Characterization of the latent K15 protein of Kaposi's sarcoma-associated herpesvirus and identification of compounds that disrupt viral latency

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology.

Chapel Hill
2008

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Abstract

Tamara Kay Nun: Characterization of the latent K15 protein of Kaposi's sarcoma-associated herpesvirus and identification of compounds that disrupt viral latency
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The Kaposi's sarcoma-associated herpesvirus (KSHV) persists in a latent state in the healthy host without apparent disease. However, in circumstances of diminished immune responsiveness, latent KSHV infection has been linked to three neoplastic diseases, including Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease. Cytokines, chemokines and growth factors play important roles in all three KSHV-associated malignancies, stimulating tumor cell proliferation and neovascularization. The restricted expression of viral proteins during latency minimizes the risk of immune recognition and also limits the number of potential therapeutic targets. Currently, no drugs successfully target KSHV latency. Thus there is no cure. One potential therapeutic target is the latent membrane protein K15. Multiple K15 isoforms result from alternative splicing of the K15 message. However, all K15 isoforms are membrane-bound and share a long cytoplasmic tail with several conserved signaling motifs. Given its location and potential signaling capacities, we investigated the function of the K15 protein in B lymphocytes. We show that K15 expression alters the cytokine milieu. K15 induces interleukin-6 (IL-6) expression by activation of AP-1 transcription factors. IL-6 secretion is increased by K15 alone or in the context of viral infection. The viral IL-6 homolog is also induced by K15, stressing the important role of IL-6 cytokine signaling in viral pathogenesis. Paradoxically, K15 also activates the STAT1 protein, normally shown to be active in the interferon response. Our studies suggest that K15 signaling may enhance cell survival and promote viral latency. Therefore, K15 might be a promising target for new pharmaceuticals. In order

to screen samples for activity against latent viral infection, we developed a fluorescence-based screening assay that we used to identify antiviral agents without bias to mechanism. Of 81 plant extracts screened, we found two potential hits that were relatively non-toxic to uninfected cells, highly toxic to naturally infected cells, and that exhibited selective viral inhibition in a latent model of infection. These extracts may achieve their antiviral effects by disrupting the latency associated nuclear antigen (LANA) which tethers the viral episome to the host cell chromosome, ensuring the latent virus is not lost from the dividing cell population.

To my family and to my husband Trevor
whose love and encouragement made this work possible.
For your sacrifices and support, I am forever indebted and truly blessed.

Acknowledgments

I would like to thank my advisor, Dr. Blossom Damania for her guidance, her willingness to allow me to work independently, and her diligence in ensuring that “no samples were switched”. Seriously, I am extraordinarily grateful for her support and her encouragement during my scientific training. Her breadth of knowledge and innovative ideas were impressive, her uncontained energy and enthusiasm for science were reassuring and have given me something to strive to achieve.

I extend deep gratitude to my committee members, Dr. Dirk Dittmer, Dr. David Margolis, Dr. Nancy-Raab-Traub and Dr. Lishan Su. Their patience, understanding, flexibility during scheduling conflicts, advice and direction attest to their commitment to assisting students to become better scientists.

Special thanks go to all the members of the Damania lab, with whom I have had the pleasure of working. Christy Tomlinson and Carlos Gonzalez were especially helpful in the early years. Thanks for pondering science with me in the lab and for sharing the dance floor outside of it. Thanks to Dr. John West, who shared a windowless room with me for the past two years without complaining about my organizational challenges. Also, to Sean Gregory, Aadra Batt, and Dr. Patrick Dillon, who made lab fun again. I will miss your camaraderie.

I would also like to thank the medical and graduate students who have shared this time with me, as well as the administrative staff of the Department of Microbiology and the MD-PhD program. I am so grateful for your continuous support and to have shared this time with you all.

Most importantly, I would like to thank my family for their unwavering support. To my parents, Vaughn and Susan Nun, my siblings, Staci Armstrong, Alicia Reeves, and Eric Nun, and my husband, Trevor Moyo—you have all been there every step of the way, not always understanding my goals but encouraging me to pursue them anyway. Your love and support have kept me sane. I am truly blessed by you all. Words cannot express how grateful I am or how much I love you.

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AIDS	Acquired Immunodeficiency Syndrome
AP-1	activating protein-1
ATCC	American type culture collection
BAC	bacterial artificial chromosome
BCBL	body cavity-based lymphoma
BCCM/LMBP	Belgian Co-ordinated Collections of Micro-organisms/ LMBP Plasmid and DNA library collection
bFGF	basic fibroblast growth factor
bp	base pairs
BTC	betacellulin
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cDNA	complementary DNA
CHCl3	chloroform
CT	cycle threshold
CTL	cytotoxic T lymphocyte
CXCL	chemokine (C-X-C motif) ligand
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	early
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
GA	glycyrrhizic acid

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GRO	growth related oncogene
GSK-3β	glycogen synthase kinase-3β
H_2O	water
HAART	highly active antiretroviral therapy
Hax-1	HCLS1 associated protein X-1
HCLS1	hematopoietic cell-specific Lyn substrate 1
HCMV	human cytomegalovirus
HHV	human herpesvirus
hIL-6	human interleukin-6
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HSV	herpes simplex virus
I-309	inflammatory cytokine 309
ICAM	intercellular adhesion molecule
IE	immediate early
IFA	immunofluorescence assay
IFN	interferon
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin G
IL	interleukin
IL-6R	interleukin-6 receptor
IV	intravenous
JAK	Janus kinase
JNK	Jun N-terminal kinase

K15g	genomic K15 construct
K15-M	minor allele of viral K15 protein
K15-P	predominant allele of viral K15 protein
Kan^R	kanamycin resistance
kb	kilobase pairs
KCP	complement control protein
kDa	kilodalton
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
KSHVΔK15	recombinant KSHV with the K15 ORF deleted
L	late
LAMP	latency-assocated membrane protein
LANA	latency-associated nuclear antigen
LB	Luria-Bertani
LMP	latent membrane protein
MAPK	mitogen-activated protein kinase
MCD	multicentric Castleman's disease
MCP	monocyte chemoattractant protein
MeOH	methanol
microRNA	micro ribonucleic acid
MIP	macrophage inflammatory protein
mRNA	messenger RNA
MSP	macrophage stimulating protein
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate

NF-κB	nuclear factor-κB
NIH	National Institutes of Health
NOD	nonobese diabetic
nt	nculeotide
NT-4	neurotrophin-4
ORF	open reading frame
oriLyt	lytic origin of replication
OSM	Oncostatin M
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-BB	platelet-derived growth factor BB homodimer
PEL	primary effusion lymphoma
PIGF	placental angiogenic growth factor
PMA	phorbol 12-myristate 13-acetate
PNG	Papua New Guinea
POEMS	polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin abnormalities
PPH	primary pulmonary hypertension
QPCR	quantitative polymerase chain reaction
Rb	retinoblastoma protein
RF	retroperitoneal fibromatosis
RFHV	retroperitoneal fibromatosis-associated herpesvirus
RIG-I	retinoic acid inducible gene-I
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-1640

RRV	rhesus monkey rhadinovirus
RTA	replication and transcription activator
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	src-homology domains
SI	selectivity index
SIV	simian immunodeficiency virus
ssDNA	single-stranded deoxyribonucleic acid
STAT	signal transducer and activator of transcription
sTNFRI	soluble tumor necrosis factor receptor type I
sTNFRII	soluble tumor necrosis factor receptor type II
Supp	supplementary
TIVE	telomerase-immortalized human umbilical vein endothelial cells
TLR	toll-like receptor
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate
TRAF	tumor necrosis receptor-associated factor
TRAIL R4	tumor necrosis factor-related apoptosis-inducing ligand receptor 4
TRE	TPA-responsive element
TRITC	tetramethyl rhodamine iso-thiocyanate
U.S.	United States
vBCL-2	viral bcl-2-like protein
vCyclin	viral cyclin
VEGF	vascular endothelial growth factor
vFLIP	viral FLICE inhibitory protein

vGPCR	viral G-protein coupled receptor
vIL-6	viral interleukin-6
vIRF	viral interferon regulatory factor
vMIP	viral macrophage inhibitory peptide
VZV	varicella zoster virus
WT	wild type
xCT	cystine:glutamate transporter protein

Chapter One

Introduction

Kaposi's Sarcoma

In 1872 the Vienna-trained physician Moritz Kaposi published the first report of the lesions that would later bear his name(109). Kaposi described five cases of aggressive idiopathic sarcomas arising on the hands and feet of middle-aged men in Austria. The lesions ranged in size from a few millimeters to large coalescing plaques. They were highly pigmented and hemorrhagic and were lethal in all cases. In at least one case, multiple metastatic tumors in the esophagus, stomach and liver were found upon autopsy. This cancer described more than 100 years ago is now known as Kaposi's sarcoma (KS).

Histologically, KS lesions are characterized by masses of spindle cells of endothelial origin (38, 62, 92, 96, 150). Hyaline bodies may be found within the spindle cells and interspersed among the cells are vascular structures and spaces filled with red blood cells and pigmented hemosiderin (159). Infiltrating inflammatory cells are also present. An infectious etiological agent was long suspected, and viral particles and inclusion bodies in tumor sections were noted as early as 1972 (89, 216). However, it was not until 1994 that Drs. Chang and Moore isolated unique viral DNA sequences from KS lesions using representational difference analysis (50). Thus, the Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8, was identified as the etiological agent of KS.

Four distinct epidemiological subtypes of KS have been described: classic KS, epidemic (or AIDS-related) KS, iatrogenic (or post-transplant) KS, and endemic KS. Common among the four types of KS is the presence of KSHV DNA sequences in the KS lesions (37, 102). However, the existence of multiple epidemiological subtypes of KS highlights the importance of environmental and/or host-specific co-factors in viral pathogenesis and oncogenesis.

