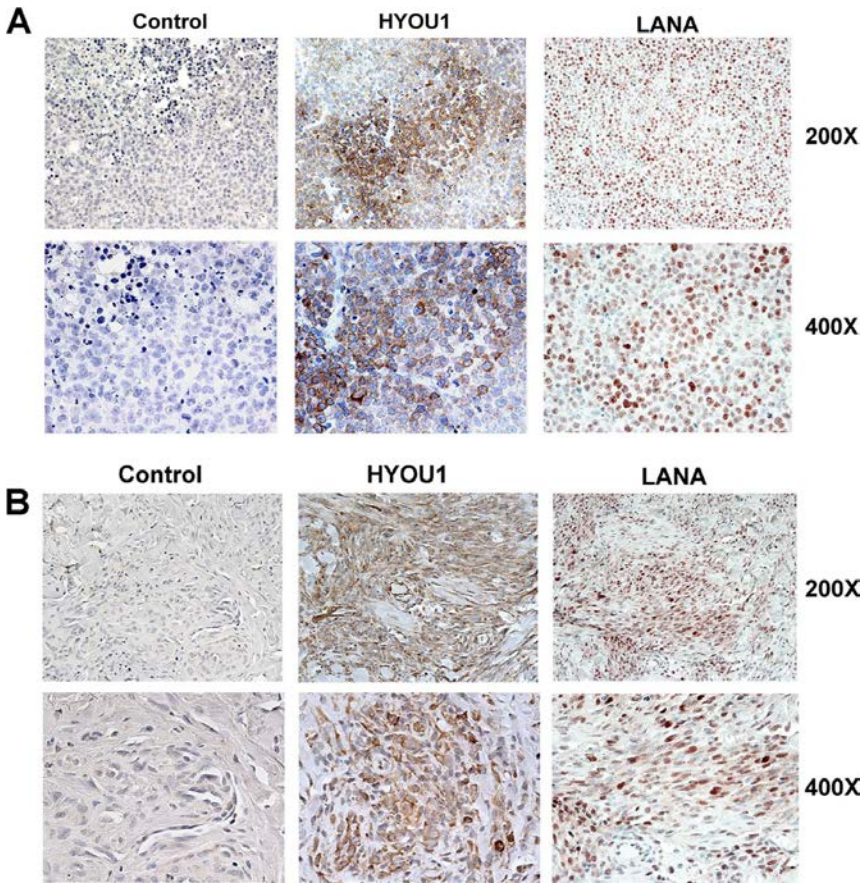


Figure 2.2: HYOU1 is expressed in tissue from KSHV-associated tumors. (A) Immunohistochemical staining for HYOU1 and LANA on BC-1 PEL xenograft sections. The control received no primary antibody. (B) Immunohistochemical staining for HYOU1 and LANA on sections of human KS lesions. The control received no primary antibody.

HYOU1 Increases vIL-6 Levels

HYOU1 is an ER chaperone protein (295) so we sought to determine if its interaction with vIL-6 influences endogenous vIL-6 protein levels. BCBL1 PEL cells were transduced with lentivirus expressing a non-targeting control (NTC) shRNA or a HYOU1-targeting shRNA. Three days post-transduction, cells were treated with

DMSO to maintain latency or 25 ng/mL 12-O-Tetradecanoylphorbol 13-acetate (TPA) to induce lytic reactivation for 24 hours. Cell lysates and media were harvested and analyzed by SDS-PAGE and Western blot. High levels of HYOU1 knockdown were achieved with the HYOU1-targeting shRNA, and as expected, vIL-6 levels were higher overall in lytic samples since vIL-6 is induced during lytic reactivation (144) (Figure 2.3 A). We found that HYOU1 knockdown decreased endogenous vIL-6 protein levels in both lytic and latent BCBL1 cells (Figure 2.3 A). Additionally, HYOU1 knockdown decreased levels of secreted vIL-6 during reactivation. While this is likely due to the increased levels of intracellular vIL-6 when HYOU1 is expressed, we cannot rule out the possibility that HYOU1 may also promote the secretion of vIL-6.

We also performed this experiment using TREx BCBL1 and TREx BCBL1-RTA PEL lines that express doxycycline-inducible empty vector and doxycycline-inducible RTA, respectively (305). The two TREx BCBL1 cell lines were transduced with lentivirus expressing a NTC or a HYOU1-targeting shRNA. After 72 hours, cells received 1 μg/mL doxycycline for 24 hours to maintain latency in the TREx BCBL1 cells and induce lytic replication in the TREx BCBL1-RTA cells. Cell lysate and media were harvested and analyzed by SDS-PAGE and Western blotting for the indicated proteins (Figure 2.3 B). Similar to the results seen in BCBL1 PEL cells, knockdown of HYOU1 in both latent TREx BCBL1 and lytic TREx BCBL1-RTA cells reduced levels of intracellular and extracellular vIL-6. Conversely, levels of LANA did not significantly change with HYOU1 knockdown in both the latent and lytic cells. We

also examined the levels of the lytic proteins RTA and K8.1 in the reactivated cells, and found that HYOU1 knockdown did not impact the levels of these lytic proteins.

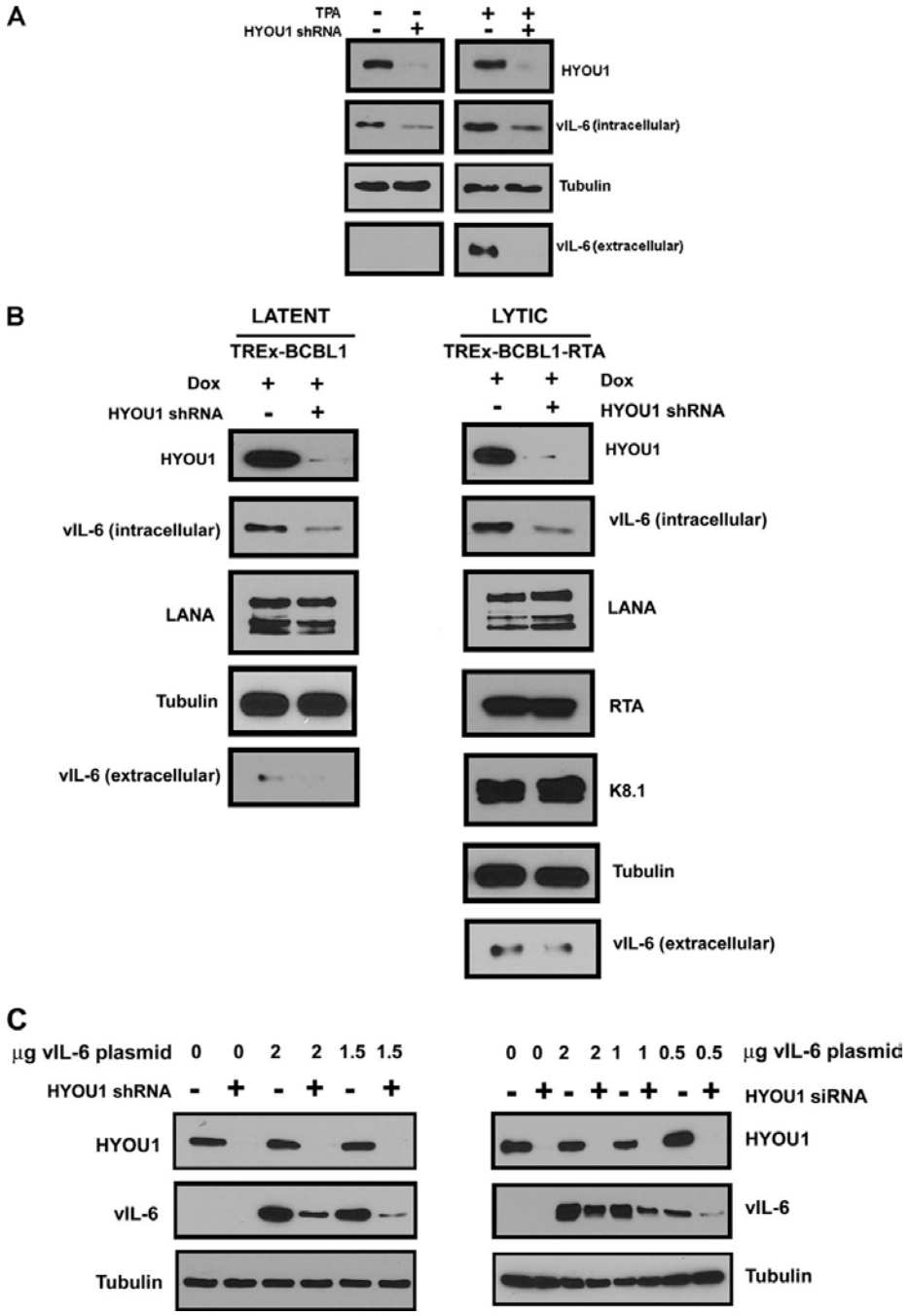


Figure 2.3: HYOU1 increases endogenous vIL-6 levels. (A) BCBL1 PEL cells were transduced with lentivirus expressing a non-targeting control (NTC) or HYOU1-targeting

shRNA. Three days later, cells were treated with DMSO or 25 ng/mL TPA to induce reactivation for 24 hours. Lysates and media were collected and analyzed by SDS-PAGE and Western blot for the indicated proteins. (B) TREx BCBL1 and TREx BCBL1-RTA PEL cells expressing doxycycline-inducible empty vector or RTA, respectively, were transduced with lentivirus expressing a NTC or HYOU1-targeting shRNA. Three days post-transduction, 1 μg/mL doxycycline was added for 24 hours. Lysates and media were harvested and analyzed by SDS-PAGE and Western blot for the indicated proteins. (C) HEK293 stably knocked down for HYOU1 (left panel) or HEK293 first transfected with NTC or HYOU1-targeting siRNA (right panel) were then transfected with a titration of FLAG-tagged vIL-6 plasmid for 48 hours. Lysates were harvested and analyzed by SDS-PAGE and Western blot for the indicated proteins.

To further investigate if HYOU1 knockdown impacts levels of vIL-6, we used HEK293 cells stably expressing a NTC or a HYOU1-targeting shRNA. These cells were then transfected with a titration of FLAG-tagged vIL-6 plasmid for 48 hours. We also performed a similar experiment by transiently transfecting HEK293 cells with NTC or HYOU1-targeting siRNA for 24 hours followed by transfection with a titration of FLAG-tagged vIL-6 plasmid for 48 hours. Lysates from both of these experiments were harvested and analyzed by Western blot (Figure 2.3 C). In both of these experiments, knockdown of HYOU1 prior to expression of FLAG-tagged vIL-6 resulted in a reduction in vIL-6 expression, as compared to cells that expressed endogenous levels of HYOU1. These results closely match the phenotype we see in PEL cells endogenously expressing vIL-6 (Figure 2.3 A and 2.3 B). Interestingly, we find that when FLAG-tagged vIL-6 is expressed prior to knockdown of HYOU1, vIL-6

levels do not appear to be significantly changed by knockdown of HYOU1 (Figures 2.4, 2.5 C, 2.6 B and 2.7 B). For subsequent experiments, we chose to overexpress FLAG-tagged vIL-6 before knocking down HYOU1 to keep the amount of vIL-6 the same and eliminate differences in vIL-6 levels as a variable contributing to the observed results. This approach allowed us to determine if HYOU1 actually affects vIL-6 function as opposed to simply affecting vIL-6 protein levels as a mechanism of action.

HYOU1 Facilitates vIL-6-Dependent STAT Signaling

Following activation of gp130, STAT3 becomes phosphorylated at Y705. This causes STAT3 to dimerize and relocate to the nucleus where it upregulates IL-6-responsive proinflammatory genes. Others have shown that vIL-6 induces STAT3 Y705 phosphorylation through activation of gp130 (142, 199). To determine if HYOU1's interaction with vIL-6 is involved in the induction of this signaling cascade, HEK293 cells were transfected with empty vector or FLAG-tagged vIL-6 plasmid followed by transfection of a NTC or HYOU1-targeting siRNA 24 hours later. Twenty-four hours after the siRNA transfection, media were replaced with serum-free media for another 24 hours. Lysates were harvested and analyzed by SDS-PAGE and Western blotting. Compared to cells expressing empty vector, vIL-6 increased STAT3 Y705 phosphorylation in the presence of HYOU1; however, knockdown of HYOU1 diminished this phosphorylation event (Figure 2.4 A). We generated hTERT-HUVEC (178) stably expressing empty vector or FLAG-tagged vIL-6 and transfected these cells with NTC or HYOU1-targeting siRNA for 3 days. These cells were treated with plain EBM-2 media containing 2% charcoal-filtered FBS for 24 hours prior to

harvest to eliminate background levels of STAT3 phosphorylation. Lysates from the hTERT-HUVEC showed a similar trend to HEK293 cells for STAT3 phosphorylation (Figure 2.4 B). This suggests that HYOU1 is involved in enhancing vIL-6-induced activation of the JAK/STAT signaling pathway in multiple cell types.

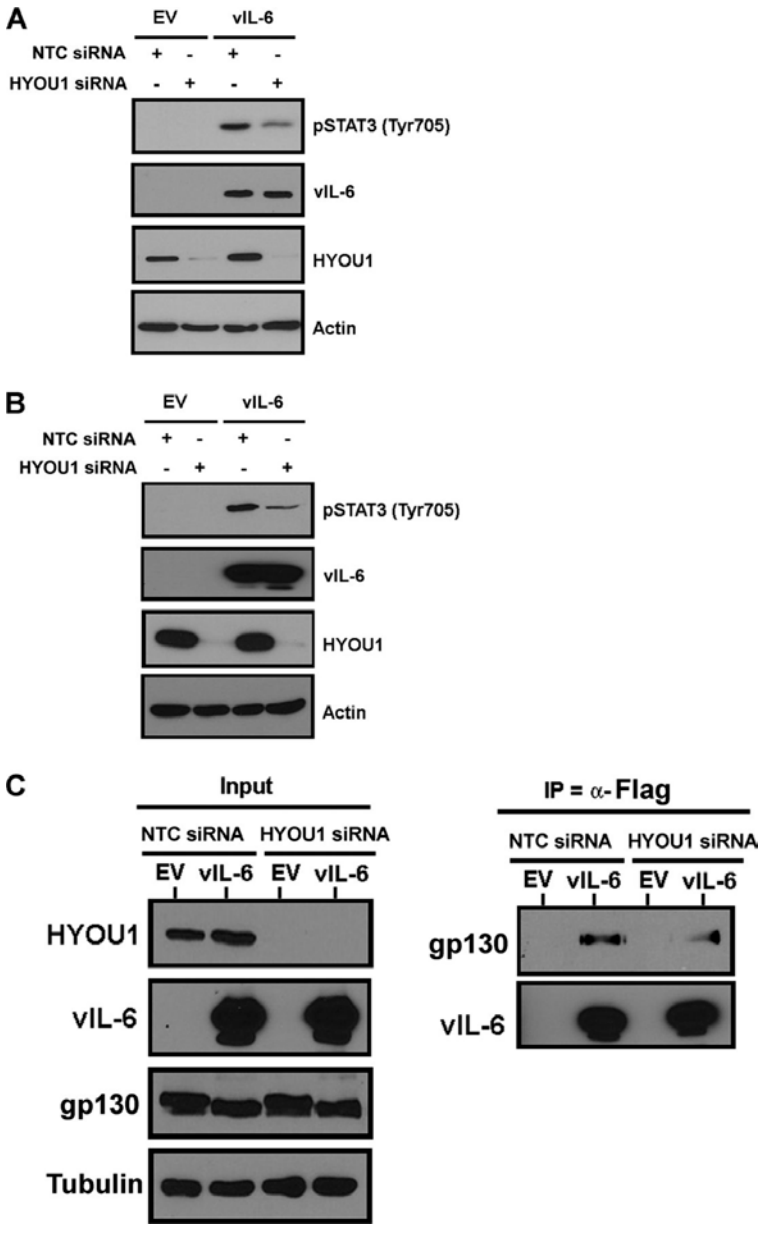


Figure 4: HYOU1 enhances vIL-6-induced STAT3 signaling. (A) HEK293 cells were transfected with empty vector (EV) or FLAG-tagged vIL-6 plasmids followed by a

transfection with non-targeting control (NTC) or HYOU1-targeting siRNA 24 hours later. Twenty-four hours post-siRNA transfection, cells were serum starved for another 24 hours. Lysates were harvested and analyzed by SDS-PAGE and Western blotting for the indicated proteins. (B) hTERT-HUVEC stably expressing EV or FLAG-tagged vIL-6 were transfected with NTC or HYOU1-targeting siRNA for 3 days. Media were replaced with plain EBM-2 with 2% charcoal filtered FBS for 24 hours. Lysates were harvested and analyzed by SDS-PAGE and Western blotting. (C) HEK293 stably expressing EV or FLAG-tagged vIL-6 were transfected with 75 nM NTC or HYOU1 siRNA for 3 days. Lysates were harvested and subjected to a FLAG immunoprecipitation overnight. Bound proteins were eluted with 3x FLAG peptide and eluates and input samples were analyzed by SDS-PAGE and Western blotting for the indicated proteins.

Since vIL-6 can bind ER-embedded gp130 to initiate JAK/STAT signaling (207), we hypothesized that HYOU1 may facilitate vIL-6-induced STAT3 phosphorylation by promoting vIL-6's interaction with gp130. To test this, we used HEK293 cells stably expressing an empty vector or FLAG-tagged vIL-6. Cells were transfected with 75 nM of a NTC or HYOU1-targeting siRNA for 72 hours. Lysates were harvested and subjected to an immunoprecipitation with FLAG beads to pull down vIL-6. Bound proteins were eluted with FLAG peptide and eluates and input samples were analyzed by SDS-PAGE and Western blotting. We found that gp130 coimmunoprecipitated with vIL-6 as expected, but knockdown of HYOU1 consistently reduced this interaction (Figure 2.4 C). This suggests that HYOU1 promotes the vIL-6-gp130 interaction, so this may be one mechanism by which HYOU1 facilitates vIL-6-mediated STAT signaling.