Classic KS occurs primarily in aging Caucasian men of eastern European or Mediterranean descent, as described by Kaposi in 1872 (109). The incidence of classic KS in Italy is two- to three-fold higher than in the U.S. and ten-fold higher than in the United Kingdom (86), with the highest incidence rate of 2.49 per 100,000 per year reported in southern Italy between 1998-2002 (16). Classic KS may follow an indolent course with spontaneous regressions reported in some cases (206). In cases with few cutaneous lesions, classic KS may be treated by excision or localized radiotherapy with great success (40, 214). In cases with multiple lesions that may be difficult to treat with conventional therapies, targeting the mTOR pathway with sirolimus (rapamycin) has also been successful (97, 141).

An outbreak of KS in San Francisco in 1981 signified the advent of a new epidemiological subtype of KS (149). *Epidemic KS* describes KS that arises in HIV-positive populations. Initially, epidemic KS was more common in men who have sex with men, but was also noted in IV drug users, Haitian natives and hemophiliacs (105). Along with *Pneumocystis carinii* pneumonia, KS was one of the first AIDS-defining illnesses (105), occurring approximately 100 times more frequently in AIDS patients than in the general population (1). Although still more common among men, as HIV continues to be spread by heterosexual contact and IV drug use, the incidence of epidemic KS among women continues to increase, as well, and may follow a more aggressive course (56, 154). Highly active antiretroviral therapy (HAART) for HIV infection has reduced the incidence of epidemic KS, and immune reconstitution following HAART is associated with regression of epidemic KS (2, 46, 72, 73, 157, 212). However KS is still the leading neoplasm seen in HIV-infected individuals today (44). HAART is the mainstay of treatment for epidemic KS, but localized treatment, conventional chemotherapeutics and immunomodulators are also considered (69).

Iatrogenic KS is a form of KS found in patients receiving immunosuppressive chemotherapy to prevent transplant rejection (168). Incidence of iatrogenic KS is relatively low in post-transplant patients in the U.S. as compared to Europe and Saudi Arabia (77, 140, 213). Regression often follows cessation of the immunosuppressive regimen (26, 140, 213, 225). Sirolimus has also been shown to cause regression of post-transplant KS, while maintaining immunosuppression (114, 145, 175, 235).

Prior to the HIV epidemic, KS was already endemic in some regions of Africa ((54) and reviewed in (104)). *Endemic KS* occurs predominantly in young African males, although females are also affected (196). The male to female incidence ratio increases with age. Usually uncommon in children, KS occurs with higher frequency in African children (60) . It typically follows a very aggressive course, often involving lymph nodes (196). Since the niveau of HIV, the incidence of KS has increased in Africa in both children and adults, and the gender disparities are equalizing (reviewed in (138)). Additionally, the geographical distribution has widened to include regions of Africa where few cases have been reported previously (reviewed in (64)). In addition to radiation and chemotherapy, recombinant interferon alpha has shown promise in treating cutaneous KS and stabilizing visceral tumors with symptomatic relief (184).

Other KSHV-associated diseases

Even before the discovery of KSHV as the etiological agent of KS, case reports were building an association between the lymphoproliferative disorder multicentric Castleman's disease (previously named multicentric giant lymph node hyperplasia) and KS (51, 63, 70, 98, 112, 113, 118, 185). A common causative agent was suspected (185). Shortly after the discovery of KSHV sequences in KS tissue, KSHV DNA was also isolated from multicentric Castleman's disease (200). In a large study to examine the prevalence of KSHV infection in

AIDS-related lymphomas, KSHV was also strongly associated with primary effusion lymphoma, previously named body cavity-based lymphoma (47). Although a definitive role for KSHV has not yet been established, KSHV has been implicated in the pathogenesis of a number of other diseases, including multiple myeloma, primary pulmonary hypertension, and atypical Type II diabetes mellitus. KSHV has been hypothesized to play a supportive role in multiple myeloma (36, 84, 181, 186). However, an increased prevalence of KSHV DNA or seroprevalence in multiple myeloma patients has been refuted in many subsequent studies (57, 136, 161, 163, 169, 188, 211, 223). In one study, KSHV infection was demonstrated in plexiform lesions and the surrounding tissue of primary pulmonary hypertension (PPH) (55), but the role of KSHV in PPH disease pathogenesis has been called into question (22, 59, 110, 124, 155). Most recently, in a study of atypical type II diabetes mellitus patients of African origin, antibodies directed against latent and lytic antigens of KSHV were demonstrated in 88% of patients prone to ketosis versus 15% of patients not prone to ketosis (197). KSHV DNA was also amplified from peripheral blood mononuclear cells in atypical ketosis-prone patients with increased frequency (197). This preliminary evidence will undoubtedly spur future studies.

Multicentric Castleman's Disease

Clinically, Castleman's disease presents as a polyclonal lymphocytic hyperplasia involving multiple lymphoid organs and cytokine dysregulation (201). In particular, interleukin-6 (IL-6) levels are highly associated with disease progression and outcome (230). Three histological types of Castleman's disease have been described: hyaline-vascular, plasma cell type, or mixed cell type. Hyaline-vascular Castleman's disease typically presents as a single mediastinal mass amenable to surgical excision with good outcomes (45). The more aggressive plasma and mixed-cell types account for 10%-40% of localized

Castleman's disease diagnoses and nearly all multicentric disease cases (99, 111). Multicentric Castleman's disease is a multifocal neoplasm associated with peripheral lymphadenopathy, hepatosplenomegaly, and constitutional symptoms (82). Multicentric Castleman's disease is a corollary of the POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin abnormalities) (88, 134, 147, 153, 199).

KSHV DNA sequences have been isolated from the plasmablastic variant of multicentric Castleman's disease (200). A strong association between HIV and KSHV positivity was established, although KSHV has also been detected in cases of HIV-negative MCD (49, 87, 160, 200). Given that KSHV infection can be detected in only a subset of cells in MCD lesions, the role of the virus is likely co-stimulatory. KSHV may contribute to cytokine dysregulation that is central to MCD by stimulating overexpression of cellular IL-6 and by expression of the virus-encoded IL-6 homologue (vIL-6) (160). Some patients have received benefit from antiviral and/or chemotherapeutic treatment strategies, but MCD has a poor prognosis and patients tend to suffer multiple relapses (25, 53, 139, 185, 191, 205).

Primary Effusion Lymphoma

Primary effusion lymphoma (PEL) may occur in peritoneal, pericardial or pleural spaces, hence it was first called body cavity-based lymphoma (47, 94). A sold mass may be undetectable. Whereas the KSHV-infected endothelial cell represents the primary cell type that drives neoplastic growth in Kaposi's sarcoma, the malignant cells in PEL are KSHV-infected B lymphocytes. Many B cell-specific surface markers are absent in PEL (151). However, gene rearrangement and surface marker studies have determined the lymphocytes are monoclonal and composed of post-germinal center B cells (76, 83, 137). KSHV sequences have been identified in >99% of AIDS-related PEL and have also been

isolated from PEL in HIV-negative hosts (15, 47, 152). While PEL are invariably KSHV-positive, co-infection with the closely related Epstein-Barr virus (EBV) is not uncommon (47, 115).

Primary effusion lymphoma is more prevalent in HIV-positive patients and is associated with poor prognosis (15). PEL is often refractory to treatment and relegated to supportive care. Patients succumb to the disease within months. In a study of HIV-associated PEL, the 1-year overall survival rate was just 39% with the mean survival just 6.2 months (32).

Success and failure: current treatment strategies for KSHV-related malignancies

Patients presenting to clinic with Kaposi's sarcoma, primary effusion lymphoma, or multicentric Castleman's disease are treated with regimens similar to any other cancer patient. Surgical excision, radiation and chemotherapeutic regimens including paclitaxel, doxorubicin, and duanorubicin are considered first-line of therapy for MCD, PEL and KS (68), as well as for many other non-virus-associated cancers. These strategies are associated with severe toxicities, since they target all dividing cells and give no regard to the viral etiology of the tumors. The incidence of KS has dramatically declined in regions where highly active antiretroviral therapy (HAART) has been available to treat concomitant HIV infection and boost the immune system (71, 75). The realization that therapeutic benefit can be achieved by targeting the virus or the immune response has likely contributed to the recent push to develop targeted therapies more tailored to the pathogenesis of KSHV.

Recently, immune modulators and antiviral drugs have come into fashion. Interferon alpha has become a mainstay of treatment for MCD and has also benefited KS and PEL patients (12, 100, 116, 117, 158, 215). However, like classic chemotherapy, interferon is associated with severe dose-limiting toxicities such as neutropenia and failed to eliminate

the latent reservoir (116). Stochastic reports of therapeutic benefit from other targeted drugs such as rapamycin and antibodies directed against IL-6, for instance, sprinkle the literature, but very few randomized trials have been conducted to determine efficacy or to assist physicians in determining which patients will likely benefit.

Classic anti-herpetic drugs such as ganciclovir, cidofavir and foscarnet have been successful in preventing KSHV-associated malignancies (91, 144), but success has been limited using these nucleotide analogs to treat existing disease (23, 130). The major impediment to treatment with nucleotide analogs is that they are only effective at blocking lytic replication, whereas the majority of cells in KSHV-associated neoplasms are latently infected. Somewhat better outcomes are achieved with these anti-herpes drugs in MCD, since MCD supports lytic replication. However, it is important to note that treatment with these anti-herpes drugs does not eradicate the virus (103). Drugs that target latent viral infection may prove more efficacious and tolerable and will be discussed in more detail in chapter 4.

Kaposi's sarcoma-associated herpesvirus classification

The unique viral DNA sequences identified by Drs. Chang and Moore in 1994 resembled sequences of the tegument and capsid genes of another herpesvirus, the Epstein-Barr virus (50). Indeed, based on sequence homology, host-specificity, viral pathogenesis and cell tropism, the Kaposi's sarcoma associated herpesvirus, also known as human herpesvirus-8, became the newest member of a large family of human herpesviruses. The *Herpesviridae* family is divided into three subfamilies. Human pathogens of the *Alphaherpesvirinae* include Herpes Simplex viruses 1 and 2 (HSV-1 and -2), as well as the Varicella-Zoster virus (VZV) (166). The *Alphaherpesvirinae* establish latency in the dorsal root ganglia. Spontaneous lytic reactivation is associated with oral or

genital herpetic lesions (HSV-1 and HSV-2) or in a dermatomal pattern on the skin or mucosae (VZV). The *Betaherpesvirinae,* which include human cytomegalovirus (HCMV) and human herpesviruses -6 and -7 (HHV-6 and -7), cause symptomatic disease in a select group of susceptible hosts (166). HCMV infection may result in graft rejection in iatrogenically immunosuppressed post-transplant patients (131) and is associated with congenital abnormalities when *in utero* transmission occurs (19). HHV-6 and HHV-7 cause the childhood disease of roseola (101, 210). The *Gammaherpesvirinae* are a collection of lymphotropic viruses with oncogenic potential. Both KSHV and the closely related virus, EBV, are members of the gamma herpesvirus subfamily (166). KSHV has been linked to the aforementioned B lymphocyte and vascular endothelial cell cancers. EBV is associated with B-lymphocytic cell neoplasms as well as epithelial cell cancers.

The Kaposi's sarcoma-associated herpesvirus

Like all herpesviruses, KSHV can establish a lifelong persistent infection in an otherwise healthy host. The mode of transmission is still under debate with theories ranging from vertical transmission to horizontal transmission through sexual, salivary exchange, transfusion or transplantation routes (171). Seroprevalence is highest in endemic regions. Despite low seroprevalence in the U.S., KSHV DNA and viral particles have been detected in oral mucosal cells of otherwise healthy hosts (74, 221) indicating that detection of KSHV-specific antibodies may not be a sensitive measure of virus exposure.

Characteristic of the *Herpesviridae* family, the KSHV is a large double-stranded DNA virus with two replication programs. The 140-150 kb unique coding region is flanked by long terminal repeat regions adding from 20-25 kb of 801 bp repeat sequences (121, 179). The genome encodes more than 85 open reading frames (ORFs) and at least a dozen microRNAs. Alignment of the sequences of multiple strains of KSHV indicates that the

regions immediately adjacent to the terminal repeats contain the most variability (156, 174). Sequence analysis in these gene regions, which encode two viral membrane proteins, has been used to further define KSHV subgroups. The linear viral genome is packaged in a proteinaceous capsid within the nucleosome, acquires the tegument, and is enveloped in a lipid membrane upon viral egress (166).

The viral life cycle

KSHV infection is initiated when viral envelope proteins couple with receptors on the cell surface. The KSHV envelope glycoproteins K8.1 and gB interact with heparan sulfate in the extracellular matrix (4, 6, 217) and may attract viral particles into the close proximity of the host cell, where the virus is more likely to bind cell surface receptors. Glycoprotein gB coprecipitates with integrin α3β1, suggesting a role for this integrin in viral attachment (5). Engagement of the cystine:glutamate transporter protein xCT on the surface of the host cell is proposed to stimulate membrane fusion and viral entry (108). After trafficking to the nucleus, the linear viral genome is released from the nucleocapsid and is transported into the nucleus. At this point, the virus may instigate a highly ordered lytic replication program, with the goal of new progeny virion production and release. In the majority of cells, however, the viral genome will circularize via its flanking terminal repeat regions and will persist in a latent state for the life of the host cell. After establishing latency, the virus may also be recruited to produce new viral particles via lytic reactivation.

Lytic Replication

During lytic replication, the complete repertoire of viral genes is expressed in a highly regulated cascading fashion (189, 209). The immediate early (IE) genes are the first transcribed during lytic replication. The IE genes include ORF50 which encodes the

replication and transcription activator (RTA) protein. RTA acts as a transcription factor, initiating transcription from the promoters of the next wave of viral early (E) genes as well as some late (L) genes. RTA targets the promoters of polyadenylated nuclear RNA, vIL-6, ORF6 (single-stranded DNA binding protein), ORF59 (DNA polymerase-associated processivity factor), thymidine kinase, and viral G-protein coupled-receptor among others (reviewed in (65)). Early genes typically include genes that are required for viral DNA replication. KSHV viral DNA replication is initiated at two lytic origins of replication (*oriLyt*) (18). Core replication proteins (including the ssDNA binding protein, DNA polymerase, primase-associated factor, helicase, primase, and polymerase processivity factor), RTA and K-bZIP assemble as a complex at the *oriLyt* to initiate viral genome replication via a rolling circle mechanism (17). Following viral genome replication, the final or late (L) wave of genes is transcribed. Late genes encode structural proteins that form the capsid, as well as tegument proteins and membrane glycoproteins. The nucleocapsids are assembled at the nucleus and the mature virions acquire an envelope from subcellular membranes as the particles exit the cell.

In addition to the genes directly involved in and required for viral genome replication and packaging, viral proteins are also expressed during the IE, E and L phases that perform auxiliary roles, enhancing the process by diverting signal transduction pathways and the host cellular machinery and subverting the immune response. Some auxiliary viral genes encode proteins with cellular homologues that may enhance proliferation (vIL-6 protein), dysregulate cellular signaling pathways (viral G-protein coupled receptor or vGPCR), modulate the host immune response (viral interferon regulatory factors or vIRFs and macrophage inhibitory peptides or vMIPs), or prevent apoptosis (viral bcl-2-like protein or vBCL-2) (43, 146, 156, 190). Other viral genes encode proteins with structural and/or functional homology to proteins encoded by other herpesviruses. For instance, the two most extreme viral ORFs K1 and K15 encode viral membrane proteins, which resemble the

latent membrane proteins (LMPs) of the EBV both structurally and functionally. Like LMP1, ectopic expression of K1 in Rat-1 fibroblasts leads to a transformed phenotype (126). Additionally K1 expression deregulates B cell receptor signaling (122, 125) and induces invasion and angiogenic factors in epithelial and endothelial cells (219), similar to LMP1. The viral membrane protein K15 is the subject of this dissertation and will be discussed in more detail. Still other viral genes have no function ascribed them, yet.

Latency

More commonly, KSHV does not proceed to productive infection but establishes latency in an infected lymphocyte or endothelial cell. In the three KSHV-associated malignancies, the majority of infected cells are in fact latently infected, with only a small percentage of cells undergoing spontaneous lytic reactivation at any given time (estimated to be 2-5% of cells in PEL but up to 25% in MCD) (14, 29, 143). During latency, a small cadre of viral genes is expressed. This contingent of viral gene products is thought to be absolutely essential for maintenance of the circularized viral genome, or episome, and also to encode viral oncogenes. Given the large size of the KSHV genome, limited expression of viral genes during latency is a highly efficient survival mechanism. Transcription of only a handful of genes is certainly less taxing than initiating the full transcriptional program and allows the virus to be maintained by the host cell throughout its lifetime without exhausting cellular resources. Furthermore, restricted gene expression may ensure that the virus persists without triggering host immune responses.

The most studied of the latent viral proteins is the latency-associated nuclear antigen (LANA). LANA tethers the viral episome to the host chromosome via the terminal repeats (58, 85), thus ensuring that the viral genome is replicated along with the host chromosome in dividing cells and is equally segregated to each daughter cell upon cell division (20).

LANA is a fairly promiscuous protein with interactions demonstrated between LANA and various cellular proteins, including p53, pRb, GSK3β, and histone subunits, among others (21, 79, 80, 127, 173, 176). LANA, with its many functions, is pivotal in maintenance of the viral genome and in the oncogenic process. LANA is transcribed along with viral cyclin (vCyclin) and viral FLICE inhibitory protein (vFLIP), which causes cell cycle perturbations and inhibits apoptosis, respectively. The viral microRNAs are also encoded at the same locus (41, 165, 170, 187). Deciphering the roles of the viral microRNAs is an area of research that is currently hotly pursued. One of the viral microRNAs, miR-K12-11 has shown similarity in seed sequence to the cellular miR-155 as well as in target sequence prediction programs and genetic profiling assays (93, 195). However, validation of target sequences remains a difficult process. Finally, kaposin and K15 are also expressed during latency. K15 is the focus of much of the work in this thesis and will be discussed in more detail in a later section.

Lytic reactivation

After the virus has established latency in the host cell, it may undergo lytic reactivation in response to cellular or environmental stimuli. In culture, lytic reactivation is achieved by chemical induction with phorbol esters, which activate protein kinase C signal transduction pathways, or with N-butyrate, which is a histone deacetylase (143, 180). Furthermore, overexpression of the lytic switch protein RTA is also sufficient to induce lytic reactivation in tissue culture (132, 208). Importantly, no treatment is 100% effective at inducing lytic replication. Chemical induction or expression of RTA overcomes repression of lytic replication in approximately 30% of PEL cells in culture. Hence, modeling lytic replication of KSHV in tissue culture is often confounded by a high background of cells remaining in a latent state. Hypoxia and changes in cytokine secretion (especially interferon

and interleukin-6) into the microenvironment have also been reported to cause spontaneous lytic reactivation of some infected cells in culture (3, 61, 198). *In vivo* stimuli are unknown, but hypoxia and cytokine secretion are valid hypotheses. Environmental stimuli have also been reported including volcanic soils, mineral deposits in the soil, and herbal remedies that might contain natural activators of lytic replication (194, 224, 233).