HYOU1 is Essential for vIL-6-Induced Endothelial Cell Migration

To further understand the impact of HYOU1 on vIL-6 activity, we developed biological assays for vIL-6 function in endothelial cells. KS is a cancer of endothelial cell origin, and vIL-6 can be detected in patients with this malignancy (194). Therefore, endothelial cells are a relevant model to investigate vIL-6 function. We first investigated if vIL-6 can influence endothelial cell migration in a scratch assay. Equivalent numbers of hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were plated as a confluent monolayer and serum starved overnight before scratching the monolayer with a P10 pipet tip. The scratch was monitored at various timepoints utilizing brightfield microscopy on a Nikon Eclipse Ti inverted microscope. Cells expressing vIL-6 were able to close the scratch faster than cells expressing empty vector (Figure 2.5 A). The influence of vIL-6 expression on endothelial cell migration has not been previously studied, so this data represents a novel biological function for vIL-6.

We extended this assay to study the effect of HYOU1 on vIL-6-induced endothelial cell migration. hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were transduced with lentivirus expressing a NTC or HYOU1-targeting shRNA. Two days post-transduction, cells were serum starved overnight prior to scratching the monolayer. Again, when HYOU1 was expressed normally the vIL-6-expressing cells closed the scratch more rapidly than the empty vector-expressing cells (Figure 2.5 B). However, vIL-6-expressing cells knocked down for HYOU1 were unable to close the gap, similar to the empty vector-expressing cells (Figure 2.5 B). The area of each scratch was quantified with ImageJ software (Figure 2.5 C).

Lysates were harvested from these cells and subjected to SDS-PAGE and Western blotting to confirm HYOU1 knockdown and vIL-6 expression (Figure 2.5 D). These data suggest that HYOU1 is critical for vIL-6-induced migration of endothelial cells.

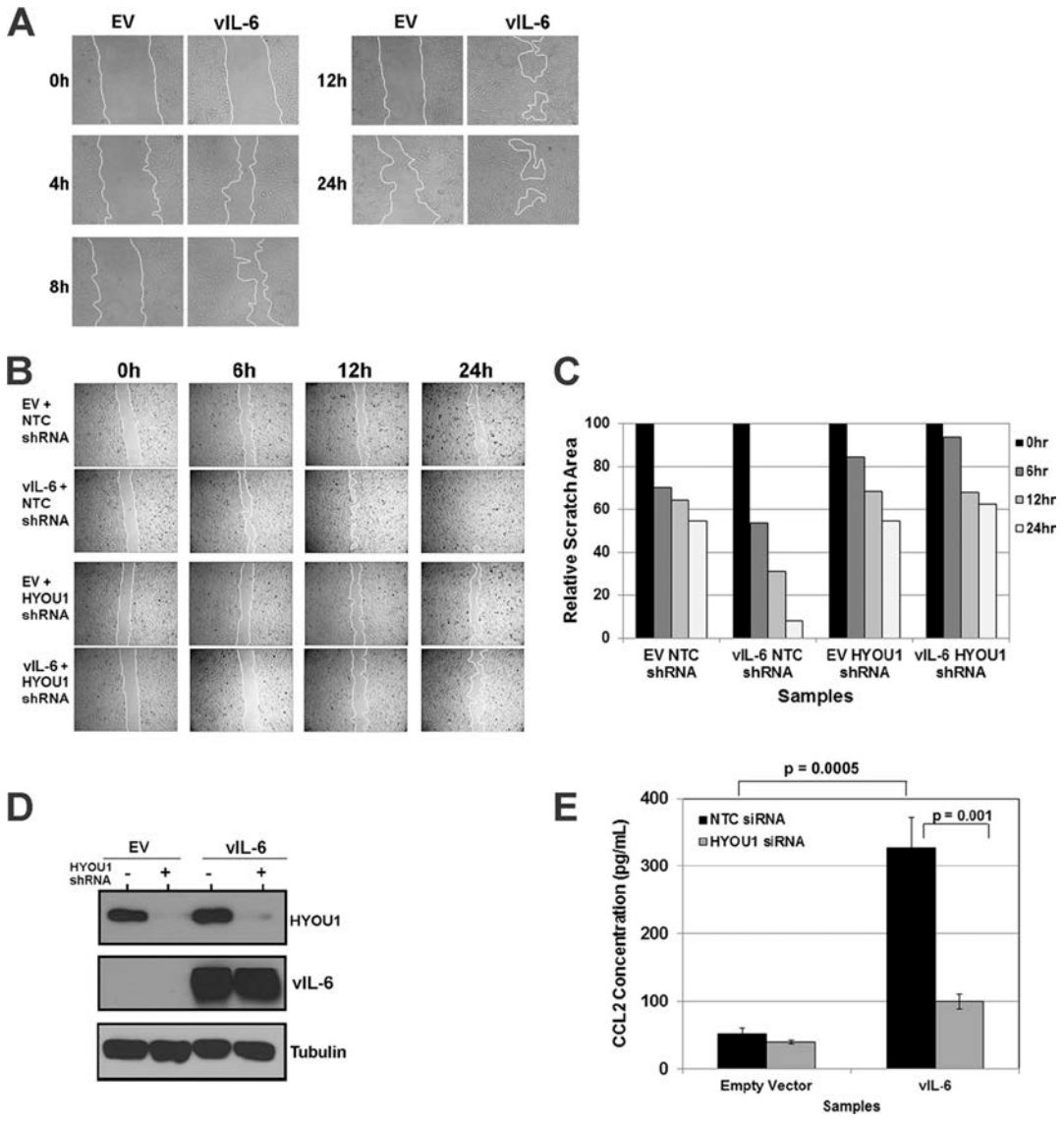


Figure 2.5: HYOU1 facilitates vIL-6-induced migration of endothelial cells. (A) hTERT-HUVEC cells stably expressing empty vector (EV) or FLAG-tagged vIL-6 were serum starved overnight and scratched with a P10 pipet. Scratch closure was monitored at 0, 4, 8,

12, and 24h by brightfield microscopy at 100x magnification using a Nikon Eclipse Ti inverted microscope. (B) hTERT-HUVEC cells stably expressing EV or FLAG-tagged vIL-6 were transduced with lentivirus expressing a non-targeting control (NTC) or HYOU1-targeting shRNA. Cells were serum starved overnight and scratched with a P10 pipet tip and monitored for scratch closure at 0, 6, 12, and 24h at 40x magnification. (C) Quantification of the area of each scratch pictured in figure 5B normalized to the 0hr timepoint. (D) Lysates from the cells described in panel B were harvested at the 24h timepoint and analyzed by SDS-PAGE and Western blot to confirm efficient HYOU1 knockdown and vIL-6 expression. (E) hTERT-HUVEC cells stably expressing EV or FLAG-tagged vIL-6 were transfected with 50 nM NTC or HYOU1-targeting siRNA for 3 days followed by serum starvation for 48 hours. Supernatants were collected and a CCL2 ELISA was performed with each sample tested in triplicate. Absorbance was read at 450 nm and a standard curve was generated using a best fit power trendline in Microsoft Excel. The concentrations of the samples were calculated and error bars represent standard deviation of the triplicates. A two-tailed Student's t test was used to compare the sample concentrations with a p value < 0.05 considered significant. Results are representative of at least 3 experiments.

To elucidate the mechanism by which vIL-6 induces endothelial cell migration, we investigated if vIL-6 affects the levels of chemokine (C-C motif) ligand 2 (CCL2, also called monocyte chemoattractant protein 1 or MCP1) since this chemokine is associated with tumor cell migration and metastasis and increased vascular permeability (303, 304). We transfected hTERT-HUVEC stably expressing an empty vector or FLAG-tagged vIL-6 with 100 nM of NTC or HYOU1-targeting siRNA for 72 hours followed by serum starvation for 48 hours. We performed a CCL2 ELISA on

supernatants from these cells and found that vIL-6 expression increases the level of extracellular CCL2 as compared to the empty vector (Figure 2.5 E). Interestingly, knockdown of HYOU1 in vIL-6 cells caused the CCL2 level to decrease substantially, although the level of CCL2 in this sample was still higher than the level seen in hTERT-HUVEC expressing empty vector (Figure 2.5 E). This suggests that there may be other factors besides CCL2 that are involved in vIL-6-mediated cell migration.

HYOU1 is Required for vIL-6-Mediated Endothelial Cell Survival

We next investigated vIL-6's role in endothelial cell survival in serum starved conditions. hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were transfected with 100 nM of NTC or HYOU1-targeting siRNA. Seventy-two hours post-transfection, cells were serum starved and brightfield images were obtained at the indicated timepoints. vIL-6 expressing cells expressing HYOU1 maintained normal morphology, remained adherent, and survived significantly longer in serum-free conditions than cells expressing empty vector (Figure 2.6 A). HYOU1 knockdown had little impact on survival in cells expressing the empty vector. However, vIL-6-expressing cells with HYOU1 knockdown displayed significantly reduced survival as compared to vIL-6 cells expressing HYOU1 (Figure 2.6 A). Lysates from a duplicate set of samples were harvested for analysis by SDS-PAGE and Western blotting to ensure adequate HYOU1 knockdown and vIL-6 expression (Figure 2.6 B).

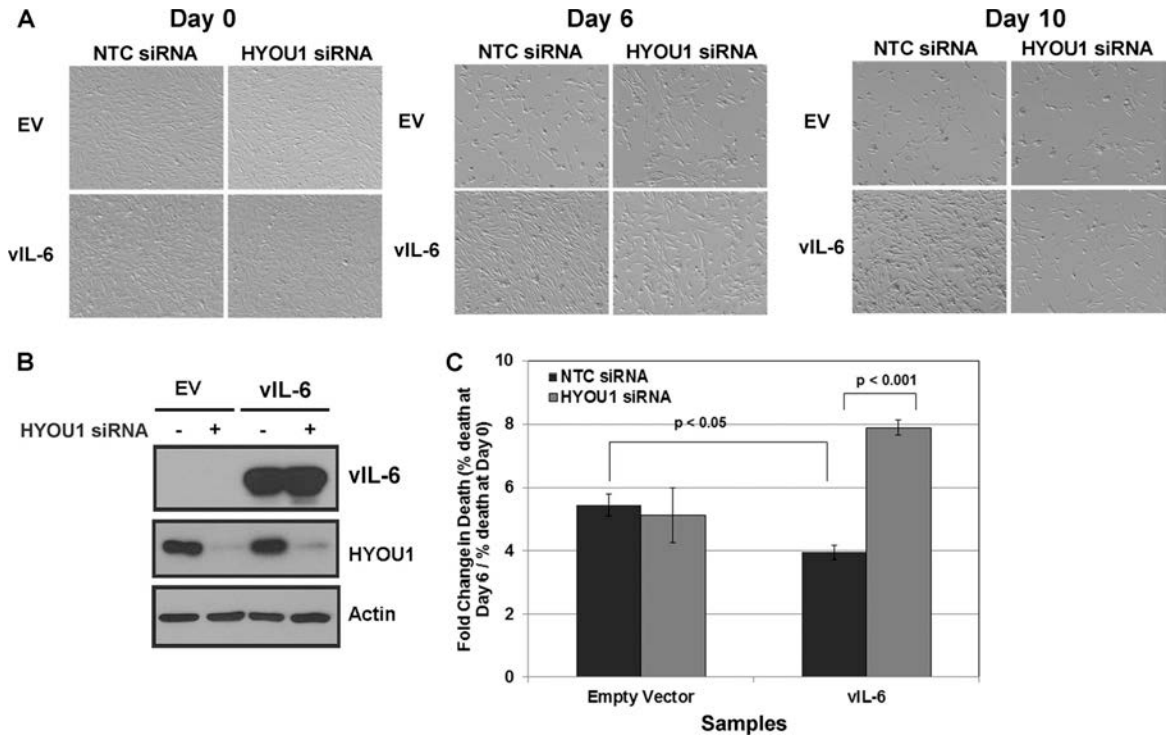


Figure 2.6: HYOU1 is required for vIL-6-induced endothelial cell survival in serum starved conditions. (A) hTERT-HUVEC stably expressing EV or FLAG-tagged vIL-6 were plated as a confluent monolayer and transfected with 100 nM non-targeting control (NTC) or HYOU1-targeting siRNA. Cells were serum starved 72 hours post-transfection and brightfield images at 100x magnification were taken at 0, 6, and 10 days post-starvation using a Nikon Eclipse Ti inverted microscope. Replicate samples were harvested 24 hours after starvation for analysis by SDS-PAGE and Western blot to confirm efficient vIL-6 expression and HYOU1 knockdown (B). (C) hTERT-HUVEC stably expressing EV or FLAG-tagged vIL-6 were reverse-transfected with 100 nM NTC or HYOU1-targeting siRNA in triplicate in two white-walled 96 well plates. Three days post-transfection, a CytoTox Glo (Promega) cytotoxicity assay was performed on the first plate and the percent of dead cells at Day 0 was calculated. The second plate was serum starved for 6 days followed by completion of a cytotoxicity assay used to calculate the percent of dead cells at Day 6. The graph represents a fold change in dead cells calculated by dividing the percentage of dead

cells at Day 6 by the percentage of dead cells at Day 0. Error bars represent standard deviation of the mean and p values were calculated using a one way ANOVA with Tukey's post-hoc test to compare all values to each other, with a p value < 0.05 considered significant. This data is representative of 3 independent experiments.

To quantify the effect of HYOU1 on vIL-6-induced survival, we performed a luminescence-based cytotoxicity assay. hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were reverse-transfected with 100 nM NTC or HYOU1 siRNA in triplicate in two 96-well white-walled plates. Three days post-transfection, the cytotoxicity assay was performed on one plate (Day 0 reading). At this timepoint, the levels of cell death were comparable between all samples (data not shown). The second plate was serum starved for 6 days and another cytotoxicity assay was performed. The percentages of dead cells at day 0 and day 6 were calculated as per the manufacturer's instructions. A fold change in dead cells was calculated by dividing the percentage of dead cells at day 6 by the percentage at day 0. Similar to trends seen in the serum starved survival assay (Figure 2.6 A), empty vector cells receiving NTC siRNA have a statistically significant increase in cell death after 6 days of serum starvation as compared to vIL-6-expressing cells receiving the NTC siRNA (Figure 2.6 C). HYOU1 knockdown had little impact on the survival of cells expressing empty vector. However, knockdown of HYOU1 in vIL-6-expressing cells caused significantly higher levels of cell death after 6 days of serum starvation than vIL-6 cells expressing HYOU1 (Figure 2.6 C). Overall, these data suggest that HYOU1 is critical for vIL-6-mediated survival of serum starved endothelial cells.

HYOU1 Influences the Signaling of Human IL-6

After confirming vIL-6's interaction with HYOU1, we wanted to determine if hIL-6 can also interact with HYOU1. hIL-6 is rapidly secreted (206) so intracellular levels are typically undetectable by Western blot. hIL-6 constructs that were either wild type or tagged with an ER-targeting KDEL motif (207, 306) were transfected into HEK293 cells. Lysates were harvested and subjected to an immunoprecipitation with HYOU1 antibody followed by SDS-PAGE and Western blot analysis. We found that hIL-6-KDEL, but not wild-type hIL-6, coimmunoprecipitated with HYOU1 (Figure 2.7 A). The fact that an immunoprecipitated band for wild type hIL-6 was not visible is not surprising since no wild type hIL-6 was identified in the input lysates, whereas high levels of hIL-6-KDEL were observed in the lysates (Figure 2.7 A). Therefore, HYOU1 can interact with hIL-6 only when this cytokine is retained in the ER.

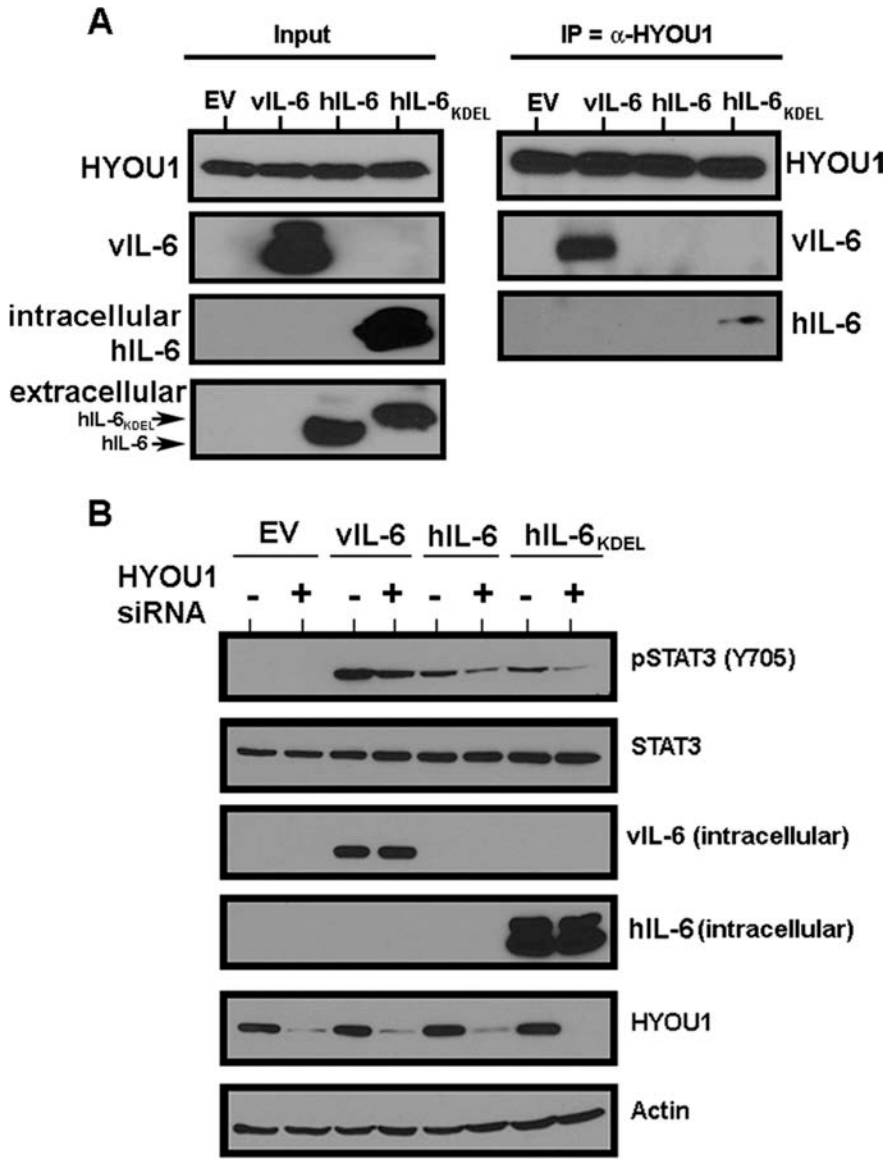


Figure 2.7: HYOU1 interacts with and influences the signaling of hIL-6. (A) HEK293 cells were transfected with empty vector (EV), vIL-6, wild type hIL-6, or hIL-6 with a KDEL motif (hIL-6-KDEL). Lysates were harvested and immunoprecipitated with a HYOU1 antibody. Eluates were subjected to SDS-PAGE and Western blotting for the indicated proteins. Input lysates and media were analyzed for intracellular expression of HYOU1, vIL-6, and hIL-6. (B) HEK293 cells were transfected with empty vector (EV), vIL-6, wild type hIL-6, or hIL-6-KDEL for 24 hours followed by transfection with 100 nM of non-targeting or HYOU1-targeting siRNA for an additional 24 hours. Cells were serum starved overnight and

lysates and media were harvested for analysis by SDS-PAGE and Western blotting for the indicated proteins.