The viral K15 protein

The K15 ORF is located at the far right end of the genome, immediately adjacent to the right terminal repeat region. Given its placement next to the terminal repeat, K15 sequences are highly variable between viral isolates, as compared to centrally located gene sequences. Two divergent alleles have been described—the P or predominant allele and the M or minor allele. Overall, the two alleles share approximately 33% sequence identity (174). The K15-M allele is thought to represent an ancestral allele that resulted from recombination with a closely related rhadinovirus and is more common in A and B strains of KSHV (119, 174). Of particular note, multiple putative signal transduction domains have been described that are conserved between the two K15 alleles, highlighting the importance of these signaling sequences to the function of K15. The signaling functions of the K15-P allele are investigated in Aim 1 of this thesis.

The viral K15 protein has been described as a latent protein and initially was named the latency-associated membrane protein or LAMP (90). Viral K15 transcripts have been isolated from latently infected cells and protein expression has been detected in uninduced PEL cell lines (52, 90, 193). We, and others, have shown that K15 expression increases upon induction of lytic replication with RTA overexpression and/or chemical induction (35, 52, 90, 227). Thus, K15 is not a latent gene in the strictest sense, since it is highly induced during the lytic phase of the viral lifecycle. Given that its expression has been detected in

both PEL and MCD lesions by immunohistochemistry, K15 likely plays an important function in latency as well as during lytic replication.

The K15 gene is transcribed from right to left and encodes multiple alternatively spliced transcripts. The full length K15 P-transcript contains eight exons and encodes a 45kDa protein with 12 predicted transmembrane domains (52, 90, 156). The smaller K15 isoforms are transcribed with an alternative first exon and share a variable number of exons with the full length isoform (52, 90, 156). All K15-P isoforms contain a long cytoplasmic tail with conserved constitutively phosphorylated tyrosine residues that constitute binding sites for proteins containing src-homology (SH)-2 domains, SH3 domains, and tumor necrosis factor receptor-associated factors (TRAFs) (Figure 1.1).

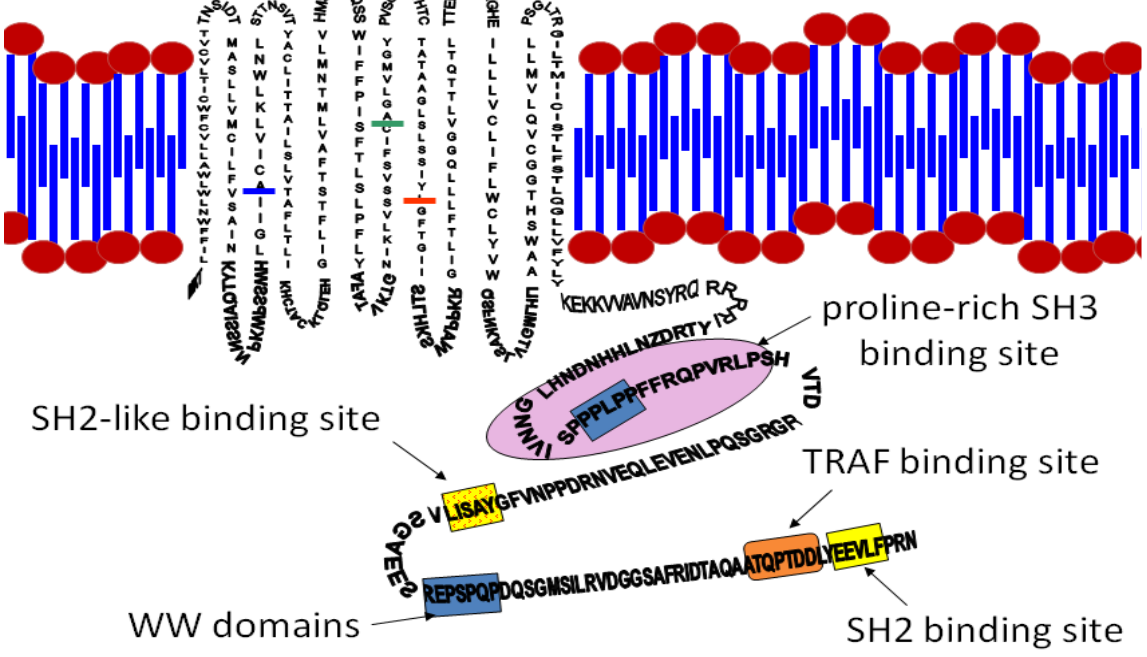

Figure 1.1 The K15 protein contains multiple transmembrane domains and conserved signaling domains in its cytoplasmic tail. The amino acid sequence of K15 is depicted in the putative structure of the full length K15 protein. The full length K15 protein encodes 12 membrane spanning domains. Smaller isoforms (designated by bars in the amino acid sequence) encode variable numbers of transmembrane domains but share multiple highly conserved signaling domains in the common cytoplasmic tail, including SH2 and SH3 (src-homology) binding sites, a TRAF (tumor necrosis factor receptor-associated factor) binding site, and two putative WW domains.

Multiple signaling pathways have been shown to be activated by the K15 protein. For instance, the YEEVL and YASIL motifs represent putative SH2-binding sites in the C-terminal cytoplasmic tail that activate multiple MAPK pathways, while interactions with TRAFs lead to induction of the NF-κB signal transduction pathway (34, 90). The SH3-binding site has been shown to bind the SH3 domain of the intersectin-2 protein, which is involved in the regulation of endocytosis (128). Like K15-P, the K15-M proteins are derived from multiply spliced transcripts and contain highly conserved putative SH2 and SH3 domains in the cytoplasmic tail. The phosphotyrosine of the conserved YEEV motif is also required for activation of the MEK/Erk2 MAPK and NF-κB signal transduction pathways by K15-M, but not for JNK activation (218). Target genes induced by K15-M are similar to those of K15-P and include inflammatory cytokines such as interleukin-6 and -8 (218).

K15 proteins are incorporated into lipid rafts (34). Various groups have shown K15 localized to multiple cellular membranes, including the plasma membrane (34, 52, 90), perinuclear or endoplasmic reticular membranes (52, 90, 193, 218), and potentially even mitochondrial membranes where it appears to bind the anti-apoptotic protein Hax-1 (193).

Expression of K15-P in epithelial cells induced the expression of multiple cytokines, including interleukin (IL)-1α/β, IL-6, IL-8, CCL2, CCL20 and CXCL3 (35). These cytokines may play a role in cell survival and proliferation and may also play immunomodulatory roles that help the virus escape detection by circulating antigen presenting cells.

KSHV and the immune response

The host organism mounts a multi-faceted defense strategy to identify and eliminate detrimental pathogens like KSHV. The consequences may be dire for the invading pathogen. Thus, in a fight for survival, evasion tactics become necessary. In the case of herpesviruses, induction of the innate immune response may select for survival of cells in

which the virus has gone latent with a restricted antigen profile. It may also exert evolutionary pressure on the virus to either actively block or subvert the innate immune response to its own advantage. For example, activation of innate the immune response may result in chemokine induction that recruits monocytes, dendritic cells and lymphocytes to the site of initial infection. These infiltrating cells may themselves be susceptible to viral infection and may serve as an easy conduit for viral spread.

Primary defenses against invading pathogens in the host incorporate multiple mechanisms for recognition and elimination of the offender. Pattern recognition receptors, such as the toll-like receptors (TLRs), and retinoic acid inducible gene-I (RIG-I) alert the cell to the presence of viral nucleic acid or protein and initiate the antiviral response. KSHV activates the toll-like receptor 3 (TLR3) during primary infection of monocyte cells, resulting in upregulation of TLR3 via a positive feedback loop and upregulation of TLR3-induced cytokines including CXCL10 and type I interferon (222). In addition to cellular pattern recognition molecules, differences in membrane structure of invading pathogens may trigger the complement cascade (234). Deposition of complement culminates in hydrolysis of the invading pathogen or infected cell through pores formed by the membrane attack complex. The complement control protein (KCP) of KSHV actively inhibits the complement cascade (202).

Following the innate immune response, a healthy host normally develops adaptive immunity to include cell-mediated and humoral immunity to invading pathogens. It is believed that a T-cell mediated immune response keeps KSHV in check during the lifetime of the asymptomatic host harboring a latent virus in his B lymphocytes. Cytotoxic T lymphocyte (CTL) epitopes have been identified in both latent (33, 142, 226) and lytic (142, 204, 220, 226) viral gene products. Memory CTL responses have been detected in otherwise healthy individuals (142). Failure of cell-mediated immunity is met with an increased prevalence of KSHV-associated malignancies, both in the setting of

immunosuppressive therapies administered to prevent graft rejection in post-transplant patients and in the setting of the Acquired Immunodeficiency Syndrome (AIDS) when CD4+ T cell counts drop to <200/ml (77, 115, 167).

Secretion of soluble factors represents an important branch of the cellular communication network. Cytokines and chemokines often signal the presence of invading pathogens and represent a request for professional assistance in elimination of the threat, either by innate immune cells or by the more experienced B- and T-lymphocyte cells. As mentioned previously, KSHV encodes multiple homologues of cellular cytokines and chemokines, such as vIL-6, vIRFs, and vMIPs (156). Regulation of the viral homologues is skewed in favor of the virus. For example, the vIRFs impede the cellular antiviral response by suppressing interferon signaling and transcriptional activity (39, 81, 129), while the vIL-6 supports growth and prevents apoptosis in IL-6 dependent cell lines (146). In addition to viral homologues, the virus also encodes proteins that increase expression of stimulatory cytokines or anti-apoptotic cytokines, which are central to pathogenesis of the KSHV-associated malignancies. For instance, the K1 protein of KSHV induces expression of the vascular endothelial growth factor (VEGF) (219), which has been shown to be important for the pathogenesis of the highly vascularized KS lesions. Cellular IL-6 and IL-10 levels are high in PEL cells (107). *De novo* viral infection and ectopic expression of LANA have been shown to cause increases in cellular IL-6 expression (11, 229).

Infection model systems

Since the discovery of KSHV in 1994, attempts to develop an efficient system for viral propagation in culture has been highly sought without much success. Cell lines derived from KS tissue samples lose the virus in serial passage (8, 9). However, multiple cell lines have been derived from PEL cells following adaptation to tissue culture (13, 31, 42, 48,

180). These PEL cell lines maintain a high copy number of viral genomes in culture (123). As expected, the majority of cells are latently infected, with 2-5% of cells undergoing spontaneous lytic replication (180). Therefore, PEL cell lines provide relatively efficient models for studying the role of viral proteins during latency. However, only a few viral genes are naturally expressed during viral latency (106, 164, 189), so the utility of the latently infected PEL cell system is pronounced in laboratories that study the functions of latent proteins. Lytic reactivation can be induced as previously described (180), but the results of such experiments must be considered in light of the high background of cells remaining latent since only a subset of cells will proceed to lytic amplification of the virus. Furthermore, this system fails to elucidate how KSHV transforms B lymphocytes, since transformation has already occurred prior to the establishment of the cell line for tissue culture. Infection of primary B lymphocytes, epithelial, and endothelial cells has been achieved and has provided useful systems to study viral infection (10, 30, 120, 177). The utility of these systems is limited, however, since they do not support sustained lytic replication or long-term viral genome maintenance.

Systems for studying the role of viral proteins by reverse genetics have recently been employed. Recombinant viruses have been constructed using a series of overlapping cosmids, in which one or more elements have been mutated or deleted. In 2002, a bacterial artificial chromosome (BAC) was created containing the entire KSHV genome, a hygromycin expression cassette for mammalian selection, and a green fluorescent protein expression cassette for visual screening (231). Mutants have been created by transposon-mediated mutagenesis or homologous recombination using this KSHV-BAC system (133, 231, 232). The KSHV-BAC system allows the viral DNA to be grown to high copy number in bacterial cells. The BAC DNA can then be transfected into cells in culture, circumventing any entry blockades to viral infection. Introduction of the KSHV-BAC into epithelial 293 cells achieves

a higher percentage of cells undergoing lytic reactivation upon chemical induction, as compared to PEL cell lines allowing more rigorous studies of lytic phase viral proteins.

In vivo infection models are complicated by the restricted host range of KSHV but many new models have been developed to study KSHV biology and pathogenesis. Long-term KSHV-infected telomerase-immortalized human umbilical vein endothelial (TIVE) cells formed tumors in nude mice modeling KS (10). Early studies modeled KSHV-associated malignancy formation by inoculating SCID mice intraperitoneally or subcutaneously with PEL cells (31, 172). Clinical hallmarks of this system include lymphomagenesis and ascites formation. This system is labor and time intensive, requiring both a long latency period and a large number of cells in order to establish detectable tumors. Injecting PEL cells with matrigel into mice has proven to hasten tumor development (203). Reverse genetics are difficult in this system, but cells stably transfected with wild-type or recombinant KSHV-BACs could be employed in a similar manner to study the contribution of single genes on tumor formation (148).

More recently SCID mice have been injected with KSHV virions to study *de novo* infection and tumor progression. SCID mice engrafted with human skin developed KS-like lesions when virus particles were injected into the graft (78). Persistent infection of humanized NOD/SCID mice following injection of purified KSHV virions revealed that KSHV targets lymphocyte and monocyte cell populations (162). 6% of KSHV-injected mice developed splenomegaly during this relatively short study, but tumor formation could not be confirmed. Nonetheless, this system represents a unique and promising model to study *de novo* KSHV infection. Long-term studies may prove the potential of this system to model KSHV-associated tumorigenesis, as well.

Attempts to study KSHV pathogenesis in a more relevant non-human primate model have been hampered by the species specificity of KSHV. SIV-infected or uninfected rhesus macaques permitted low levels of persistent KSHV infection, but did not seroconvert or

exhibit KSHV-related disease progression (178). These findings suggest that KSHV infection may be abortive in rhesus macaques and therefore is not a good *in vivo* model for KSHV pathogenesis. The closely related gammaherpesviruses rhesus monkey rhadinovirus (RRV) and retroperitoneal fibromatosis-associated herpesvirus (RFHV) may serve as *in vivo* models of KSHV biology and pathogenesis, since they are associated with similar diseases in their natural hosts.

The organization of the genomes of RRV and KSHV are markedly similar, with each ORF of RRV represented in KSHV (7, 192). For most genes, some degree of sequence homology is observed between the viruses. However, KSHV does contain some unique sequences that are not present in RRV. An estimated 90% of captive rhesus macaques have antibodies directed against RRV antigens (24, 66). Experimental infection studies revealed that RRV causes lymphadenopathy resembling MCD and implicated RRV may play a role in the generation of KS-like lesions in SIV-infected macaque s (135, 228). The major advantage of using the RRV system is that the virus can be grown lytically to high titers in rhesus fibroblasts or can be used to model latency in other cell types, such as B lymphocytes (27, 67). Thus, *in vivo* studies that require large amounts of virus are possible by growing large amounts of virus *in vitro* in the lytic RRV culture system. Furthermore, for studying the contribution of individual genes to viral pathogenesis and oncogenesis, reverse genetic systems have been described which increase the utility of this system considerably (28, 67), especially given that the system allows the investigation of lytic as well as latent viral protein functions.

RFHV was isolated from simian AIDS-associated KS-like retroperitoneal fibromatosis (RF) tissue samples harvested at necropsy from macaques during an epidemic in experimental primate colonies (183). RFHV has not been detected in peripheral blood mononuclear cells of living RF-negative macaques. Sequence analysis reveals that RFHV is very closely related to KSHV (182) and may be an ideal virus to study KSHV-related

diseases in a non-human primate model. Additionally, more recent studies have identified other viruses that are closely related to both KSHV and RFHV (95, 207), presenting new options for the pursuit of a suitable animal model of infection.

Project outline

As discussed previously, KSHV is associated with multiple neoplastic diseases, including two B-cell malignancies and one of endothelial origin. Although multiple treatment strategies have been tried and met with variable success, no strategy available today has the ability to cure the latent reservoir of infection with KSHV. The only specific antiviral agents in wide use are nucleotide analogs that selectively target the viral thymidine kinase and block lytic DNA replication. These antiviral agents have generally been more efficacious in MCD, where a higher percentage of infected cells are engaged in the lytic cycle, but the latently infected cells persist regardless. Thus, the overarching theme of this dissertation is to elucidate potential mechanisms for disrupting KSHV latency. Our approach is two-pronged. Our first approach was to investigate the function of the relatively understudied latent viral membrane protein K15. By understanding the role of K15 in viral infection, we may reveal new "druggable targets" or a means to swing the pendulum back in the favor of cell survival and out of the favor of viral proliferation and pathogenesis. The second approach was to devise a method to screen potential antiviral agents (regardless of mechanism) for activity against latent viral infection.

1) Characterization of signal transduction pathways activated by K15 in B lymphocytes

Prior to this work, little was known about the function of K15 in B lymphocyte cells. An early report indicated that when fused to the extracellular domain of the CD8 molecule, K15's cytoplasmic tail could inhibit B-cell receptor signaling as measured by calcium

mobilization experiments (52). Other than that study, the function of K15 had only been investigated in epithelial or endothelial cell lines. K15 expression has been detected in both B-cell malignancies (52, 90, 193). Therefore, studying the function of K15 in B lymphocytes seemed highly relevant. Given that the K15 protein localizes to membranes and its signaling functions have been demonstrated in epithelial and endothelial cell lines, we suspected that K15 might also activate signaling pathways in B lymphocyte cells and that the signals initiated by K15 may promote changes in the cytokine milieu that promote viral pathogenesis and/or oncogenesis. Our suspicions instigated the studies undertaken in Aim 1. In addition to studying K15-mediated changes in cytokine expression, we also investigated the JAK-STAT pathway, a signaling pathway activated in response to multiple cytokines, including IL-6 and Oncostatin M.

2) Characterization of K15 function in the KSHV viral life cycle.

In addition to studying K15 function in overexpression experiments in B lymphocytes, we also used the KSHV-WT and KSHV-ΔK15 bacterial artificial chromosomes to examine the function of K15 in the context of viral infection. We examined K15-mediated changes in cytokine expression. We studied cell growth and performed viral load assays in B lymphocytes harboring WT-KSHV or KSHVΔK15 genomes to determine the effect of K15 expression on cellular proliferation and infection status.

3) Development of an assay to screen antiviral drugs against KSHV

In order to identify antiviral agents with activity against latent viral infection, we employed the KSHV-BJAB system to model latent viral infection of B lymphocytes. A beneficial feature of this system was that cells harboring the virus expressed the green fluorescent protein, thus providing an easy method for screening the samples with sequential fluorescence measurements from the infected cultures.

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Chapter Two

The K15 protein of Kaposi's sarcoma-associated herpesvirus induces the expression of IL-6 and vIL-6 and activates STAT1 in B lymphocytes

Abstract

Growth factors and cytokines play important roles in two B cell malignancies linked to Kaposi's sarcoma-associated herpesvirus (KSHV). IL-6, IL-10, and Oncostatin M are secreted in large amounts in primary effusion lymphoma. IL-6 dysregulation and over-production contribute to multicentric Castleman's disease pathogenesis. In addition, the virus encodes a viral IL-6 (vIL-6) homolog that is highly expressed and stimulates proliferation of infected cells. We investigated the effect of the viral membrane protein K15, which has been detected in both B cell malignancies, on cytokine expression in B cells. Among other cytokines, in B lymphocytes K15 induced expression of Oncostatin M and IL-6, which bind their respective receptors in complex with gp130 to initiate JAK-STAT signaling. Given the well-defined role of IL-6 in KSHV-associated diseases, we probed the mechanism of K15-mediated IL-6 induction and the downstream JAK-STAT pathway. Four K15 isoforms induced cellular IL-6. Reporter assays demonstrated that K15 signaling via the AP-1 pathway activates the cellular IL-6 promoter. The viral IL-6 promoter is also activated by K15. Additionally, we found that K15 expression leads to phosphorylation of the activating tyrosine residue of the signal transducer and activator of transcription 1.