Since we detected an interaction between HYOU1 and hIL-6-KDEL, we investigated if HYOU1 impacts wild type and KDEL-tagged hIL-6 signaling through the JAK/STAT pathway. HEK293 cells were transfected with empty vector, FLAG-tagged vIL-6, wild type hIL-6, or hIL-6-KDEL for 24 hours followed by an additional transfection with either NTC or HYOU1-targeting siRNA. One day later cells were serum starved for 24 hours and lysates were harvested and subjected to SDS-PAGE and Western blotting. Expression of vIL-6 and wild type hIL-6 increased STAT3 Y705 phosphorylation (Figure 2.7 B). hIL-6-KDEL also induced STAT3 phosphorylation, which is likely due to leaky expression of hIL-6-KDEL into the media in our experimental system (Figure 2.7 A). Knockdown of HYOU1 decreased STAT3 phosphorylation mediated by vIL-6, wild type hIL-6, and hIL-6-KDEL (Figure 2.7 B). This suggests that HYOU1 not only impacts vIL-6 signaling events, but may also influence signaling by hIL-6.

DISCUSSION

KSHV expresses several homologs of human cytokines and chemokines, including vIL-6 and multiple viral CC chemokines (vCCLs, previously called vMIPs) (195). vIL-6 shares considerable structural and functional homology to hIL-6 and is detectable in all KSHV-associated malignancies (64, 194, 197). Patients with these

malignancies also have elevated hIL-6 levels (60), and vIL-6 signaling likely promotes the proinflammatory signaling of hIL-6 to exacerbate disease. Inhibition of hIL-6 signaling to treat KSHV-associated lymphomas has been successful (310), so it is plausible that targeting vIL-6 signaling may also be a viable treatment for KSHV-associated malignancies.

Many previous reports on vIL-6 used exogenously applied vIL-6 (61, 62, 198-200). Although this has given valuable insight into the role of this viral cytokine, recent publications suggest that vIL-6 is primarily retained within the host cell in the ER (206, 207). Evidence that supports this intracellular retention include the fact that vIL-6 is secreted at a much slower rate than hIL-6, has glycosylation patterns distinct from those seen on secreted cytokines, and is capable of binding intracellular gp130 to induce signaling (206, 208).

We utilized affinity purification and mass spectrometry to identify cellular vIL-6 binding partners. HYOU1 stood out as a potential hit because it had the same number of unique peptides as were seen for gp130. HYOU1 is an ER chaperone that facilitates protein processing, is involved in the ER stress response, and protects cells from hypoxia-induced cell death (295, 296, 301). KSHV-associated malignancies often persist in hypoxic environments, such as KS lesions on the lower extremities and PEL in oxygen-deprived pleural cavities. Hypoxia is able to induce lytic replication due to hypoxia response elements present in the promoter region of multiple lytic genes (300). Hypoxic conditions in KSHV-infected cells or tumors may create a favorable environment for high HYOU1 expression and function, making HYOU1 a relevant protein to investigate in KSHV pathogenesis.

Based on HYOU1's role as a chaperone protein for other secreted factors (302), we tested if it modulates the level of vIL-6 in the cell. We found that knockdown of HYOU1 reduces endogenous vIL-6 protein levels in latent and lytic PEL cells as well as in HEK293 cells when HYOU1 is knocked down prior to transfection with a vIL-6 expression construct. Interestingly, in cells that exogenously express vIL-6 before knockdown of HYOU1, the effect of HYOU1 on vIL-6 expression is not significant. Based on HYOU1's role as a known processing factor for VEGF (302), we speculate that HYOU1 may be involved in the processing and/or stability, as well as the biological function, of vIL-6.

Our data also show that HYOU1 enhances vIL-6-mediated STAT3 phosphorylation, and it is known that vIL-6 can initiate STAT signaling through ER-associated gp130 (207). We hypothesized that HYOU1's interaction with vIL-6 could place vIL-6 in closer proximity to gp130 as a mechanism to promote signaling. To test this, we performed an immunoprecipitation, and found that HYOU1 expression does appear to increase the ability of vIL-6 to bind gp130. In addition to increasing intracellular vIL-6 levels, promoting the vIL-6-gp130 interaction may be yet another way that HYOU1 is able to positively influence vIL-6 function.

KS lesions are of endothelial cell origin, and exist in a highly inflammatory and vascularized environment (29, 32). vIL-6 expression is detectable in the serum of a proportion of KS patients (194), and vIL-6 has been shown to influence important biological functions of endothelial cells such as differentiation, proliferation, and angiogenesis (211, 311). Our data further show that vIL-6 is able to induce the migration of endothelial cells in a HYOU1-dependent manner. For this assay, we

used serum starved cells to minimize the possibility that proliferating cells could be filling the gap as opposed to migrating cells, since vIL-6 has previously been shown to increase cell proliferation (61, 198, 207, 211). HYOU1 has a known role in facilitating processing of secreted factors including VEGF (302). We therefore hypothesized that HYOU1 could modulate chemokines involved in vIL-6-mediated cell migration. CCL2 is associated with increased migration of tumor cells and immune cells (303, 304). We found that vIL-6 can induce CCL2 expression and that knockdown of HYOU1 substantially reduced this vIL-6-mediated increase in CCL2, but not quite to the level of CCL2 in cells expressing an empty vector. This suggests that HYOU1 may partly influence vIL-6-mediated migration by modulating CCL2 expression, but that other chemokines or factors are likely involved. Overall, our data suggest that HYOU1 may have multiple mechanisms by which it promotes vIL-6 function in signaling, endothelial cell migration, and endothelial cell survival in serum-starved conditions.

Since vIL-6 is a homolog of hIL-6, we tested if HYOU1 can also bind the human cytokine. Because hIL-6 is rapidly secreted, it is present at levels that are undetectable by Western blotting in cell lysates. Therefore, we speculated that it would be difficult to demonstrate an interaction between wild type hIL-6 and HYOU1, since such an interaction would likely be too transient to detect. We circumvented this issue by using a hIL-6 construct with an ER-targeting KDEL motif that increases the intracellular retention of hIL-6. We detected an interaction between HYOU1 and hIL-6-KDEL, although we could not detect an interaction between wild type hIL-6 and HYOU1. However, we found that STAT3 signaling mediated by wild type hIL-6

was diminished when HYOU1 was depleted from cells. Since HYOU1 can impact wild type hIL-6 signaling, we speculate that HYOU1 might transiently interact with hIL-6 when the cytokine is transiting through the ER, despite being unable to see an interaction between HYOU1 and wild type hIL-6 by immunoprecipitation. Alternatively, HYOU1 might have an effect on other components of the hIL-6 signaling pathway to impact signaling. Overall, our data suggest that HYOU1 may play a role in both vIL-6 and hIL-6 signaling function.

During lytic reactivation of KSHV, vIL-6 is highly expressed and some is secreted from infected cells which is likely why patients with MCD and other KSHV-associated malignancies have detectable vIL-6 in their serum (55, 64). Conversely, during latency, vIL-6 is expressed at very low levels that are mainly retained within the cell. Importantly, it has been demonstrated that this low level of latent expression is functional (207). Inhibition of proinflammatory hIL-6 signaling with an anti-IL-6 receptor antibody has been moderately successful for the treatment of MCD (310); however, inhibiting vIL-6 signaling in a similar fashion may be challenging since much of vIL-6 signaling occurs inside the cell beyond reach of an IL-6R-targeting antibody. The identification of HYOU1 as a cellular binding partner that is required for facilitating multiple facets of vIL-6 function and hIL-6 activity presents an alternative druggable target for inhibiting IL-6 function to treat KSHV-associated malignancies.

CHAPTER 3: KSHV VIRAL INTERLEUKIN 6 MODULATES ENDOTHELIAL CELL MOVEMENT BY UPREGULATING CELLULAR GENES INVOLVED IN MIGRATION[4]

OVERVIEW

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. KSHV utilizes a variety of mechanisms to evade host immune responses, promote cellular transformation and growth, and persist for the lifetime of the host. A viral homolog of interleukin 6 called viral interleukin 6 (vIL-6) is expressed by KSHV and KSHV-associated cancers. Unlike host interleukin 6, vIL-6 is retained within the endoplasmic reticulum (ER) where it can initiate functional signaling through part of the interleukin 6 receptor complex. We sought to determine how intracellular vIL-6 modulates the host cell environment by analyzing vIL-6's impact on the endothelial cell transcriptome. vIL-6 significantly altered the expression of many cellular genes associated with cell migration. In particular, vIL-6 upregulated the host factor carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1) at the protein and message level. CEACAM1 has been implicated in tumor invasion and

[4] Louise Giffin, John A. West, and Blossom Damania (*Manuscript submitted.)* BD and LG designed experiments. All experiments performed by LG with assistance from JAW for viral preparations and infections. Manuscript was written by LG and edited by BD. Grants to BD provided funding for all experiments.

metastasis and promotes migration and vascular remodeling in endothelial cells. We report that vIL-6 upregulates CEACAM1 by a STAT3-dependent mechanism, and that CEACAM1 promotes vIL-6-mediated migration. Furthermore, latent and *de novo* KSHV infection of endothelial cells also induces CEACAM1 expression. Collectively, our data suggest that vIL-6 modulates cell migration by upregulating expression of cellular factors including CEACAM1.

INTRODUCTION

Kaposi's sarcoma associated herpesvirus (KSHV, HHV8) is the eighth human herpesvirus identified and is the etiological agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (3, 4, 6). KSHV-associated malignancies typically present in immunosuppressed patients such as HIV-positive individuals, and because of the high AIDS incidence in sub-Saharan Africa, KS has become the most common cancer amongst African men (17, 20). KSHV is a gammaherpesvirus that has a double stranded DNA genome and enveloped virion (40) and is able to transition between a latent phase and an actively replicating lytic phase. The virus expresses over eighty open reading frames (ORFs), many of which inhibit various host immune defenses or promote growth and transformation of host cells. These strategies allow KSHV to persist for the lifetime of the host and induce pathogenesis in immunocompromised individuals.

The KSHV protein expressed by ORF K2 is known as viral interleukin 6 (vIL-6) because of its high sequence and structural similarity to the human cytokine interleukin 6 (hIL-6) (195-197). vIL-6 is expressed at low, but functional, levels during viral latency and becomes highly upregulated during lytic induction (143, 144, 207).

Importantly, vIL-6 can be detected in either serum or tissue of patients with KSHV-associated malignancies, and in MCD, higher vIL-6 levels correlate with poor prognosis (55, 64, 194). vIL-6 expression is transforming in NIH-3T3 cells (61), and a transgenic mouse line expressing vIL-6 from an MHC class I promoter developed a MCD-like disease in a manner dependent on mouse IL-6 (312). vIL-6 drives expression of hIL-6 and VEGF (61, 200), and promotes angiogenesis, migration, survival, and activation of hIL-6-dependent signaling cascades such as the JAK/STAT, MAPK, and PI3K pathways (62, 198, 199, 211, 313).

Despite their structural similarities, vIL-6 differs from hIL-6 in that vIL-6 is secreted from the cell more slowly and accumulates in the endoplasmic reticulum (ER) where it can signal intracellularly through the gp130 subunit of the IL-6 receptor (142, 206). To better understand how vIL-6 interacts with host proteins to function inside the cell, we previously identified a host protein called hypoxia upregulated protein 1 (HYOU1) that plays a critical role in vIL-6-mediated signaling, survival, and migration (313). Two other host proteins, VKORC1v2 and calnexin, have also been identified as vIL-6-interacting partners and these cellular proteins appear to play a role in vIL-6-mediated cell survival and vIL-6 folding and intracellular retention, respectively (142, 292, 294).

We wanted to investigate how intracellular expression of vIL-6 impacts the global transcriptional profile of endothelial cells since these cells can be infected with KSHV *in vivo* and are the cells that drive the development of KS lesions (31, 32). To explore the impact of intracellular vIL-6 on gene expression, we performed microarray analysis on endothelial cells stably expressing vIL-6. We identified some

genes known to be upregulated by vIL-6 (61, 200, 313), and also found a number of genes that were previously not known to be upregulated by vIL-6. In particular, vIL-6 increased expression of a number of genes significantly associated with cell invasion and endothelial cell movement and chemotaxis, including a gene called carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1).

CEACAM1 (also known as CD66a and biliary glycoprotein) is a transmembrane adhesion molecule that is expressed in a number of different cell types, including endothelial, epithelial, and hematopoietic cells (314). Although some earlier studies reported that CEACAM1 expression decreases in colorectal cancer (315), more recent clinical studies and profiling in a wide variety of human tumors, including colon (316), melanoma (317-319), lung (320), pancreas (321), bladder (322, 323), and thyroid (324) cancers found that high levels of CEACAM1 are expressed on tumor cells and that CEACAM1 expression directly correlates with poor prognosis and tumor metastasis. CEACAM1 may also play a role in dampening the immune response to cancer, which may contribute to cancer progression (325). In addition, CEACAM1 has consistently been shown to augment angiogenesis, increase migration of endothelial cells, and induce vascular remodeling (326-330). Interestingly, human cytomegalovirus (hCMV) encodes a viral homolog of CEACAM1 which has also been shown to be important in promoting angiogenesis (331).

We found that expression of vIL-6 and *de novo* and latent KSHV infection upregulate CEACAM1 transcript and protein levels in endothelial cells. We determined that CEACAM1 expression is likely driven by vIL-6-mediated STAT3

signaling. Finally, we found that knockdown of CEACAM1 abrogated vIL-6-mediated migration of endothelial cells, indicating that CEACAM1 may play a critical role in promoting invasion and pathogenesis of KSHV-infected endothelial cells.

METHODS

Cell Culture, Generation of Stable Cell Lines, and KSHV Infection

hTERT-immortalized human umbilical vein endothelial cells (hTERT-HUVEC) and primary HUVEC were grown in EBM-2 (Lonza) with the EBM-2 bullet supplement (Lonza) as described (178). TREx BCBL1 and TREx BCBL1-RTA PEL cells (305) were kindly provided by Dr. Jae Jung's lab and were grown in RPMI 1640 medium (Corning) containing Tet System Approved FBS (Clontech) and 20 μM hygromycin B (Roche). All media were supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin (PS), and 1% L-glutamine. hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were generated by lentiviral transduction as described previously (313). KSHV was produced by reactivating KSHV-Vero cells with 2 mM sodium butyrate (Sigma) and ORF50-expressing baculovirus. Supernatants were clarified and filtered and purified KSHV was isolated by ultracentrifugation on a sucrose cushion, as described previously (223). Stable, latently infected KSHV-HUVEC were made by infecting hTERT-HUVEC with recombinant KSHV as described previously (332).

Plasmids, Lentiviral vectors, and siRNAs

The pcDNA3.1-vIL-6-His clone was a kind gift from Yuan Change and Patrick Moore. A C-terminal FLAG tag was added to vIL-6 and cloned into the pcDNA3.1

eukaryotic expression vector (Life Technologies). The non-targeting control (NTC) siRNA duplex (D001810-01) was purchased from Dharmacon. CEACAM1-targeting Stealth siRNA duplexes were purchased from Life Technologies (HSS101005). FLAG-tagged vIL-6 was cloned into the lentiviral vector pSuper-CMV Puro (Life Technologies) and all lentiviruses were produced using the ViraPower Lentiviral Expression System (Life Technologies) as per the manufacturer's instructions.

Microarray and Ingenuity Pathway Analysis

RNA was harvested from hTERT-HUVEC stably expressing empty vector or vIL-6-FLAG using TRIzol, and checked for quality with an Agilent Bioanalyzer. One µg of RNA was submitted to Mogene for use with the 4x44K Human Microarray (Agilent). Samples were run in duplicate with Cy3 and Cy5 dyes switched for each sample to correct for any fluorescence dye bias. Preliminary analysis was completed by Mogene and fold changes for each gene were reported. Gene names and corresponding fold changes were input into Ingenuity Pathway Analysis and analyzed using a fold change cutoff of 2.0 with "experimentally observed" confidence and a stringent filter for mammalian species specificity. Top diseases and biological functions were analyzed and gene lists and p-values for cell invasion and endothelial cell movement and chemotaxis were exported.

Reagents and Antibodies

The S3I-201 STAT3 inhibitor (Selleck Chem) was dissolved in DMSO (Sigma) and used at a concentration of 60-75 µM for 48-72 hours. Recombinant hIL-6 (Peprotech) was resuspended in distilled deionized water and used at a concentration of 100 or 250 ng/mL. Primary antibodies used were ECS (FLAG -

Bethyl), vIL-6 (Advanced Biotechnologies), Tubulin, CEACAM1, phospho-STAT3 Tyr705, and Total STAT3 (all from Cell Signaling unless otherwise stated). Secondary anti-rabbit and anti-mouse antibodies were purchased from Cell Signaling.