Introduction

Although the absolute numbers of cancer deaths in HIV-positive patients has declined since the advent of the highly active antiretroviral therapy (HAART), Kaposi's sarcoma (KS) is still the leading cancer in the HIV positive population (8). KS caused 13% of all deaths of HIV-positive patients in the post-HAART era (17). In 2000, KS was responsible for 27% of deaths due to AIDS-associated malignancies in France. The Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8, is the etiological agent of KS (11). In addition to KS, KSHV has also been implicated in two B

cell malignancies— primary effusion lymphoma (PEL) (9), and the plasmablastic variant of multicentric Castleman's disease (MCD) (75). KSHV-associated malignancies are more common in post-transplant patients and patients with AIDS (9, 10, 47, 55, 75). Currently there is no cure for viral infection.

The majority of infected cells in all three KSHV-associated malignancies remain latent, with a small percentage of cells undergoing spontaneous lytic replication at any given time (91). During latency only a few viral genes are expressed (35, 73, 78, 91), allowing the virus to escape immune surveillance and to persist without symptoms for the lifetime of an otherwise healthy host. One gene expressed during latent infection in PEL and MCD encodes the viral K15 membrane protein (14, 29, 65, 74).

Multiple K15 isoforms arise from alternatively spliced transcripts (14, 29, 65). The full-length transcript contains eight exons and encodes a protein with a predicted molecular mass of 45-50 kDa and 12 membrane-spanning domains. Three smaller K15 isoforms (Clones 1, 15 and 20) have a common alternatively spliced first exon, that is distinguished from the first exon of the full-length isoform (Clone 35), and share a variable number of exons with Clone 35 (Figure 2.1A). Clones 1 and 35 share exons 2 through 7, Clones 20 and 35 share exons 4 through 8, and Clones 15 and 35 share exons 5 through 8 (14). Thus, the isoforms encode a variable number of transmembrane domains, but they all maintain identical cytoplasmic tails that contain several conserved signaling motifs.

SH2, SH3 and TRAF binding sites are present in the cytoplasmic tails of all K15 isoforms (3, 41, 65, 88). In epithelial cells, K15 activation of the Ras and JNK MAPK signaling pathways is dependent on Src-mediated phosphorylation of the tyrosine residue in the Y^{481}EEV potential SH2 binding site. K15 initiates NF-κB signaling through the TRAF binding site, which has been shown to recruit TRAFs 1, 2 and 3 (3). Highlighting the importance of the SH3 binding sequence PPLP of K15, a recent study found an interaction between the PPLP residues and an SH3 domain of the Intersectin 2 protein, a cellular

regulator of endocytosis (41). An interaction between K15 and the inhibitor of apoptosis Hax-1 suggests a potential anti-apoptotic role for K15 (74).

Despite demonstrating the signaling capabilities of K15, few studies have investigated the consequences of K15 signaling. Recently, K15 signaling has been shown to cause cytokine perturbations in epithelial cell cultures. The expression of K15 in HeLa cells induced expression of IL-8, IL-6, CCL2, CCL20, IL1α/β, and CXCL3 (4). In this chapter, we investigate the role of K15 in cytokine production and signaling in B lymphocyte cells. Although K15 caused increases in multiple cytokines, we focused on IL-6 expression and JAK-STAT signaling, given the central role for this pathway in KS, PEL and MCD pathogenesis. cDNA constructs of four previously described K15 isoforms and a genomic K15 construct exhibited increases in IL-6 transcription and protein expression. We identified an AP-1 site in the IL-6 promoter that was required for activation by K15 and an NF-κB site that augmented activation. Activation of NF-κB and AP-1 transcription factors in B lymphocyte cells required tyrosine phosphorylation of the Y^{481}EEV motif in the cytoplasmic tail of K15. This motif was also required for IL-6 promoter activity. In addition, K15 expression stimulated transcription from the viral IL-6 promoter and resulted in increased vIL-6 protein expression in BCP-1 cells. Finally, K15 also activated the downstream signal transducer and activator of transcription 1 (STAT1) protein.

Materials and Methods

Cell Maintenance

BJAB (KSHV-negative B cell lymphoma) cells (49) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. KSHV-positive but EBV-negative primary effusion lymphoma BCP-1 cells (ATCC CRL-2294) were maintained in RPMI-1640 medium supplemented with 10% fetal

bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 0.1% 2-mercaptoethanol, and sodium bicarbonate solution. Growth medium and supplements were obtained from Cellgro.

Expression of K15 cDNA and genomic constructs

Flag epitope-tagged cDNA expression constructs of four K15 isoforms (Clones 1, 15, 20 and 35)(14) were PCR amplified and cloned downstream of the SRα promoter in the pFJAE plasmid using BamHI and EcoRI restriction enzymes. The Flag epitope-tagged genomic K15 construct was PCR amplified from viral DNA isolated from BCBL-1 cells. The genomic K15 construct was inserted into the pFJAE expression plasmid using KpnI and EcoRV restriction enzymes. 6×10^6 BJAB cells were transfected with 5µg of K15 expression plasmid or the pFJAE parent plasmid using the B cell transfection kit from Amaxa and the nucleofection program T-16. Following transfection cells were grown in complete growth medium for 48h, then lysed, subjected to SDS-PAGE and transferred to nitrocellulose membranes for detection of K15 proteins with an HRP-conjugated anti-ECS antibody (Bethyl).

Cytokine Array

BJAB cells were transfected as described. 48h post-transfection, cells were washed and serum starved for 24h. Conditioned growth medium was collected from the cells and centrifuged to remove cellular debris, before incubation with two cytokine antibody arrays (Raybiotech). A total of 84 cytokines are represented on two membranes VI and VII. The membranes were blocked, and then incubated with conditioned medium from BJAB cells expressing K15 or a vector control. Membranes were incubated with a primary antibody mixture, then an HRP-conjugated secondary antibody and finally with chemiluminescent

substrate. Membranes were exposed to film and individual dot intensity was measured using the ImageJ software available through NIH.

IL-6 ELISA

BJAB cells were transfected as described. 48h post-transfection, cells were washed and serum starved for 24h. Conditioned growth medium was collected from the cells and centrifuged to remove cellular debris. In some experiments, medium was concentrated by centrifugation with a centricon-10 protein concentrator. Secreted IL-6 was detected using a typical sandwich ELISA (eBioscience) as per manufacturer's instructions.

RT-PCR

Following 24h serum starvation, RNA was isolated from BJAB cells expressing K15 or a vector control using an RNeasy (Qiagen) RNA isolation kit. 1µg RNA was reverse transcribed with the Reverse Transcription System (Promega). PCR was performed with the following primer pairs: β-actinF 5'- GGCATCGTGATGGACTCCG-3' and β-actinR 5'-GCTGGAAGGTGGACAGCGA-3', K15 SeqF2 5'-GCTGTGTTGATGACAAACATGCTGG-3' and K15 SeqR2 5'- GACTTAATCCTGCAGCGGTGG-3', or IL6F 5'-GGTACATCCTCGACGGCATCTC-3' and IL6R 5'-GTTGGGTCAGGGGTGGTTATTG-3'. PCR products were resolved by electrophoresis through 1-2% agarose gel and visualized with ethidium bromide staining.

Human (hIL-6) promoter luciferase assays

Human IL-6 promoter luciferase plasmids were obtained from the Belgian Co-ordinated Collections of Micro-organisms/ LMBP Plasmid and DNA library collection (BCCM/LMBP) (Figure 2.5A)(84). The full length promoter reporter plasmid p1168huIL6P-luc+ (or LMBP

4495) consists of 1168nt upstream of the IL-6 gene transcription initiation site driving expression of the luciferase reporter gene. Promoter deletion mutants p50huIL6P-luc+ (or LMBP 4692), p110huIL6P-luc+ (or LMBP 4693) and p234huIL6P-luc+ (or LMBP 4694) contain 50, 110 and 225bp of the human IL-6 promoter. Point mutants of an NF-κB responsive element (p1168hIL6mNFkB-luc+ or LMBP 4496) and AP-1 responsive elements (p1168hIL6m3AP1-luc+ or LMBP4492) were created in the context of the full length IL-6 promoter.

6 x10^6 BJAB cells were transfected with 5µg K15 expression construct, a K15 Y481F mutant, or pFJAE vector as a control plus 0.5µg of a human IL-6 promoter luciferase reporter plasmid using the B cell nucleofection kit (Amaxa) and the nucleofection program T-16. Cells were incubated for 48h in complete medium, then serum-starved for 24h. Luciferase activity was assessed in 50µl of lysate following addition of luciferase substrate (Promega) and normalized to total protein content in the sample.