Western Blotting

Cells were washed in cold PBS and harvested in NP-40 lysis buffer (0.1% NP-40, 150 mM NaCl, 50 mM Tris HCl pH 8.0, 30 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 Roche protease inhibitor tablet per 50 mL) for 10-30 minutes on ice. Lysates were frozen and thawed and clarified by centrifugation at 16,000 x *g* for 10 minutes. Protein content was determined by Bradford assay (Bio-Rad) using a CLARIOstar Optima plate reader (BMG Labtech). Equal amounts of protein were resolved on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in 1x TBS/0.1% Tween followed by overnight incubation with primary antibody at 4^{o}C. HRP-conjugated secondary antibodies were incubated for 1 hour at room temperature. Blots were developed with PicoWest chemiluminescent reagent (Thermo).

RNA Isolation and Real Time qPCR

Cells were washed in cold PBS and resuspended in TRIzol (LifeTechnologies) and RNA was harvested as per the manufacturer's instructions. To generate cDNA, 1 µg of RNA was treated with amplification grade DNAse I (Invitrogen) followed by reverse transcription with Superscript III reverse transcriptase (Invitrogen) and oligoDT primers (Invitrogen). Quantitative real-time PCR (qPCR) was performed with an Advanced Biotechnologies 7300 instrument

using cDNA as a template in a SYBR green PCR master mix (Bio-Rad). Primers for Total CEACAM1 (Forward: 5'AATGTTGCAGAGGGGAAGGA 3'; Reverse: 5' TCTGGGTGACGTTCTGGATC 3') , CEACAM1-Long (Forward: 5'CCCACCTAACAAGATGAATGAA 3'; Reverse: 5' TTTCTGTGGCTGTTAGGGATG 3'), vIL-6 (Forward: 5' CGGTTCACTGCTGGTATCTG 3'; Reverse: 5' CAGTATCGTTGATGGCTGGT 3'), and ORF57 (Forward: 5' TGGACATTATGAAGGGCATCCTA 3'; Reverse: 5' CGGGTTCGGACAATTGCT 3') were used with GAPDH as an endogenous control. PCR reactions were run in triplicate.

KSHV Infection and Reactivation

De novo infections were performed by infecting primary HUVEC (Clonetics) with purified KSHV by spinoculation in serum-free media in the presence of 8 μM polybrene (Sigma) at 2500 rpm for 1.5 hours at 30^{o}C in a table top centrifuge as previously described (223). GFP was visualized on a Nikon T*i* Eclipse fluorescent microscope to confirm infection and samples were harvested 30-72 hours post infection. TREx-BCBL1 were reactivated by supplementing media with 1 μg/mL doxycycline for 24-30 hours.

Migration Assay

hTERT-HUVEC expressing empty vector or FLAG-tagged vIL-6 were seeded at 0.9 x 10^5 cells per well of a 6 well dish coated with collagen IV (Corning Biocoat) since collagen IV is a known adhesion substrate of CEACAM1 (324). The following day cells were transfected with 60 nM of an NTC or CEACAM1-targeting siRNA using Lipofectamine RNAimax as per the manufacturer's instructions. Media were

replaced 24 hours post-transfection and at 40 hours post-transfection cells were serum starved for 8 hours. Monolayers were then scratched with a P200 pipette tip and brightfield images were obtained at 0, 15, and 24 hours post-scratch at 40x magnification with a Nikon Eclipse Ti inverted microscope. Following the assay, cells were harvested in lysis buffer and analyzed by SDS-PAGE and Western blotting to test CEACAM1 knockdown efficiency. Scratch closure was quantified with ImageJ software (NIH) (333).

RESULTS

Intracellular vIL-6 Induces Expression of Genes Involved in Cell Migration

To investigate the effect of intracellular vIL-6 expression on the transcriptome of human endothelial cells, an Agilent 4x44K human microarray was performed in duplicate using RNA harvested from hTERT-HUVEC expressing empty vector (EV) or FLAG-tagged vIL-6 (vIL-6F). Ingenuity Pathway Analysis (IPA) software was utilized to identify major disease pathways affected by vIL-6 expression. IPA found that vIL-6 significantly impacted pathways associated with cancer, cellular movement, and cell-cell signaling and interaction (Figure 3.1 A). We further investigated the impact of vIL-6 on genes associated with cellular movement since we previously reported that vIL-6 can promote the migration of endothelial cells (313). We found that there were a significant number of genes associated with endothelial cell movement, endothelial cell chemotaxis, and cell invasion that were altered by vIL-6 expression (Figure 3.1 B). Furthermore, based on how vIL-6 influenced the expression of these migration-associated genes, IPA predicted that

vIL-6 should induce cellular migration, thus confirming our previous findings (313). In addition to the identification of many previously unidentified vIL-6 target genes, we also found some genes known to be upregulated by vIL-6, including the host chemokine CCL2 (313), VEGF (61), and hIL-6 (200) (Figure 3.1 C).

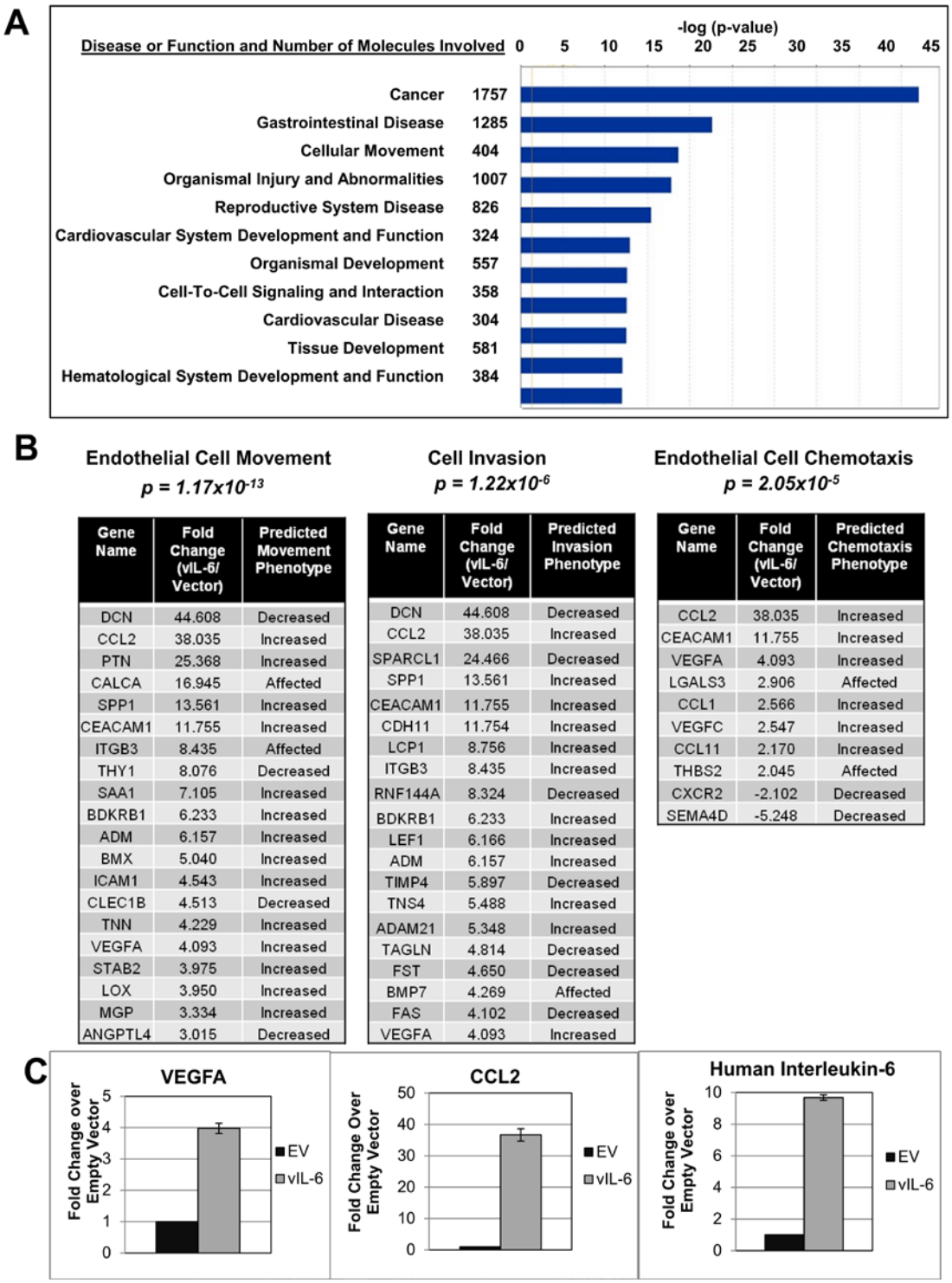


Figure 3.1: vIL-6 modulates expression of host genes associated with cell movement.

(A) RNA was harvested from HUVEC expressing empty vector or FLAG-tagged vIL-6 and used in an Agilent 4x44K human microarray. Gene fold changes were imported into

Ingenuity Pathway Analysis (IPA) and top vIL-6 associated diseases and biological functions were analyzed. (B) Top modulated genes from selected cell movement-associated sub-pathways from IPA. Fold change values represent vIL-6-expressing cells as compared to empty vector cells, and predicted cell movement phenotype represents IPA's prediction of how cells will respond based on the expression pattern of that particular cellular gene and the IPA literature database. p values calculated by IPA represent how significantly vIL-6 expression impacts genes associated with that particular sub-pathway. (C) Microarray data for genes known to be upregulated by vIL-6: VEGF, CCL2, and human IL-6.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a gene associated with cellular movement and invasion that was almost 12-fold upregulated in our vIL-6-expressing hTERT-HUVEC (Figure 3.1 B). We confirmed that expression of FLAG-tagged vIL-6 in hTERT-HUVEC significantly upregulates CEACAM1 mRNA message as compared to empty vector by qPCR (Figure 3.2 A). Lysates from hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 indicate that vIL-6 expression also upregulates CEACAM1 protein in endothelial cells (Figure 3.2 B). CEACAM1 has numerous isoforms that arise from alternative splicing of the transcript (Figure 3.2 C) (314). These isoforms exist in three general categories: secreted CEACAM1 that lacks a transmembrane domain, long isoforms of CEACAM1 (CEACAM1-L) that contains an intracellular immunoreceptor tyrosine inhibitory motif (ITIM) that can modulate downstream signaling, and short isoforms (CEACAM1-S) that lacks this intracellular signaling domain on the cytoplasmic tail. The ratios of each isoform can also differ between cell types, and dictate the outcome of CEACAM1 expression (334, 335). Higher amounts of CEACAM1-L in

cancer correlate with metastatic spread and shorter patient survival (336, 337). We speculated that the two species of CEACAM1 visible by Western blot represent the long and short isoforms of this protein, but to confirm that the long isoforms are expressed we performed additional qPCR experiments with primers specific to the nucleotides encoding the ITIM region of CECAM1-L. We found that vIL-6 induces not only total CEACAM1 message, but also CEACAM1-L (Figure 3.2 D).

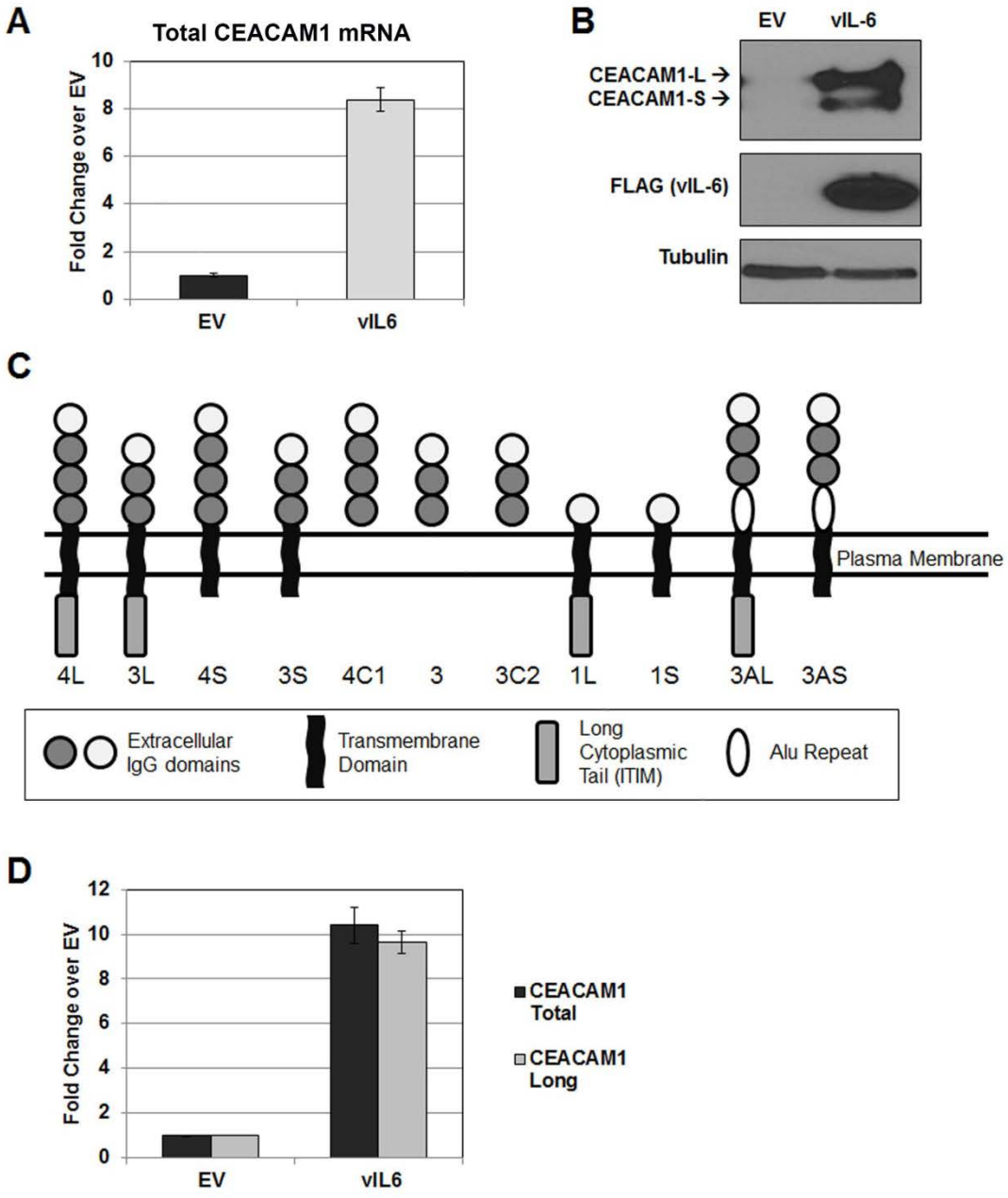


Figure 3.2: vIL-6 upregulates long isoforms of CEACAM1 in endothelial cells. (A) cDNA was generated from HUVEC stably expressing empty vector or FLAG-tagged vIL-6 and used for qPCR analysis of total CEACAM1 message. (B) Lysates were harvested from HUVEC stably expressing empty vector or FLAG-tagged vIL-6. SDS-PAGE and Western blotting was performed for the indicated proteins. (C) CEACAM1 exists in a number of transmembrane and secreted isoforms that differ in the number and type of extracellular Ig

domains, and presence or absence of a transmembrane domain, a long cytoplasmic tail, and an Alu repeat. (D) cDNA was generated from HUVEC stably expressing empty vector or FLAG-tagged vIL-6 and used for qPCR analysis of total CEACAM1 message and CEACAM1-L message.

vIL-6 Upregulates CEACAM1 Through Activation of STAT3

vIL-6 is a potent activator of STAT3 (142, 199, 205, 313), so we sought to determine if vIL-6-mediated STAT3 signaling is the mechanism by which vIL-6 upregulates CEACAM1 message and protein. S3I-231 is a small molecule that inhibits the ability of STAT3 to dimerize and become activated (338). We treated hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 with 60 uM S3I-231 or vehicle control (DMSO) for 48 hours. RNA was harvested and qPCR was performed. In vehicle-treated cells, CEACAM1 levels were much higher in vIL-6 cells as compared to empty vector cells; however, treatment with S3I-201 greatly reduced CEACAM1 transcript levels in vIL-6 cells (Figure 3.3 A). Furthermore, treatment of cells with S3I-201 for 72 hours significantly reduced CEACAM1 protein in vIL-6 cells as compared to the vehicle control (Figure 3.3 B). Western blots confirmed that Tyr705 phosphorylation of STAT3 was increased by expression of vIL-6, and that treatment with S3I-201 reduced STAT3 phosphorylation, as expected (Figure 3.3 B). This data suggests that STAT3 signaling may play a role in vIL-6-mediated upregulation of CEACAM1.

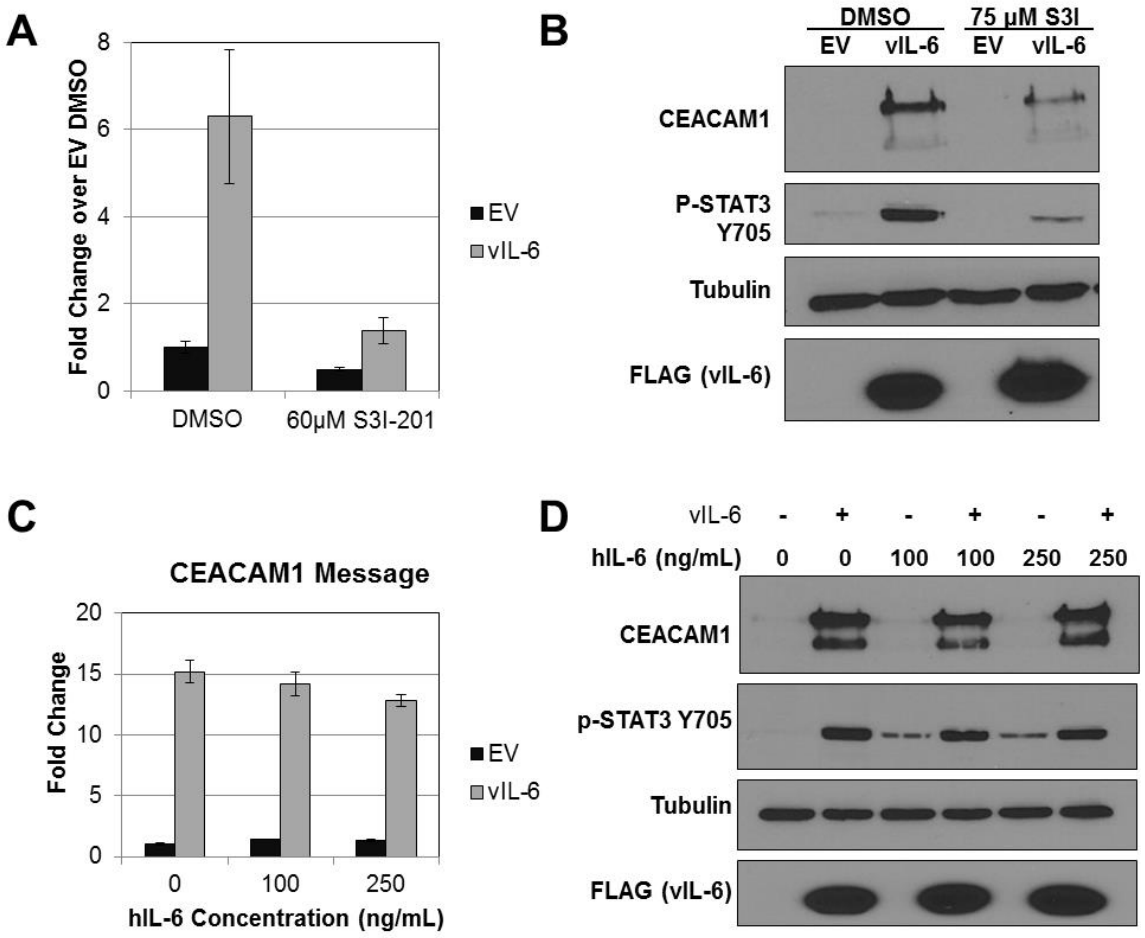


Figure 3.3: vIL-6, but not hIL-6, upregulates CEACAM1 in a STAT3-dependent manner. (A) cDNA was generated from HUVEC stably expressing empty vector or FLAG-tagged vIL-6 that were treated with DMSO or 60 μM S3I-201 for 48 hours in media containing 2% serum. qPCR was performed to assess levels of total CEACAM1 message. (B) Lysates were harvested from HUVEC stably expressing empty vector or FLAG-tagged vIL-6 that were treated with DMSO or 75 μM S3I-201 for 72 hours in media containing 10% serum. SDS-PAGE and Western blotting for the indicated proteins was performed. (C) HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were treated with exogenous hIL-6 for 48 hours. cDNA was generated and analyzed for total CEACAM1 message by qPCR. (D) HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were treated with exogenous

hIL-6 for 48 hours. Lysates were harvested and subjected to SDS-PAGE and Western blotting for the indicated proteins.

Human Interleukin-6 Does Not Influence CEACAM1 Expression

Since vIL-6 and human interleukin-6 (hIL-6) share homology, we wanted to determine if hIL-6 alone, or in combination with vIL-6, can drive CEACAM1 expression. We treated hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 with 0, 100, or 250 ng/mL of recombinant hIL-6 for 48 hours total in 2% serum, with fresh media and fresh hIL-6 supplemented at 24 hours. RNA and lysates were harvested from these cells and Western blotting and qPCR was performed. Phosphorylation of STAT3 Tyr705 was induced in empty vector cells upon hIL-6 treatment, although not nearly to the high levels seen in vIL-6-expressing cells (Figure 3.3 D). Interestingly, exogenous hIL-6 did not increase CEACAM1 expression either in the empty vector cells or the vIL-6-expressing cells at either the protein (Figure 3.3 D) or transcript (Figure 3.3 C) level. We also treated cells with 100 ng/mL hIL-6 treatment for 72 hours, and similar trends were seen (data not shown). This suggests that intracellular vIL-6 is unique in its ability to drive CEACAM1 expression in endothelial cells, despite its similarities to hIL-6.

CEACAM1 Is Required for Migration of vIL-6 Expressing Endothelial Cells

Based on CEACAM1's proposed role in invasion and metastasis of a number of cancers (319, 323, 324), we wanted to determine if it plays a role in the biological function of vIL-6. We utilized hTERT-HUVEC stably expressing empty vector or FLAG-tagged vIL-6 that were plated onto dishes coated with collagen IV as

previously described (324). We transfected cells with a non-targeting or CEACAM1-targeting siRNA, and 40 hours post-transfection, cells were serum starved for 8 hours. Monolayers were scratched with a P200 pipette tip and triplicate images of each scratch were captured at 0, 15, and 24 hours post-scratch (Figure 3.5 A). ImageJ software was utilized to trace and quantify the remaining area of each scratch at 15 and 24 hours post-scratch as compared to the area of each scratch at 0 hours (Figure 3.5 B). vIL-6 cells expressing CEACAM1 rapidly migrated and closed the scratch as compared to empty vector cells; however, knockdown of CEACAM1 reduced the ability of vIL-6-expressing cells to close the gap as fast. Following completion of the assay, lysates were harvested for SDS-PAGE and Western blotting to confirm CEACAM1 knockdown (Figure 3.5 C). This suggests that CEACAM1 plays a role in vIL-6-mediated migration of endothelial cells.

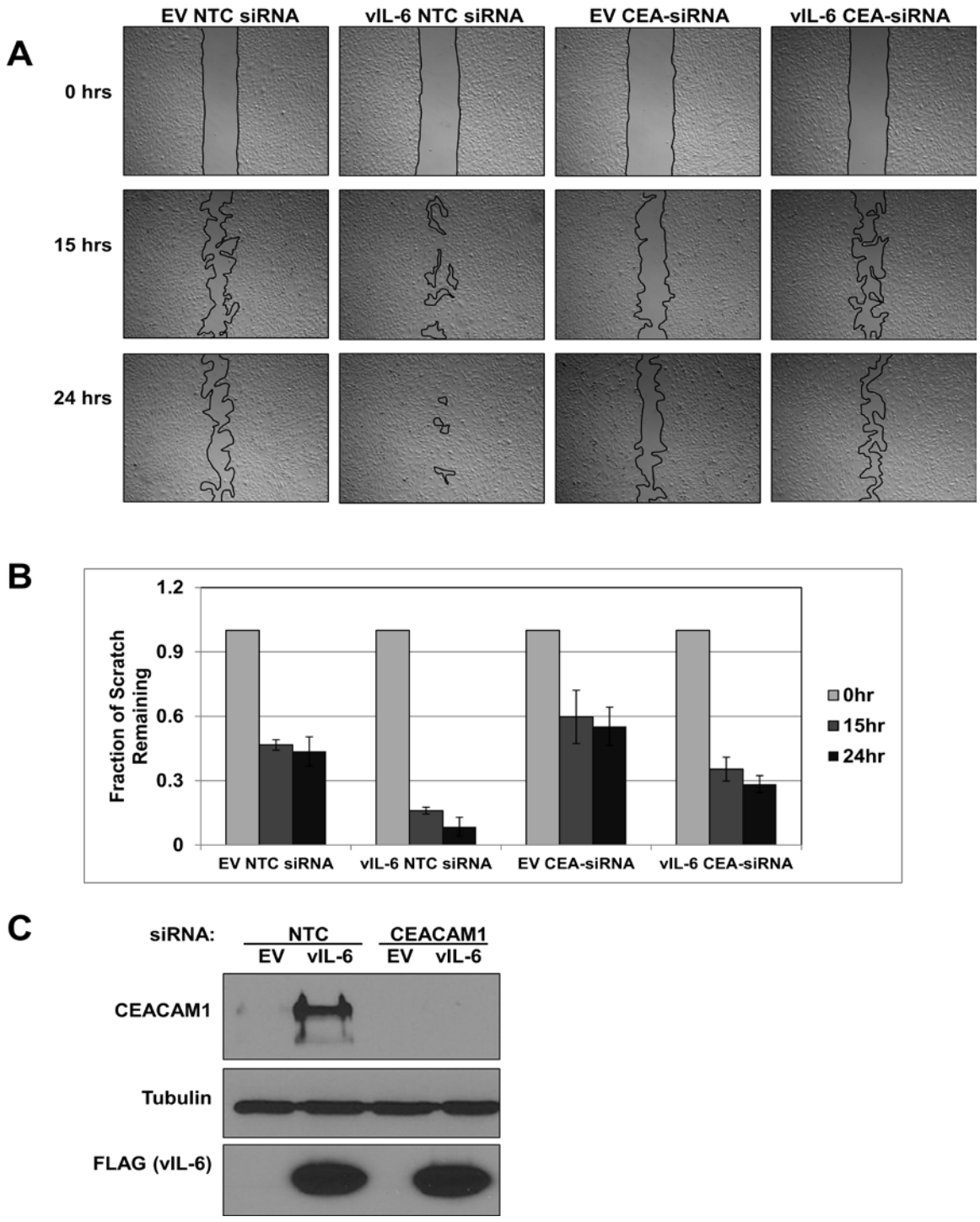


Figure 3.4: CEACAM1 facilitates vIL-6-mediated endothelial cell migration. (A) HUVEC stably expressing empty vector or FLAG-tagged vIL-6 were plated on collagen IV coated plates. Cells were transfected with 60 nM non-targeting (NTC) or CEACAM1-targeting siRNA (CEA-siRNA) for 40 hours. Cells were serum starved for 8 hours and the monolayers

were scratched with a P200 pipette tip. Brightfield images were obtained at 40x magnification with a Nikon Eclipse Ti inverted microscope at 0, 15, and 24 hours post-scratch. Three images were taken of each individual scratch, with representative images shown. (B) Scratch assays were quantified using ImageJ software. The starting scratch area was determined and set at a value of 1, and at subsequent time points, scratch areas were divided by the original scratch area to normalize for variation in the original size of each scratch. Three images and measurements were taken for each time point for each scratch and averaged, and the graph and error bars were determined by the average of two independent experiments. (C) After the 24 hour scratch assay time point, cell lysates were harvested and subjected to SDS-PAGE and Western blot for the indicated proteins to confirm CEACAM1 siRNA knockdown.

CEACAM1 is Upregulated by KSHV Infection in Multiple Cell Types

We next wanted to determine if CEACAM1 is upregulated following KSHV infection. vIL-6 is expressed at low but functional levels during latency (207) and is highly upregulated during lytic infection or lytic reactivation (143, 144). We first investigated if *de novo* infection of primary HUVEC with a recombinant KSHV virus expressing GFP (339) could induce CEACAM1 upregulation. Upon KSHV infection in endothelial cells, the virus undergoes several brief rounds of lytic infection before establishing latency (340). At 30 hpi, almost 100% of cells receiving KSHV were GFP positive whereas the mock control was not (Figure 3.5 A). RNA and lysates were harvested and analyzed by qPCR and Western blotting, respectively. Ample vIL-6 message was detected in addition to another lytic transcript, ORF57, further confirming successful KSHV infection (Figure 3.5 B). CEACAM1 message was

upregulated over 6 fold in KSHV-infected cells as compared to mock control (Figure 3.5 C), and we were also able to detect significant upregulation of CEACAM1protein in infected cells by Western blot (Figure 3.5 D).

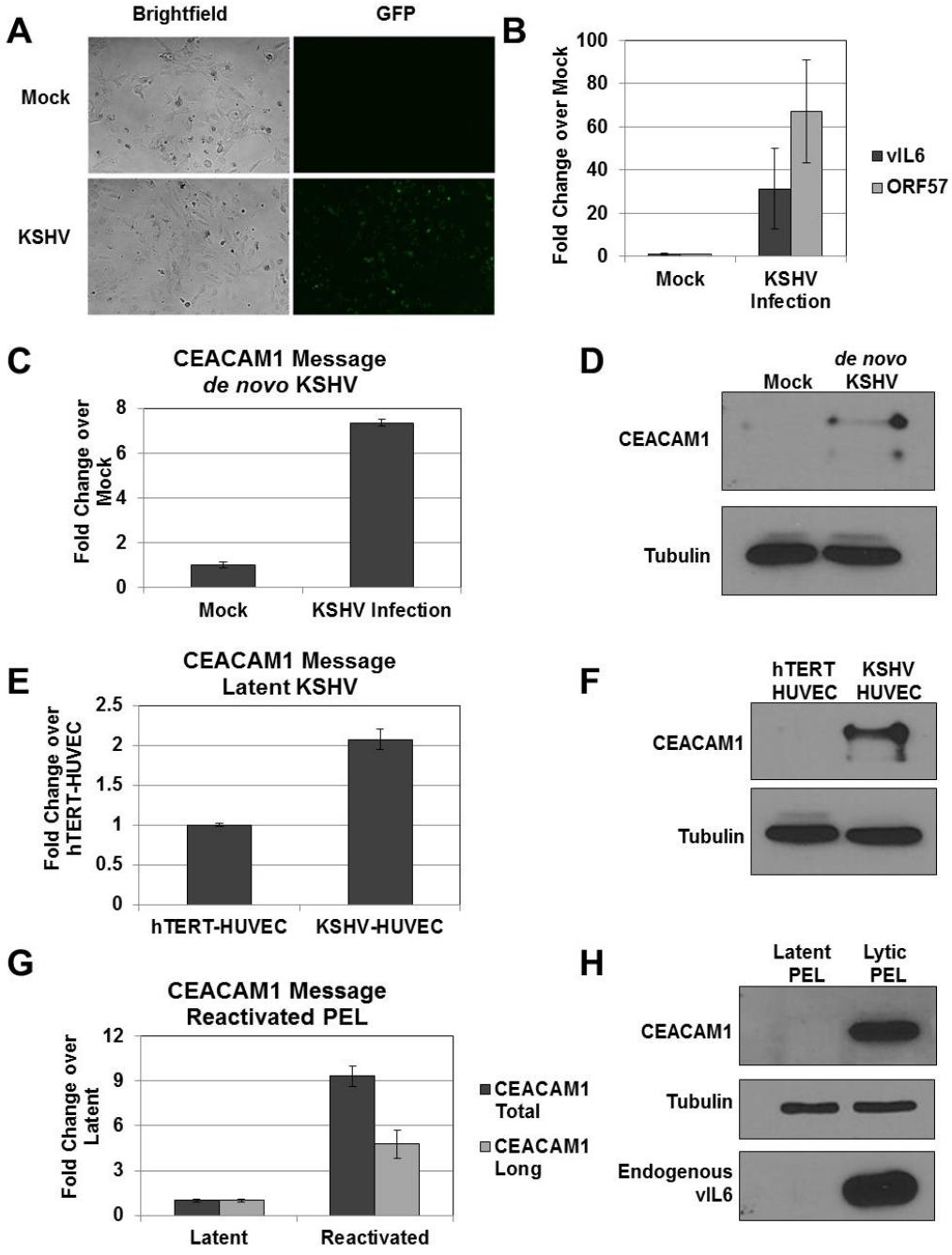


Figure 3.5: CEACAM1 is upregulated during *de novo* and latent KSHV infection of endothelial cells and during reactivation in PEL. (A) PBS (mock) or 150 µL purified

KSHV was added to primary HUVEC in the absence of serum followed by spinoculation. The following morning serum was added to 10% and 30 hours post-infection brightfield and GFP images at 100x magnification were obtained using a Nikon Eclipse Ti inverted microscope. (B) RNA was harvested and cDNA was generated from mock and KSHV-infected primary HUVEC at 30 hours post infection. qPCR was performed for the lytic transcripts vIL-6 and ORF57 and total CEACAM1 (C). (D) Lysates were harvested from mock and KSHV-infected primary HUVEC at 30 hours post infection and subjected to SDS-PAGE and Western blotting for the indicated proteins. (E) RNA was harvested and cDNA was generated from uninfected hTERT-HUVEC and latent KSHV-HUVEC. qPCR was performed for total CEACAM1. (F) Lysates were harvested from uninfected hTERT-HUVEC and latent KSHV-HUVEC and subjected to SDS-PAGE and Western blotting for the indicated proteins. (G) TREx-BCBL1 and TREx-BCBL1-RTA were treated with 1μg/mL doxycycline for 24 hours. RNA was harvested and cDNA was generated. qPCR was performed to analyze levels of total CEACAM1 and CEACAM1-L. (H) TREx-BCBL1 and TREx-BCBL1-RTA were treated with 1μg/mL doxycycline for 24 hours. Lysates were harvested and resolved by SDS-PAGE followed by Western blotting for the indicated proteins.

We then analyzed CEACAM1 message and protein levels in uninfected hTERT-HUVEC or latently infected KSHV-HUVEC that we had previously established and described (332). We found that CEACAM1 was also upregulated at the message and protein level in the latent KSHV-HUVEC (Figure 3.5 E and F).

To test another physiologically relevant cell line for the impact of KSHV infection on CEACAM1 expression, we used a latently infected PEL cell line, BCBL1. TREx BCBL1-RTA stably express a doxycycline inducible RTA expression plasmid,

and are reactivated within 24 hours of doxycycline addition as previously described (305). The control line, TREx BCBL1, expresses a doxycycline inducible empty vector, and maintains a latent infection upon doxycycline addition. These two cell lines were treated with 1 μg/mL Doxycycline for 24 hours prior to harvest of RNA and lysates. Interestingly, we found that reactivation of these cells caused a large increase in a single discrete species of CEACAM1 by Western blotting as compared to latent cells (Figure 3.5 H). We performed qPCR on RNA harvested from the TREx BCBL1, and noted that total CEACAM1 message was upregulated in reactivated cells (Figure 3.5 G). We also quantified the long isoform of CEACAM1 using an ITIM-specific primer set and found that the long isoforms of CEACAM1 are not dramatically upregulated in lytic cells as compared to latent cells, although total CEACAM1 is still highly increased in lytic cells. Collectively, our data suggest that CEACAM1 expression is increased during both primary and latent KSHV infection and in the context of reactivation in multiple physiologically relevant cell types.