Measurement of NF-κB and AP-1 activity

NF-κB activity was measured in BJAB cells transfected with 2-5µg of the genomic K15 construct and 100ng of the NF-κB -luciferase reporter plasmid pNF-κB luciferase (Clontech). AP-1 activity was measured in BJAB cells transfected with 5µg of the genomic K15 construct and 500ng of the TRE (TPA-responsive element)-luciferase reporter plasmid (kind gift of Dr. Al Baldwin). In order to determine the effect of K15 signaling on transcription factor activation, 5µg of K15 Clone 35, K15 Y481F or a vector control were co-transfected with either 100ng of pNF-κB luciferase or 500ng of TRE-luciferase into BJAB cells. Cells were grown in RPMI + 10% fetal bovine serum for 48h, then were washed and grown in serum-free medium for an additional 24h. Cells were lysed in reporter lysis buffer

(Promega) and NF-κB -driven luciferase activity was measured in 50µl of lysate. A Bradford assay was performed in tandem to determine the total protein concentration of each sample. Relative luciferase units were normalized to total protein content.

pSTAT1 detection

BJAB cells were transfected as described previously. Lysates were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with phospho-Tyr701 STAT1-specific antibody, phospho-Ser727 STAT1-specific antibody, or total STAT-1 specific antibody (Cell Signaling) to assess phosphorylation status of the downstream STAT1 molecule.

vIL-6 promoter luciferase assays

6 x10^6 BJAB cells were transfected with 5µg K15 expression construct or pFJAE vector as a control plus 0.5µg of a viral IL-6 promoter luciferase reporter plasmid using the human B cell nucleofection kit (Amaxa) and the nucleofection program T-16. Cells were incubated for 48h in complete medium, then serum-starved for 24h. Luciferase activity was assessed in 50µl of lysate following addition of luciferase substrate (Promega) and normalized to total protein content in the sample.

vIL-6 protein detection in KSHV-positive BCP-1 cells

6 x10^6 BCP-1 cells(25) were transfected with 5µg K15 expression construct or pFJAE vector as a control using the human B cell nucleofection kit (Amaxa) and the nucleofection program T-16. Cells were incubated for 48h in complete medium. 10µg total protein per sample were separated by electrophoresis through an 8% SDS-PAGE gel. Protein was transferred to nitrocellulose membranes. The viral IL-6 protein and exogenous Flag-tagged

K15 proteins were detected with rabbit anti-vIL6 antibody (Advanced Biotechnologies) followed by HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-ECS antibody (Bethyl) respectively.

Results

Expression of K15 in BJAB cells

Multiple K15 isoforms have been identified as the result of alternative splicing of the K15 mRNA (14, 29, 65). The four isoforms described by Choi, et al are referenced here. The largest isoform, Clone 35, has a reported molecular weight of 49-55 kDa when ectopically expressed in epithelial cells (3, 14). The smaller isoforms, Clones 1, 15 and 20, have predicted molecular weights of 42 kDa, 31 kDa and 28 kDa respectively (14). KSHV-negative B lymphoma cells (BJAB) were transfected with cDNA constructs of four alternatively spliced Flag-tagged K15 isoforms (Clone 1, 15, 20 or 35) or a genomic Flag-tagged K15 construct (Figure 2.1A). At 48h after transfection, BJAB cells were harvested, lysed and subjected to SDS-PAGE followed by Western blot with an antibody against the Flag epitope-tagged K15 constructs. In the case of BJAB cells transfected with K15 cDNA constructs, major protein bands were detected that were consistent with previous reports (Figure 2.1B). Specifically, a protein band of approximately 50 kDa was detected in cells transfected with Clone 35, whereas 45 kDa, 39 kDa and 35 kDa bands were detected in BJAB cells transfected with Clone 1, 15, or 20 respectively. None of these bands were detected in BJAB cells transfected with a vector control. In BJAB cells transfected with the genomic K15 construct, all four isoforms were detected with the two largest isoforms (Clone 35 and Clone 1) expressed preferentially. The smaller isoforms could only be detected after long exposure times.

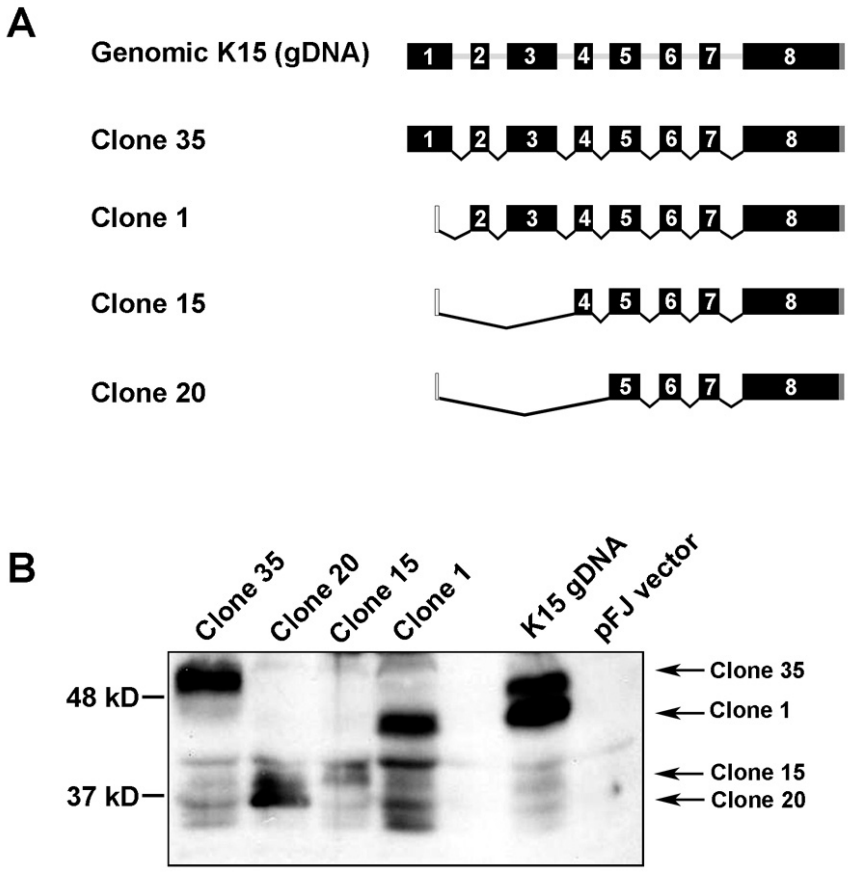

Figure 2.1 Expression of K15 in BJAB cells. A) At least four K15 isoforms arise from multiple alternative splicing events. B) The Flag-tagged K15 cDNA expression constructs Clone 1, Clone 15, Clone 20, and Clone 35 or the genomic K15 construct were transfected into BJAB cells. K15 was detected in cellular lysates by immunoblotting with an anti-ECS antibody to detect the Flag epitope tag.

Cytokine profile of K15-expressing B cells

Since K15 is a membrane protein expressed on the cell surface and is capable of initiating signal transduction cascades, including activation of both the MAPK and NF-κB pathways (3, 14, 88), we sought to determine whether K15 caused any changes in the cytokine milieu when expressed in B lymphocytes. To this end, BJAB cells were transfected with the genomic K15 construct or a vector control. Following 24h of serum starvation, conditioned medium was collected. The presence of 84 cytokines was assessed by an antibody array. Indeed K15 caused multiple perturbations in the cytokine environment,

including at least 2-fold increases in GRO, IL-2Ra, IL-6, IL-12 p40, IL-17, I-309, IGFBP-1, leptin, MCP-1, MCP-2, MCP-3, MIP-3b, MSP-1, NT-4, Osteoprotegrin, Oncostatin M, PDGF-BB, PIGF, TRAIL R4, sTNFRI, sTNFRII, and VEGF-D (Table 1). K15-expressing BJAB cells also exhibited at least a 2-fold decrease in the expression of some cytokines, including Eotaxin, ICAM-1 and BTC (Table 2.1). Given that there are high levels of circulating IL-6 found in KSHV-associated malignancies and the demonstrated role for both viral IL-6 (vIL-6) and human IL-6 (hIL-6) in PEL (12, 20, 23, 36, 59), we pursued human IL-6 as an important downstream target of K15 signaling.

Table 2.1 K15 alters cytokine expression in BJAB cells

Growth Factor/ Cytokine	Fold Change		Growth Factor/ Cytokine	Fold Change
Eotaxin	-9.6		sTNFRII	2.3
ICAM-1	-4.8		IGFBP-1	2.5
BTC	-3.3		MIP-3b	2.6
MCP-2	2.0		TRAIL R4	2.7
VEGF-D	2.0		Osteoprotegrin	2.9
IL-12 p40	2.0		NT-4	3.0
sTNF-RI	2.0		Oncostatin M	3.2
GRO	2.0		IL-6	3.6
MCP-1	2.0		MSP-1	4.6
leptin	2.1		IL-17	8.3
IL-2Ra	2.2		PIGF	8.3
I-309	2.2		MCP-3	20.7
PDGF-BB	2.3			

K15 expression upregulates hIL-6 in BJAB cells

In order to confirm that K15 expression increases hIL-6 expression, an ELISA for hIL-6 was performed using conditioned medium collected from BJAB cells expressing K15 cDNA constructs or a vector control (Figure 2.2A). When expressed individually, all four K15 constructs increased hIL-6 expression, although to varying degrees. In one representative experiment, K15 Clone 1, 15, 20, and 35 showed 3.9-, 10.1-, 10.3-, and 5.3-

fold increases in hIL-6 secretion respectively, as compared to the vector control (Figure 2.2A). In repeated experiments, the smaller K15 clones 15 and 20 exhibited the greatest induction of hIL-6 secretion.

K15 increases gene expression levels of human IL-6 in BJAB cells

In order to determine if IL-6 is regulated by K15 at the level of transcription, RNA was isolated from BJAB cells expressing a K15 cDNA construct, a genomic K15 construct or a vector control. IL-6 mRNA levels were assessed by RT-PCR (Figure 2.2B). Although all four K15 isoforms could increase IL-6 expression, the smaller K15 isoforms exhibited the most dramatic increases in IL-6 mRNA (Figure 2.2B). When normalized to β-actin and as compared to the vector control, K15 Clone 1, Clone 15, Clone 20 and Clone 35 increased mRNA levels by 4.7-fold, 11.1-fold, 6.8-fold and 2.2-fold respectively. In contrast, no IL-6 was detected in the "no RT" controls. Furthermore, all four K15 isoforms drove expression of a luciferase reporter gene, that was cloned downstream of the full-length hIL-6 promoter (p1168huIL6P-luc+). As compared to the empty vector control, K15 clones 1, 15, 20 and 35 activated the hIL-6 promoter four- to six-fold (Figure 2.2C). Additionally, the K15 genomic construct activated the hIL-6 promoter nearly seven-fold as compared to its vector control (Figure2.2C). Since the genomic K15 construct behaved similarly to the K15 cDNA constructs in both direct measurement of IL-6 expression and in reporter assays, the genomic K15 construct was used for all subsequent experiments.