DISCUSSION

The human herpesviruses are unique from most other viral classes because they persist for the lifetime of the host. To accomplish this, herpesviruses must intricately modulate their surroundings to create a favorable environment for propagation. The KSHV genome encodes an arsenal of proteins and small RNAs that help the virus persist via a number of mechanisms including direct and indirect evasion of the host immune system, modulation of host cell signaling, and mimicry of host chemokines and cytokines. For example, KSHV encodes several viral

homologs of cellular interferon regulatory factors (IRFs) that abrogate host IRF signaling to diminish an interferon response to the virus (225). In addition to the viral IRFs, KSHV encodes other homologs to cellular proteins including vIL-6, which is a viral cytokine that has functional and structural similarity to hIL-6 (195, 197).

vIL-6 and elevated hIL-6 are detectable in all KSHV-associated malignancies (60, 64, 194) and these two cytokines likely work in a synergistic manner to create a pro-inflammatory environment that promotes disease. Despite their similarities, hIL-6 and vIL-6 have differential requirements for signaling through the IL-6-receptor (IL-6R): the gp80 and gp130 IL-6R subunits are essential for hIL-6 signaling, but vIL-6 can signal through gp130 in the absence of gp80 (201, 203). Since gp80 is predominantly on the cell membrane, this limits hIL-6 to extracellular signaling, whereas vIL-6 is able to signal intracellularly through ER-embedded gp130 (207). Furthermore, hIL-6 is rapidly secreted from cells, but vIL-6 is primarily retained within the ER (206). Based on its unique intracellular localization and intracellular signaling capabilities, we were curious as to how vIL-6 might use or modulate the intracellular host environment to promote its function.

We previously reported that vIL-6 interacts with a cellular ER protein called HYOU1, and that HYOU1 was important for vIL-6-induced JAK/STAT3 signaling, migration, and serum-starved survival in endothelial cells (313). Interestingly, vIL-6-mediated endothelial cell migration was dependent in part on HYOU1-facilitated expression of the host chemokine CCL2. We speculated that vIL-6 may influence expression of a number of additional host genes to promote vIL-6 mediated biological processes such as endothelial cell chemotaxis and angiogenesis. A

previous study analyzed the impact of vIL-6 on the expression of several hundred cellular genes in a hepatoma cell line; however, this group used exogenously applied bacterially-derived vIL-6 and only a limited number of transcripts were analyzed (62). vIL-6 requires glycosylation derived from mammalian cell machinery for it to be fully functional (341), and it is now known that vIL-6 functions intracellularly (142, 206), so exogenous application of vIL-6 is less physiologically relevant. The impact of intracellular vIL-6 expression specifically on the endothelial cell transcriptome is a previously unexplored area, and endothelial cells are extremely relevant to KSHV infection since these cells are infected *in vivo* and can give rise to KS (31, 32).

We performed a human microarray on hTERT-HUVEC stably expressing an empty vector or FLAG-tagged vIL-6. Our data confirmed previous reports that vIL-6 upregulates the host genes CCL2, VEGF, and hIL-6 (Figure 3.1 C). We also found that vIL-6 expression altered genes associated with endothelial cell movement and chemotaxis and cell invasion (Figure 3.1 B). One gene, CEACAM1, was highly upregulated by vIL-6 expression. CEACAM1 is an adhesion protein that is implicated in a number of human cancers and can promote angiogenesis, migration, and vascular remodeling in endothelial cells (326-330). Because KS is a highly angiogenic tumor, we wanted to further investigate the induction of CEACAM1 expression by vIL-6.

We sought to determine the mechanism by which vIL-6 mediates the increase in CEACAM1 transcription. It has been previously shown that knockout of STAT3 can reduce CEACAM1 expression in *APC*^{min} mice (342), and vIL-6 is a known

inducer of STAT3 signaling (142, 199, 205, 343). Inhibition of STAT3 activity with S3I-201 reduced the level of both CEACAM1 message and protein in vIL-6-expressing endothelial cells. Interestingly, hIL-6 did not upregulate CEACAM1 despite its known ability to activate STAT3. There are several possibilities that could explain this: first, exogenously applied hIL-6 activates STAT3 to a lesser degree than intracellularly-expressed vIL-6, so it is possible that hIL-6-induced STAT3 activity is not adequate to impact CEACAM1 expression. Another possibility is that vIL-6 is inducing a unique intracellular pathway capable of upregulating CEACAM1. hIL-6 can only signal from gp80-containing IL-6R complexes found exclusively on the plasma membrane, so it is possible that vIL-6 can activate an intracellular mechanism from the ER that hIL-6 does not have access to. Pathways uniquely activated by intracellular vIL-6 as compared to extracellular hIL-6 could be an interesting avenue of investigation for KSHV treatment, since this would allow for specific targeting of virally-activated pathways.

Since CEACAM1 can promote the movement and invasive capability of endothelial cells (323, 327), we investigated whether vIL-6-mediated upregulation of CEACAM1 plays a role in cell movement. In the presence of CEACAM1, vIL-6-expressing endothelial cells migrated rapidly, but knockdown of CEACAM1 abrogated the ability of these cells to migrate. Knockdown of CEACAM1 in empty vector HUVEC also reduced migration to a small degree, which is expected since CEACAM1 is known to affect cell migration outside the context of vIL-6. These data suggest a significant biological function of vIL-6 through its ability to upregulate

CEACAM1 expression to induce increased migration of endothelial cells.

Previous microarray studies using immortalized dermal microvascular endothelial cells with a predominantly latent KSHV infection also reported that CEACAM1 was one of multiple genes affected by KSHV infection (344).Furthermore, CEACAM1 was found to be upregulated in the lungs of mice infected with the murine gammaherpesvirus MHV68 (345, 346). In the present study, we found that both KSHV latent and *de novo* infection in endothelial cells upregulated CEACAM1 as compared to uninfected cells, and reactivation of PEL also resulted in increased CEACAM1 expression. It was recently shown that CEACAM1 expression is important for survival of naïve B cells and for proliferation of activated B cells, so KSHV's induction of CEACAM1 expression in PEL may be a mechanism by which the virus promotes survival of infected B cells in PEL and/or MCD (347). In endothelial cells, however, we speculate that vIL-6's upregulation of the long isoforms of CEACAM1 contributes to the ability of these infected cells to promote vascular remodeling, migration, and invasion.

Collectively, our data have uncovered many previously unidentified cellular genes whose expression is changed when vIL-6 is expressed in endothelial cells. We have also identified a novel host protein, CEACAM1, induced by KSHV vIL-6, as well as in the context of latent and *de novo* KSHV infection in endothelial cells and during reactivation in PEL cells. Based on CEACAM1's established role in angiogenesis, vascular remodeling, cell migration, and cancer metastasis, vIL-6-induced CEACAM1 may be a very important player in promoting KSHV-associated

pathogenesis, particularly for endothelial cell-derived Kaposi's sarcoma. Our results further elucidate the function of intracellular vIL-6 in KSHV biology, and have identified CEACAM1 as a migration-associated factor that may be a novel therapeutic target for KSHV-associated disease.

CHAPTER 4: ALTERATION OF THE HOST CELL KINOME BY LYTIC AND LATENT KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS INFECTION[5]

OVERVIEW

Kinases regulate many sensitive signaling pathways and processes within the cell. Cancerous cells often have dyregulated kinase activity, and numerous selective kinase inhibitors are on the market or under investigation with the goal of restoring normal kinase activity in cancer cells or selectively eliminating these cells. Primary effusion lymphoma (PEL) is a B cell neoplasia associated with Kaposi's sarcoma-associated herpesvirus (KSHV) infection. PEL cells typically harbor a latent KSHV infection, but can undergo lytic reactivation in which a wider array of viral proteins is expressed and viral progeny are produced. We utilized a novel kinome profiling technique using multiplex inhibitor beads coupled with mass spectrometry (MIB/MS) to characterize the activated kinome in PEL cells undergoing latent or lytic infection. We identified numerous kinases that were differentially activated during these stages of the viral lifecycle. This work has uncovered novel kinases that may be effective targets for the treatment of KSHV-associated malignancies

[5] Experiments were designed by Blossom Damania and Louise Giffin. LG prepared all samples for MIB/MS and Gary Johnson and members of his lab, including Deborah Granger, Tim Stumiller, Noah Sciaky, Rachel Reuther, and Trang Pham, performed MIB/MS and critical analysis. Grants to BD provided funding for these experiments.

INTRODUCTION

The human genome encodes over 500 protein kinases (collectively called the kinome) which regulate the activation of numerous signaling pathways in the cell by catalyzing the phosphorylation of cellular proteins and substrates. Upon phosphorylation by a kinase, a substrate's conformation changes, which may then alter that substrate's enzymatic properties, cellular localization, and ability to interact with and modulate other proteins and substrates. Abnormal activation or inactivation of particular kinases is thought to be one of the major driving forces by which cells escape growth regulation and become cancerous (348). Because of this, kinase inhibitors have become an intense focus of investigation in drug development, and there are currently over 130 selective kinase inhibitors in development as cancer chemotherapeutics (349). However, many studies have reported that cancer cells have the unique ability to reprogram their kinome to compensate for chemical inhibitor treatments or radiation therapy (350-353) which highlights the important issue of drug resistance in tumors. To compete in this war against cancer, it will be crucial to understand the baseline activation status of the kinome in various cancers, and also to understand what kinase or kinases need to be targeted to effectively and permanently inhibit cancer cell growth. Kinome profiling has recently identified promising novel kinase targets in triple negative breast cancer and acute lymphoblastic leukemia (354, 355). The application of this technology to other types of cancer has huge potential for the development of more efficacious chemotherapeutic drugs.

KSHV modulates the activation of many kinase-regulated cellular signaling pathways. Upon entry into the cell, KSHV infection activates numerous kinases and pathways including focal adhesion kinase (FAK), PI3K, Src, MAPK, and NFκB (356-360). Activation of these pathways allows the viral genome to be transported to the nucleus and facilitates viral gene expression (361, 362). KSHV expresses many viral signaling proteins during the lytic cycle that can activate various cell signaling pathways. For example, the JAK/STAT pathway is activated by vIL-6 (62, 199), the PI3K/AKT/mTOR pathway is activated by K1 and vGPCR (171, 189), the NFκB pathway is activated by vGPCR and K15 (218, 363), and the MAPK pathway is activated by vGPCR, vIL-6, and K15 (198, 218, 364). During latency, KSHV expresses a limited repertoire of viral proteins and viral microRNAs that also modulate cell signaling pathways. For example, LANA can interact with the cellular kinase, GSK3, to facilitate expression of cyclin and myc to drive cell proliferation (94), and vCyclin activates cyclin dependent kinase 6 (CDK6) and phosphorylates histones to promote entry into S phase (98, 101).

PEL is an aggressive cancer with a median survival time of only 6 months (46). Currently, there is no standard therapy for PEL and patients are typically treated with traditional chemotherapy combined with anti-retroviral therapy (ART) if patients are HIV positive (365). On a case-by-case basis, patients have been treated with preclinical targeted therapeutics to inhibit NFκB, PI3K/AKT/mTOR, JAK/STAT, VEGF, and CD30, and there has been limited success with some of these strategies (365). Despite these advances, PEL is notoriously resistant to chemotherapy, so novel targeted or combination approaches will be essential to treat this KSHV-

associated lymphoma. There is also an interest in inducing reactivation of latent infection because available herpesvirus drugs such as acyclovir are effective during lytic infection. A viral kinase expressed only during the lytic cycle phosphorylates acyclovir, then cellular kinases can further modify the compound with the addition of one or two more phosphate groups to create a nucleoside analog. This analog competes with GTP for incorporation into DNA, and preferentially inhibits the viral polymerase, thus impeding replication (366). Therefore, one strategy to cure the cell of the virus is to induce lytic reactivation while simultaneously treating with acyclovir-like drugs. This has been done for other herpesvirus infections such as EBV (367).

An siRNA screen targeting each of the cellular kinases was recently completed to identify cellular kinases that could cause reactivation of KSHV from latency (368). A cellular kinase called tousled-like kinase 2 (TLK2) was identified and found to be critical for maintenance of latent infection (368). Therefore, an objective of this work is to build on this study and compare the activated kinome in PEL with either a latent or lytic KSHV infection to identify differentially activated signaling pathways, some of which may be potential targets for driving infection towards a lytic state.

To profile the kinome during latent and lytic KSHV infection, we utilized a novel technique called multiplex inhibitor bead/mass spectrometry (MIB/MS) (350). Activated kinases in a sample are selectively isolated by binding to broad spectrum kinase inhibitors attached to beads. Bound kinases can then be eluted from the beads and subjected to mass spectrometry to identify individual active kinases that are present in the sample (350, 352). We harvested lysates from a KSHV-infected

TREx-BCBL1 PEL cell line that has stable expression of doxycycline-inducible empty vector or RTA expression vector to maintain or induce the latent or lytic KSHV lifecycle, respectively (305). In our comparison between latent and lytic PEL, we found numerous differentially activated kinases. The MIBs also isolated the two KSHV-expressed protein kinases, indicating that they are bound by the kinase inhibitors used in the bead preparation.

METHODS

Cell Culture

TREx BCBL1 and TREx BCBL1-RTA PEL cells (305) were kindly provided by Dr. Jae Jung's lab and were grown in RPMI 1640 medium (Corning) containing 10% heat inactivated Tet System Approved FBS (Clontech), 1% penicillin-streptomycin, 1% L-glutamine, and 20 μM hygromycin B (Roche). TREx BCBL1-RTA were reactivated by supplementing media with 1 μg/mL doxycycline for 24hours.

Sample Harvest and Preparation

Suspension PEL cells were pelleted and washed three times in cold PBS. Pellets were lysed on ice in MIB lysis buffer [50 mM HEPES (pH 7.5), 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, 2.5 mM sodium orthovanadate, 1X protease inhibitor cocktail (Roche), and 1% each of phosphatase inhibitor cocktail 2 and 3 (Sigma)]. Cell lysates were sonicated 3×10 seconds on ice and centrifuged at 16,000×g in microfuge tubes for 10 minutes at 4°C. The supernatant was syringe-filtered through a 0.2 μm SFCA membrane.

Protein concentration was determined by Bradford assay (Biorad) and equal amounts of protein, around 2.5 mg, were used for each sample.

MIB/MS Technique and Analysis

The filtered lysate was brought to 1 M NaCl and passed through a column of multiplexed kinase inhibitor-conjugated beads (MIBs) consisting of Sepharose-conjugated Purvalanol B, PP58, CTx-0294885, VI16832, and two novel custom-synthesized pan-kinase inhibitor compounds (352). The MIBs were washed with 5 mL of high-salt buffer and 5 mL of low-salt buffer [50 mM HEPES (pH 7.5), 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 10 mM sodium fluoride, and 1 M NaCl or 150 mM NaCl, respectively]. The columns were washed a final time with 1 mL 0.1% SDS before elution in 1 mL of 0.5% SDS (100°C, 5 min). Eluted kinases were reduced (dithiothreitol) and alkylated (iodoacetamide) prior to being concentrated with Amicon Ultra centrifugal filters (Millipore) and detergent was removed from the concentrated eluate by chloroform/methanol extraction. Protein pellets were resuspended in 50 mM HEPES (pH 8.0) and were digested overnight with sequencing grade modified trypsin (Promega). Peptides were dried down in a speed-vac and cleaned with C-18 spin columns (Pierce) according to the manufacturer instructions.

Peptides were resuspended in 5% acetonitrile and 0.1% formic acid, and 25-50% injected onto a Thermo Easy-Spray 75uM x 25cm C-18 column via an Easy nanoLC-1000. Peptides were separated as a single fraction on a 300 minute gradient (5-40% acetonitrile) and identified by a Q-Exactive mass spectrometer. Parameters: 3e6 AGC MS1, 80ms MS1 max inject time, 1e5 AGC MS2, 100ms MS2

max inject time, 20 loop count, 1.8 m/z isolation window, 45s dynamic exclusion. Spectra were identified using MaxQuant software and the Uniprot/Swiss-Prot database. Peptide abundance was calculated using label-free quantification (LFQ).

Western Blotting and Antibodies

Either MIB lysates were used directly for Western blots, or cells were washed in cold PBS and harvested in NP-40 lysis buffer [0.1% NP-40, 150 mM NaCl, 50 mM Tris HCl (pH 8.0), 30 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 Roche protease inhibitor tablet per 50 mL] for 10-30 minutes on ice. Lysates were frozen and thawed and clarified by centrifugation at 16,000 x *g* for 10 minutes. Protein content was determined by Bradford assay (Bio-Rad) using a CLARIOstar Optima plate reader (BMG Labtech). Equal amounts of protein were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in 1x TBS/0.1% Tween followed by overnight incubation with primary antibody at 4^{o}C. HRP-conjugated secondary antibodies were incubated for 1 hour at room temperature. Blots were developed with PicoWest (Thermo) or ECL Prime (GE Healthcare) chemiluminescent reagent. Phospho-Src Y416, phospho-p70 S6K T421/S424, phospho-IKKα/β S176/S180, phospho-p90 RSK S380/ T359/ S363/ T573, Total p70 S6K, and Tubulin were purchased from Cell Signaling. vIL-6 and LANA antibodies were from Advanced Biotechnologies. HRP-conjugated secondary antibodies were from Cell Signaling.

RESULTS

The activated kinome in latent and lytic TREx BCBL1 PEL were compared by averaging values obtained from two independent experiments, and dividing the latent average over the lytic average and the lytic average over the latent average. Values were ranked, and the top 20 differentially activated kinases were reported for both latent and lytic cells (Table 4.1).

A

Kinase	Latent	Lytic
MET	95.040	1
STK17A	17.813	1
EIF2AK2	8.239	1
STK39	6.807	1
INSR	6.145	1
SGK196	4.931	1
NUAK2	4.575	1
EEF2K	4.410	1
RPS6KA1	4.103	1
RPS6KA5	3.773	1
PRKCB	3.502	1
EIF2AK4	3.202	1
TYK2	3.066	1
IGF1R	2.990	1
PLK4	2.990	1
PFKM	2.972	1
EPHA3	2.760	1
ACVR1B	2.369	1
RPS6KA3	2.369	1
MELK	2.366	1

B

Kinase	Latent	Lytic
CAMK1D	1	6.147
PRKCQ	1	5.378
CASK	1	3.649
CDK6	1	3.594
ARAF	1	3.523
CDK3	1	3.374
MAP2K4	1	3.149
FRK	1	3.073
DSTYK	1	2.438
CDK17	1	2.226
PRKD1	1	2.166
NME4	1	1.975
PRKG1	1	1.954
ULK1	1	1.922
KSR1	1	1.849
ROCK2	1	1.834
MASTL	1	1.819
FN3KRP	1	1.814
PAK2	1	1.759
TEC	1	1.757

Table 4.1: Top twenty differentially activated kinases in latent and lytic PEL. (A) Kinases activated in latent TREx-BCBL1 PEL. (B) Kinases activated in lytic TREx-BCBL1 PEL.

We also picked up the KSHV encoded kinases, ORF21 and ORF36 (vPK), in the MIB/MS profiling. As expected, spectral hits for these kinases were only

identified in the lytic cells since they are both expressed exclusively during lytic reactivation (Figure 4.1).

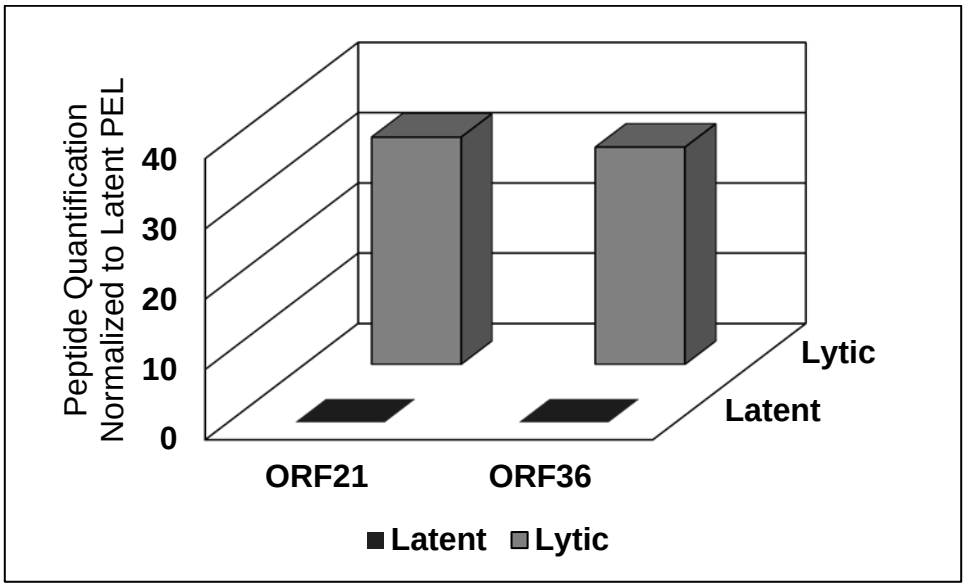


Figure 4.1: Viral kinases are isolated by MIB/MS. Quantification of peptides isolated and identified with MIB/MS that correspond to the viral kinases, ORF21 and ORF36, in latent and lytic TREx-BCBL1 samples. Samples were normalized to latent PEL.

We also wanted to confirm changes in kinase activity by Western blot. Left over MIB lysates were analyzed by SDS-PAGE and Western blotting for the activation status of Src, p70 S6K, IKKα/β, and p90 RSK (Figure 4.2).

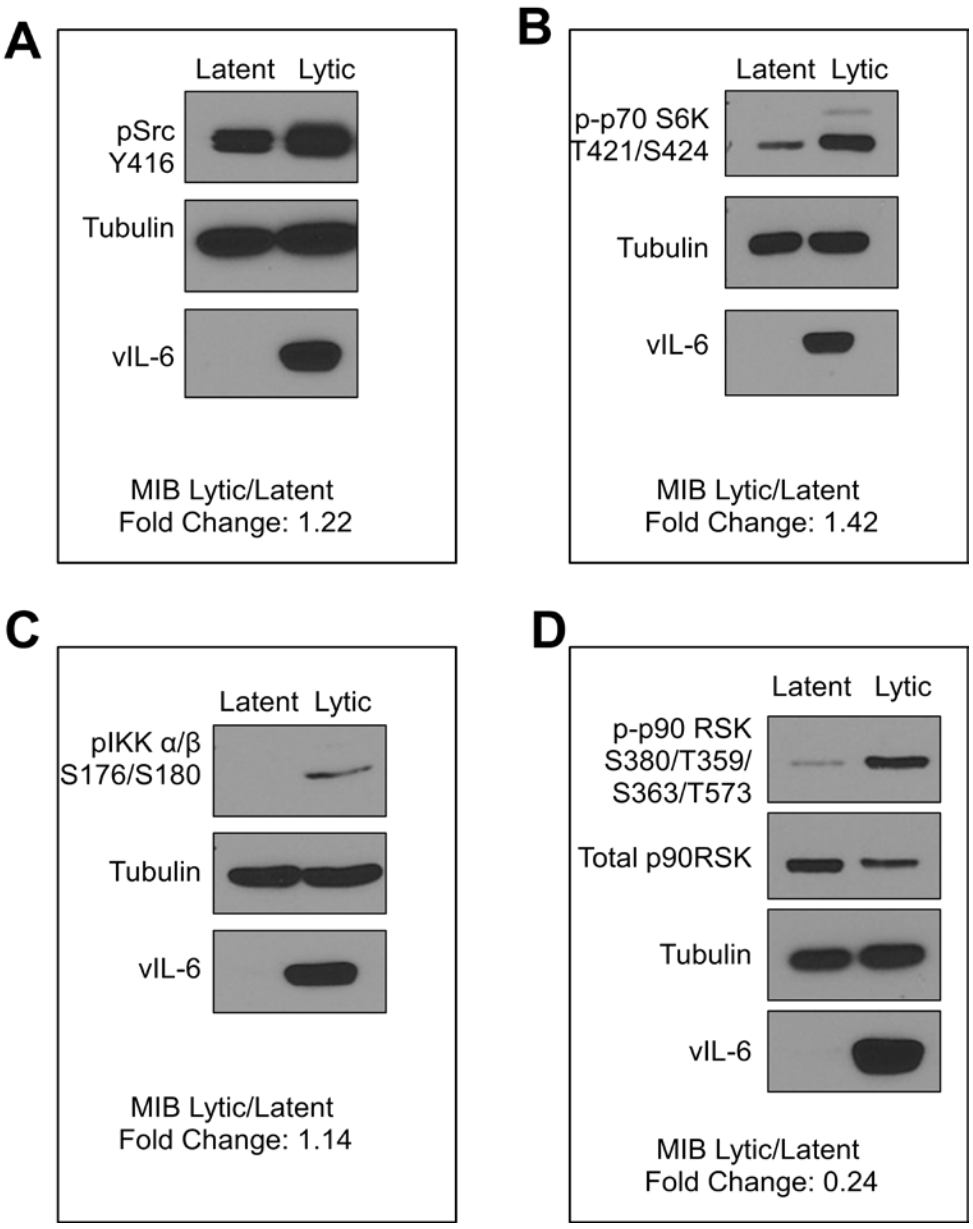


Figure 4.2: Analysis of specific kinase activation in latent and lytic TREx-BCBL1 PEL. MIB lysates were subjected to SDS-PAGE and Western blotting for phospho-Src Y416 (A), phospho- p70 S6K T421/S424 (B), phospho-IKKα/β S176/S180 (C), and phospho-p90 RSK S380/T359/S363/T573 (D). Tubulin was used as a loading control, and vIL-6 was used as a measure of lytic reactivation. Fold change values indicate the level of predicted kinase activation in lytic cells as compared to latent cells by MIB/MS.

DISCUSSION

MIB/MS is a powerful tool to profile the active kinome in cells or tissues. There are a number of other ways of assessing global kinase activation, each with their own caveats. RNA sequencing can be used to determine which kinase genes are actually being transcribed in cells, however this does not indicate which kinases are expressed at the protein level, or if the kinase proteins are actually activated. Identifying kinases with proteomic mass spectrometry is challenging because they make up a relatively small portion of the expressed proteome (369). Furthermore, the presence of a kinase does not indicate its activation status. Adenosine triphosphate (ATP) affinity probes have been utilized for profiling the kinome expression of cells since kinases bind ATP. The limitations of this method are that it is not selective for activated kinases, and kinases have inherently different ATP-binding affinities which may skew quantified data (370). Titanium dioxide can be used to enrich for phospho-proteins prior to mass spectrometry (371); however this method can also enrich for non-kinase proteins that can be phosphorylated, such as STAT3. Furthermore, many kinases have inactivating phosphorylation sites, such as Tyr527 of Src kinase (372), so it is possible that inactive kinases are also enriched by titanium dioxide prior to mass spectrometry. Antibody arrays are available to quantify levels of active kinases with phospho-specific antibodies, but these are largely limited by the quality and availability of these antibodies as well as the low-throughput nature of these arrays.

The multiplex inhibitor bead method used for this work is unique and overcomes some of the caveats of other kinome profiling methods. The beads used

are coupled to several moderately selective kinase inhibitors and several pan-kinase inhibitors, which collectively can bind to the active site of the majority of expressed human kinases (350). The advantage of these beads is that they selectively bind only activated kinases, since kinase inhibitors are inherently designed to bind to the active site of active kinases. Samples are run over a stacked column of these beads, and bound active kinases are then eluted and subjected to mass spectrometry.
We began by profiling the active kinome of TREx-BCBL1 PEL that harbor either a latent or lytic KSHV infection to determine how kinases are differentially activated in these two lifecycles. Understanding how various kinases are activated in different stages of the viral lifecycle may uncover reasonable kinase targets to drive infection towards the lytic lifecycle which is treatable with acyclovir and related compounds (366). Our kinome profiling recovered hundreds of active kinases present in latent and lytic infection, including the two kinases encoded by KSHV, ORF21 and ORF36 (vPK) (Figure 4.1). We reported the top kinases that are differentially activated in latent and lytic cells (Table 4.1).

CAMK1D is a member of the calcium/calmodulin-stimulated protein kinase family, and plays a role in cell cycle progression. CAMK1 has specifically been implicated in regulating the transition into G_1 phase, and is thought to activate cdk4 and cyclin D1 to promote entry into G_2/S (373, 374). Our MIB/MS data indicate that CAMK1D is activated in lytic cells (Table 4.1). KSHV does not rely on host DNA replication machinery to copy its genome since it expresses a viral DNA polymerase; however, promoting entry into the cell cycle may still be advantageous for the virus since the production of nucleotides, amino acids, and lipids - all important

components of infectious progeny virions - are ramped up by the host cell during entry into the cell cycle. It would be interesting to investigate if CAMK1D is activated by a specific KSHV lytic protein to initiate the cell cycle. On a related note, the MIB/MS data also identified several host cyclin dependent kinases (CDKs) that were highly activated during lytic replication, including CDK3 and CDK17. CDK3 is a key cell cycle regulator, and its inhibition results in cell cycle arrest (375). Interestingly, inhibition of CDKs that are critical for promoting the cell cycle also inhibits replication of another herpesvirus, herpes simplex virus (HSV) (376). It would also be interesting to investigate the lytic KSHV proteins that can induce activation of CDKs to promote entry into the cell cycle and successful lytic viral replication.

In latent PEL, the MIB/MS analysis suggests that the tyrosine kinase c-MET is highly activated as compared to lytic PEL. The ligand for c-MET is hepatocyte growth factor (HGF), and a previous study indicated that both HGF and c-MET are expressed and functional in PEL (377). Activation of HGF/c-MET is implicated in the development of many cancers and the inhibition of MET activity is an active area of focus for the development of cancer therapeutics (378). Interestingly, another group recently reported preliminary studies indicating that the HGF/c-MET pathway is highly active in PEL, and that use of a novel selective HGF/c-MET inhibitor induces PEL apoptosis via cell-cycle arrest and DNA damage *in vitro* and reduces PEL tumor expansion *in vivo* (Dai, Qin, *et al*. Unpublished results, Abstract presented at the 18th International Workshop on KSHV and Related Agents, Miami FL, July 2015). This work not only confirms our MIB/MS findings, but also emphasizes the potential impact of inhibiting kinases that are found to be highly active in our MIB/MS analysis.

The MIB/MS data, as well as confirmatory Western blots, also found higher activation of p70 S6 kinase (S6K) in lytic PEL. Additionally, Western blots indicated higher p90 ribosomal S6 kinase (RSK) activation in lytic PEL as compared to latent PEL. The S6 kinases regulate and promote protein translation, an essential process for viral replication, so higher levels of these kinases would be expected during lytic replication. A recent publication investigating phosphorylated kinases in latent and lytic KSHV-infected epithelial cells also noted higher S6K and RSK activation in lytic cells (379). They found that ORF45 plays a role in the activation of both of these S6 kinases, and that activation of RSK is crucial for the production of infectious progeny (379, 380). Since S6K is downstream of a number of signaling pathways activated by various KSHV-encoded proteins, there are likely many viral mechanisms in addition to ORF45 that contribute to the activation of both S6K and RSK.

MIB/MS and subsequent Western blotting indicated higher activation levels of the tyrosine kinase Src and the NFκB regulators IKKα/β in lytic PEL. Src family kinases are a group of non-receptor tyrosine kinases that regulate cell growth and promote cellular transformation. They interact with a variety of receptor tyrosine kinases (RTKs) to induce diverse downstream signaling pathways. Src and the Src family member Lyn have been shown to be activated by several KSHV lytic proteins, including K1 and vGPCR, as well as by binding between the KSHV virion and EphrinA2, a known KSHV receptor (173, 381, 382). The NFκB pathway is implicated in inflammation, cell survival, and tumorigenesis, and activation of the IKKs is required for initiation of both canonical and non-canonical NFκB signaling. There are several KSHV lytic proteins known to activate NFκB, including K1, vGPCR and K15

(173, 218, 383). Since expression of these KSHV-encoded signaling proteins is induced during lytic reactivation, this provides a possible mechanism by which Src and IKK activation occurs during lytic reactivation.

Some of the kinases we analyzed for phosphorylation status by Western blot did not match the activation status suggested by the MIB/MS results, such as p90 RSK. There are several possibilities for this disparity: the first is that kinases often have multiple phosphorylation sites, some of which are activating and some of which are inhibitory. By probing for a single phosphorylation site on a particular kinase with a single antibody, we are not seeing the entire picture of that kinase's activity. Another explanation for inconsistent data is that MIB/MS cannot normalize for differences in total levels of a kinase. For example, in Figure 4.2 D the total levels of p90 RSK vary substantially amongst samples. MIB/MS analyzes the total amount of activated kinase regardless of whether the activated form makes up 3% of that particular kinase or 99% of that particular kinase, which is a bias that must be taken into consideration when evaluating MIB/MS data.

In conclusion, we profiled the activated kinome of latent and lytic BCBL1 PEL and identified many kinases that are differentially activated in latent and lytic infection. These data are interesting and intriguing, and leave much to be explored. Understanding how KSHV modulates the kinome of infected cells will have huge implications not only for viral infection, but also for potential therapeutics for KSHV-associated malignancies.

CHAPTER 5: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

GENERAL SUMMARY

My dissertation research, performed under the guidance of Dr. Blossom Damania, is focused on the broad goal of further understanding how KSHV interacts with and modulates the host cell environment to promote pathogenesis. My project took advantage of several different approaches to investigate this question. I first performed detailed characterization of how a single KSHV protein, vIL-6, interacts with and modulates the cellular environment. This project utilized both proteomics and transcriptomics to unravel the intricacies of vIL-6 function inside the cell. My project then investigated the impact of latent and lytic KSHV infection on the host cell kinome. Cellular kinases regulate nearly every cellular function, and dysregulated kinase signaling is implicated in the development of many cancers. Understanding further how KSHV and all of its encoded components interact with and modulate the host cell environment and host kinases will be essential to developing efficacious strategies to combat infection and associated malignancies.

In chapter one, the general biology and manifestations of KSHV infection are discussed. Chapter two investigates the interaction of the KSHV protein vIL-6 with cellular proteins, and focuses on an interaction of vIL-6 with the protein HYOU1. HYOU1 was found to be important for maintaining vIL-6 protein levels in the cell and facilitating vIL-6-mediated STAT3 signaling, cell migration, and nutrient-deprived

survival in endothelial cells. Chapter three delves into the impact of intracellular vIL-6 expression on the endothelial cell transcriptome. My work uncovered that vIL-6 upregulates expression of the host adhesion and angiogenesis factor CEACAM1, and that CEACAM1 facilitates vIL-6-mediated migration of endothelial cells. Additionally, I found that KSHV infection also upregulates CEACAM1, further implicating this host protein in viral pathogenesis. In chapter four, I utilized a novel kinome profiling technique to probe the activated kinome in latent versus lytic KSHV infection in PEL. This work could shape the development of targeted therapeutics to counteract dysregulated kinase activation in KSHV-associated cancers.

These diverse projects have come together to achieve my objective of further characterizing how KSHV interacts with and manipulates the host cell. Part of my graduate work investigates how one specific viral protein manipulates the host cell, and the other part broadly probes how the entire virus alters global signaling pathways within infected cells. This final chapter meshes these distinct approaches into the overall objective of my graduate research, and highlights future directions that will extend this work to bring a fuller understanding of KSHV biology and pathogenesis.

KSHV-ASSOCIATED MALIGNANCIES AND MECHANISMS OF PATHOGENESIS

Since its discovery in 1994, KSHV has been linked to three human malignancies, KS, PEL, and MCD. KS is an endothelial cell tumor that presents as highly vascularized skin lesions, PEL is a clonal expansion of latently infected plasma cells in the pleural cavities, and MCD is an expansion of plasmablasts within

B cell follicles. KSHV is also associated with two rare immune activation syndromes called KSHV-associated inflammatory cytokine syndrome and KSHV immune reconstitution inflammatory syndrome, which present with high KSHV viral loads and immune system flare ups.

KSHV infection exists in a latent or lytic lifecycle, and it is thought that its ability to transition between these stages is one mechanism by which the virus evades host immune detection. During latency, KSHV expresses a limited set of viral proteins and viral miRNAs, many of which have oncogenic and transforming properties. KSHV primarily defaults to a latent infection in the host, so understanding the mechanisms by which latency proteins, such as LANA, drive pathogenesis in the host cell has been the focus of many scientific studies. LANA tethers the viral episome to the host chromosome and influences the oncogenic capability of latently infected cells by modulating the tumor suppressor p53, GSK3β, myc, and telomerase (91, 94, 95, 97). The viral miRNAs are also highly expressed in latency, and have diverse functions ranging from latency maintenance to immune evasion and cell proliferation.

During lytic reactivation, the viral replication and transcription activator (RTA) protein initiates a complex transcriptional cascade that results in expression of all viral non-structural and structural proteins. Many lytic proteins have been characterized as pro-growth signaling proteins, including K1, vIL-6, vGPCR, and K15, amongst others (199, 384). KSHV lytic proteins have been shown to activate major host proliferation and pro-inflammatory pathways, including the MAPK, PI3K/Akt/mTOR, JAK/STAT, PKC, and NF-κB pathways. Identifying cellular

pathways that are stimulated by lytic KSHV proteins and unraveling the underlying mechanisms by which they activate these pathways will not only enrich our knowledge of KSHV biology, but may also guide the development of targeted therapeutics to reduce pathogenic signaling.

In addition to utilizing latency to hide from the host immune system, KSHV has an arsenal of factors that also prevent its detection by and activation of antiviral immune defenses. Although KSHV has several mechanisms of evading the adaptive immune response, numerous studies have focused on the interplay between KSHV and the innate immune system. KSHV can be detected by various innate immune sensors, including the TLRs, RLRs, NLRs, and cGAS/STING (281, 385), but it also encodes viral proteins including the viral IRFs, ORF63, and ORF45, that potently blunt these responses to reduce expression and release of interferon β and IL-1β (225, 278). KSHV also expresses proteins that inhibit complement activation and detection by NK cells (242, 288). Further investigation of how KSHV evades immune detection may lead to alternative therapeutic strategies that boost the innate immune system to allow the host to successfully defend against the virus and eliminate infection.

MODULATION OF KSHV VIRAL INTERLEUKIN 6 FUNCTION BY HYPOXIA UPREGULATED PROTEIN 1

KSHV encodes a homolog of the human interleukin 6 cytokine (hIL-6), called viral interleukin 6 (vIL-6). These proteins have significant structural and functional similarity, so many early studies on vIL-6 investigated how it influenced cells after

being exogenously applied in culture media. Elegant studies by Meads *et al* in 2004 identified some striking differences between hIL-6 and vIL-6 secretion characteristics: vIL-6 was found to be secreted over 8 fold slower than hIL-6, and vIL-6 had a glycosylation profile distinct from typical secreted cytokines (206). A subsequent study discovered the ability of vIL-6 to form tetramers with the gp130 subunit of the IL-6 receptor within the endoplasmic reticulum (ER) compartment to induce functional intracellular signaling in the absence of the IL-6R gp80 subunit (207). Collectively, these studies strengthened the evidence that vIL-6 largely functions inside the cell. We therefore hypothesized that vIL-6 must interact with host proteins that help promote the function of vIL-6, so the objective of this project was to identify and characterize novel cellular interaction partners of vIL-6.

The results of this work were published in the *Journal of Virology* (313). I initiated this project by performing a vIL-6 immunoprecipitation followed by mass spectrometry to identify bound proteins. Protein mass spectrometry is extremely sensitive, and data sets are notorious for containing many false positive hits. Proteins that had higher spectral counts in the vIL-6 samples as compared to an empty vector control sample were categorized as potential hits, and we became interested in one particular protein that had a comparable number of spectra identified in the vIL-6 samples to gp130. This protein was called hypoxia upregulated protein 1 (HYOU1), which is an ER chaperone protein that promotes angiogenesis and prevents hypoxia-induced cell death.

After confirming that vIL-6 and HYOU1 interact by coimmunoprecipitation experiments, I determined that HYOU1 is expressed in tissue from KSHV

malignancies. I then found that HYOU1 plays a role in maintaining the level of secreted and intracellular vIL-6 protein. To determine if HYOU1 modulates vIL-6 function, I examined the impact of HYOU1 on vIL-6-induced signaling, and found that in the absence of HYOU1, vIL-6 was less able to interact with gp130 and activate STAT3. I then performed biological assays in endothelial cells and found that HYOU1 was critical for vIL-6-mediated migration and serum-starved survival. Finally, I showed that HYOU1 also facilitates hIL-6-induced STAT3 signaling, and that HYOU1 can bind hIL-6 when the human cytokine is in a modified form that restricts its localization to the ER compartment. I speculate that HYOU1 interacts transiently with wild type hIL-6 as it transitions out of the ER to be secreted, but because hIL-6 remains in the cell so briefly I was unable to detect it in a co-immunoprecipitation experiment.

While investigating the mechanism by which HYOU1 affects vIL-6 mediated migration, I performed a chemokine array and identified CCL2 as a chemokine that is upregulated by vIL-6 in a HYOU1-dependent manner. Since vIL-6 has been implicated in migration and angiogenesis, it is likely that there are other important chemokines and cytokines whose expression is modified by vIL-6. An interesting future direction is to perform a more thorough profiling of secreted factors from vIL-6-expressing cells, which may give further insight into the mechanisms by which vIL-6 promotes increased movement, survival, and angiogenesis in endothelial cells. Another future direction could be to explore the vIL-6 residues or domains at the interface of the HYOU1-vIL-6 interaction. I attempted to mutate sizeable domains of vIL-6 but was unable to ablate the interaction, which suggests that there may be

multiple points of contact between vIL-6 and HYOU1. This would not be surprising, since HYOU1 is a large chaperone protein, but understanding where it interacts with vIL-6 may give insight into HYOU1's possible interaction with and effect on hIL-6. Finally, there have been no other reports investigating HYOU1 in KSHV biology, so it is unknown whether KSHV infection is impacted by HYOU1 expression or if HYOU1 plays a role in the processing or function of other viral proteins. These would be exciting avenues for future research.

KSHV VIRAL INTERLEUKIN 6 MODULATES ENDOTHELIAL CELL MOVEMENT BY UPREGULATING CELLULAR GENES INVOLVED IN MIGRATION

This work was a continuation of my first project that investigated protein binding partners of vIL-6 that promoted vIL-6 function. While some vIL-6 functions have been elucidated by our lab and others, an area that has not been thoroughly explored is how this viral cytokine impacts the transcriptional profile of endothelial cells to modulate the host cell environment or host cell function. The objective of this work was to identify major pathways altered by vIL-6 expression, and identify if there are host genes that vIL-6 induces to manipulate cellular function.

A previous study examined expression of a limited set of host genes with the exogenous application of bacterially-derived vIL-6 (62). More recently, it has been discovered that vIL-6 requires mammalian glycosylation machinery for optimal function, and that an appreciable amount of vIL-6 signaling occurs intracellularly (207, 341). My work therefore revisited the impact of vIL-6 on host gene expression, but took an approach using intracellular vIL-6 expression and endothelial cells,

which are a relevant cell type for KSHV biology. Microarray results and subsequent analysis with Ingenuity Pathway Analysis identified numerous biological function pathways that were significantly altered by vIL-6 expression, including cellular movement.

A host gene called CEACAM1 was identified in several of the cellular movement sub-pathways, and this protein is known to promote endothelial cell migration and angiogenesis. I confirmed that this gene is upregulated at both the message and protein level in endothelial cells that express vIL-6, as well as in endothelial cells with latent or *de novo* KSHV infection. Chemical inhibition of STAT3 reduced vIL-6-mediated upregulation of CEACAM1, suggesting that vIL-6 induces CEACAM1 expression via the JAK/STAT pathway. Interestingly, exogenously applied hIL-6 did not induce CEACAM1, which could be due to the inherently weaker activation of STAT3 signaling by hIL-6, or to a unique intracellular mechanism employed by vIL-6 that results in CEACAM1 upregulation. Finally, I show that CEACAM1 plays a role in promoting vIL-6-mediated migration of endothelial cells.

There are a number of interesting questions that remain for this project. Despite the similarities between vIL-6 and hIL-6, there are also striking differences between these cytokines with regard to localization, secretion, and glycosylation. It would be interesting to evaluate and compare the microarray profiles of cells with intracellular expression of hIL-6 or vIL-6 and exogenously applied hIL-6 or vIL-6. This may elucidate not only differences in how vIL-6 and hIL-6 modulate cellular gene expression, but also whether these changes are impacted by where signaling

initiates (extracellular or intracellular). This work could also provide insight into why exogenously applied hIL-6 does not induce CEACAM1 expression.

A number of other interesting genes associated with cellular movement were modulated by vIL-6. Pleiotrophin (PTN) was upregulated about 25 fold by vIL-6 in the microarray, and about 8.5 fold with follow-up qPCR (data not shown). PTN is a secreted factor in the midkine chemokine family that promotes endothelial cell migration and angiogenesis by inducing expression and secretion of VEGF and CCL2 (386, 387). Tumor necrosis factor superfamily member 15 (TNFSF15; also called TL1A) is a protein secreted by endothelial cells to negatively regulate neovascularization, and its expression has been shown to be decreased by IFNγ in endothelial cells and decreased by VEGF and CCL2 in macrophages and T regulatory cells (388, 389). The microarray data as well as subsequent qPCR analysis of TNFSF15 indicated that this host transcript was approximately 10 fold reduced in vIL-6-expressing endothelial cells (data not shown). vIL-6 is known to increase VEGF and CCL2, so it is possible that modulation of PTN by vIL-6 causes induction of VEGF and CCL2 which then downregulates TNFSF15 to collectively contribute to the increased migration and angiogenesis observed with vIL-6 expression. A more thorough investigation of these pathways may further elucidate the mechanism by which vIL-6 modulates these host genes.

While performing *de novo* KSHV infections in endothelial cells, I observed that knockdown of CEACAM1 increased viral gene expression at 30 hours post infection of primary HUVEC (data not shown). An *in vivo* study of MHV-68 in CEACAM1-knockout mice found less robust lytic infection but increased viral load

and splenomegaly during latent infection in CEACAM1-null mice (345). This phenotype was later found to be mediated by natural killer cells (346). An interesting future direction would be to probe the reason for increased viral gene expression following *de novo* infection in CEACAM1-knockdown primary HUVEC. It is possible that CEACAM1 impedes viral entry, or that signaling from the cytoplasmic CEACAM1 ITIM domain blocks signaling through pathways such as FAK, PI3K, Src, and MAPK to abrogate delivery of capsids to the nucleus. Alternatively, it is possible that CEACAM1 somehow promotes latency, and KSHV upregulates CEACAM1 to drive infected cells away from lytic replication and into latency, to evade immune detection. Pursuing these questions may elucidate why CEACAM1-knockdown endothelial cells have increased viral gene expression following *de novo* infection, which could uncover interesting and important implications for CEACAM1 in KSHV biology.

Collectively, the work discussed in chapters 2 and 3 identify novel ways in which vIL-6 interacts with and modulates the host cell environment. I identified a host protein, HYOU1, which contributes to vIL-6 function by supporting vIL-6 signaling through the gp130 subunit of the IL-6 receptor. I also found that intracellular vIL-6 modulates the expression of many host genes associated with cell movement, including the host adhesion factor CEACAM1, which contributes to vIL-6-mediated endothelial cell migration. My working model suggests that intracellular ER-localized vIL-6 is stabilized by the chaperone HYOU1, which promotes vIL-6 signaling through the IL-6 receptor. This increases STAT3 activation, which drives

expression of a number of host genes that promote vIL-6-mediated biological functions, such as migration, angiogenesis, and survival (Figure 5.1).

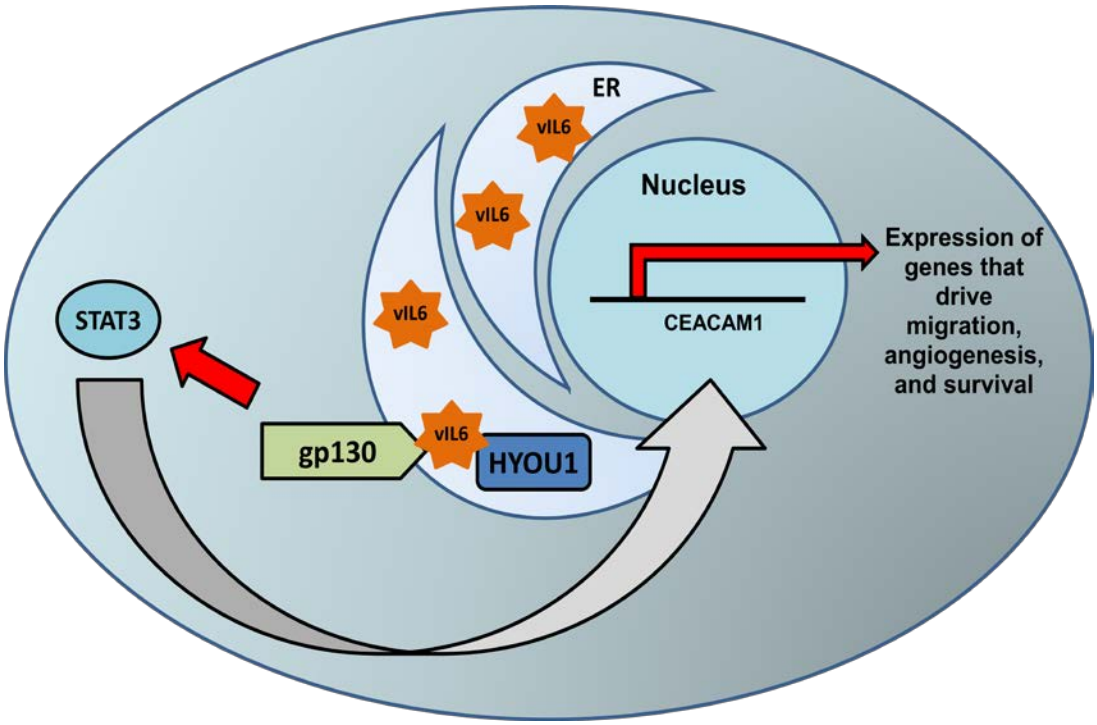


Figure 5.1: A model for the mechanism of action of intracellular vIL-6.

Since KSHV expresses over eighty open reading frames in addition to viral miRNAs and long non-coding RNAs, determining the significance and contribution of each viral component with regard to disease pathogenesis is often complex. In the context of cancer pathogenesis, I speculate that the main role of vIL-6 is to induce a pro-inflammatory environment that results in the increased secretion of chemokines, cytokines, and growth factors. Collectively these factors may promote the growth and migration of infected cells, thus encouraging transformation and tumorigenesis. The presence of a pro-inflammatory environment may also provide the necessary

cellular stresses to induce viral reactivation in some cells to allow for the propagation of the virus. Additionally, vIL-6-induced chemokines may result in the recruitment of other susceptible immune cells, thus providing new host cells to help perpetuate KSHV infection. It is also important to consider the role of vIL-6 during asymptomatic KSHV infection, since only a fraction of patients with KSHV develop KSHV-associated malignancies. I speculate that vIL-6 levels are likely much lower (if expressed at all) during asymptomatic KSHV infection since the level of vIL-6 positively correlates with prognosis in MCD (59, 60). It has not been investigated if vIL-6 is present in asymptomatic KSHV infection, but if expressed, I hypothesize that small amounts of vIL-6 would still promote a mildly inflammatory environment: enough to promote a low level of viral persistence without inducing pathogenesis. KSHV and closely related herpesviruses are the only human viruses known to express a homolog of human IL-6, and while my work has advanced the field's understanding of vIL-6, the comprehensive role of this unique, multifaceted viral protein in disease and KSHV biology remains to be elucidated.

ALTERATION OF THE HOST CELL KINOME BY LYTIC AND LATENT KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS INFECTION

This research was performed in collaboration with Dr. Gary Johnson's lab at the University of North Carolina at Chapel Hill. His lab developed a novel method to specifically profile the activated kinome of cell or tissue samples, and the goal of our collaboration was to identify differentially activated kinases in lytic and latent PEL.

The MIB/MS analysis picked up several hundred kinases among our samples, and I ranked the top differentially activated kinases in the latent and lytic PEL (Table 4.1). The MIBs also isolated the two KSHV-encoded kinases, ORF21 and ORF36, which suggests that they are activated in lytic cells and can be bound by known kinase inhibitors. There are a number of future directions for this project, the first of which is confirming activation of top kinases identified in the MIB/MS comparison of latent and lytic PEL. Ideally, radioactive kinase assays should be used to probe the activation of selected kinases since Western blots analyzing phosphorylation at particular kinase residues have caveats, as described in chapter 4.

Due to the rarity of PEL and the lack of an established treatment protocol, clinicians will sometimes treat PEL patients with a variety of novel drugs that target signaling pathways, with some efficacy. These include chemical inhibitors of NFκB, PI3K/AKT/mTOR, and JAK/STAT, VEGF, and CD30. Using kinase inhibitors to reactivate KSHV from latency will induce lysis of infected cells or sensitize cells to treatment with acyclovir-like compounds. Analyzing the kinome of latent versus lytic PEL may identify key kinases that can be targeted to accomplish lytic reactivation. For example, inhibition of TLK2 by siRNA knockdown induces lytic reactivation (368). Therefore, another future direction of this project is to determine if inhibition or activation of specific kinases in latent PEL can drive cells towards lytic reactivation that is treatable with acyclovir or related compounds.

Collectively, this project has provided preliminary evidence that there are differentially activated kinases in latent and lytic PEL. This project has numerous

future directions that will be necessary to confirm and characterize the activation of these cellular kinases, since this will provide critical direction for the development and use of specific kinase inhibitors to treat patients with PEL.

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