K15-mediated IL-6 expression leads to hyperphosphorylation of STAT1

IL6-IL6 receptor signaling has previously been shown to activate the JAK/STAT pathway resulting in the activation of signal transducer and activator of transcription 1 (STAT1) (28, 46). Thus, we investigated K15's ability to activate the downstream IL-6

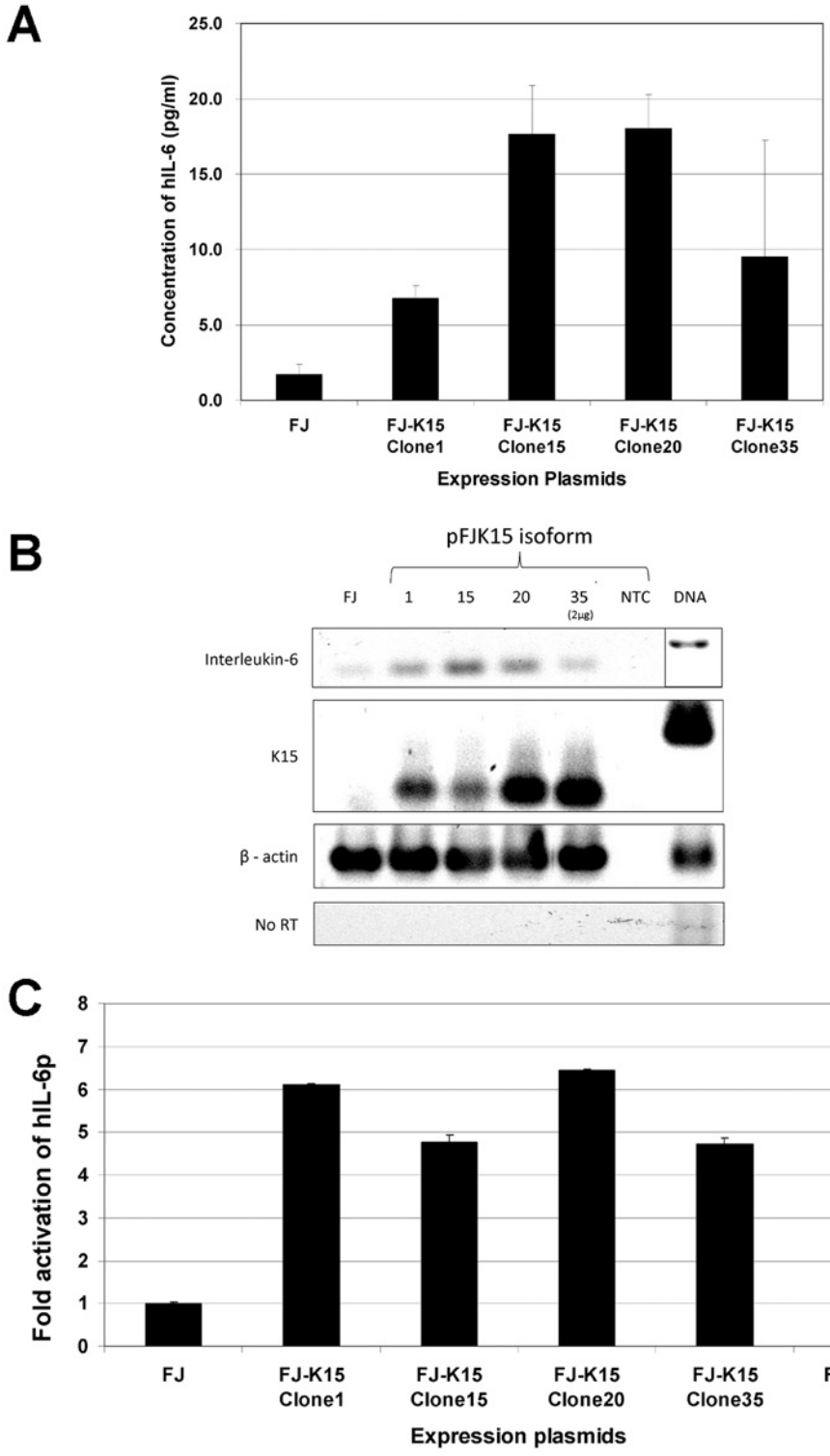

Figure 2.2 K15 induces the expression of cellular interleukin-6 in B lymphocytes. Interleukin-6 (IL-6) was one of many cytokines and growth factors induced by a genomic K15 construct in BJAB cells as identified by a cytokine expression array. The induction of IL-6 by the smaller K15 isoforms and the genomic K15 construct was confirmed by ELISA (A), RT-PCR (B), and IL-6 promoter luciferase reporter assays (C).

signaling molecule STAT1. Serum-starved BJAB cells transfected with K15 cDNA constructs or a vector control were lysed and subjected to SDS-PAGE followed by immunoblot using antibodies that specifically react with STAT1 or activated phosphoTyr701-STAT1. Increased levels of phosphorylated STAT1 (218-378% as compared to the vector control) were detected in cells expressing K15 as compared to the vector control, although no changes in total STAT1 were evident (Figure 2.3).

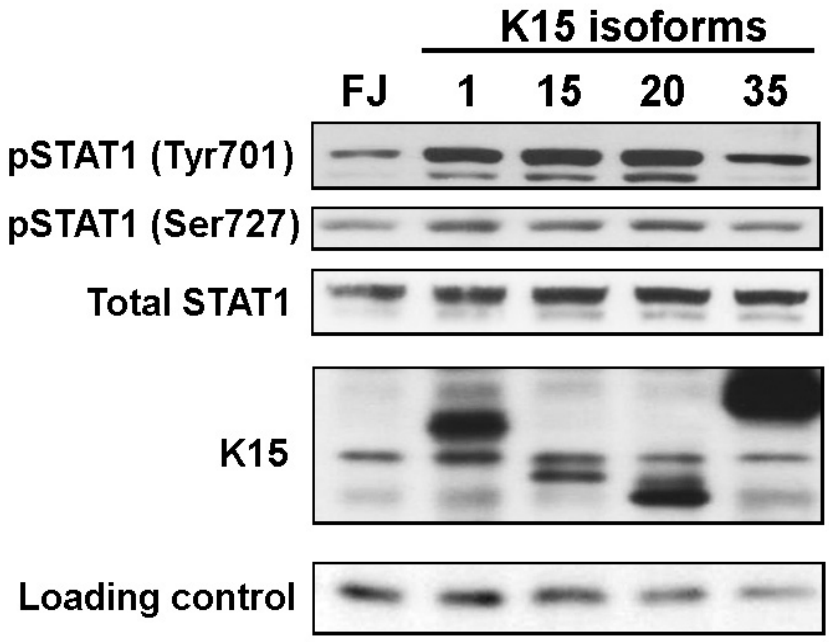

Figure 2.3 K15 expression results in hyperphosphorylation of STAT1. K15 expression in B cells causes hyperphosphorylation of the activating tyrosine residue 701, and to a lesser extent of the serine residue 727, of the Signal Transducer and Activator of Transcription-1 (STAT1). STAT1 is a downstream effector of the IL-6/JAK-STAT signaling axis. Thus, its activation may be a result of IL-6 overexpression.

K15-mediated IL-6 upregulation requires K15 signaling

Since K15 mediates IL-6 upregulation at the transcriptional level, we investigated the requirement for K15 signaling. K15 is a membrane protein expressed on the cell surface and the cytoplasmic tails of all K15 isoforms contain highly conserved putative signaling motifs (29). It has been previously reported that K15 mobilizes multiple transcription factors

including AP-1 and NF-κB in epithelial cells and that the putative SH2 binding site is important for its signaling functions (3, 88). We first determined if K15 expression activates NF-κB signaling pathways in B lymphocytes by co-transfecting a genomic K15 expression construct or a vector control with an NF-κB –responsive luciferase plasmid (Figure 2.4A). Expression of the genomic K15 construct invoked a 2.6-fold increase in NF-κB-driven luciferase activity as compared to a vector control. Thus, K15's ability to activate NF-κB in B lymphocytes is lower than in epithelial cells (3). Mobilization of AP-1 transcription factors was also examined by co-expression of a genomic K15 expression construct or a vector control with a luciferase reporter plasmid containing a TPA-responsive element (TRE) in the promoter (Figure 2.4A). AP-1 activity, as determined by the TRE-luciferase construct, was 3.6-fold higher in BJAB cells expressing the genomic K15 construct as compared to the vector control.

Tyrosine residue 481 in the cytoplasmic tail of K15 has been shown to be important for NF-κB and AP-1 signalling in epithelial cells (3, 88). Therefore, we examined if tyrosine residue 481 is a requirement for K15-mediated NF-κB and AP-1 activation. The full length K15 isoform (Clone 35), a full length K15 isoform with the tyrosine 481 residue mutated to phenylalanine (K15 Y481F), or a pFJ vector control was transfected into BJAB cells with an NF-κB responsive luciferase reporter or with the TRE-luciferase construct. Expression of the full length wild-type K15 Clone 35 resulted in a 2.1-fold increase in NF-κB driven luciferase activity as compared to the vector control, while K15 Y481F showed no increase in NF-κB activity (Figure 2.4B). Similarly, K15 Clone 35 caused an 8.4-fold increase in AP-1 activity in contrast to the K15 Y481F mutant which exhibited a minor increase (1.6-fold) as compared to the vector control (Figure 2.4B).

Finally, to determine if signaling through the putative SH2 binding site in the cytoplasmic tail of K15 is necessary for activation of the cellular IL-6 promoter, the K15 Clone35, K15 Y481F, or a pFJ vector control was transfected into BJAB cells with the full-

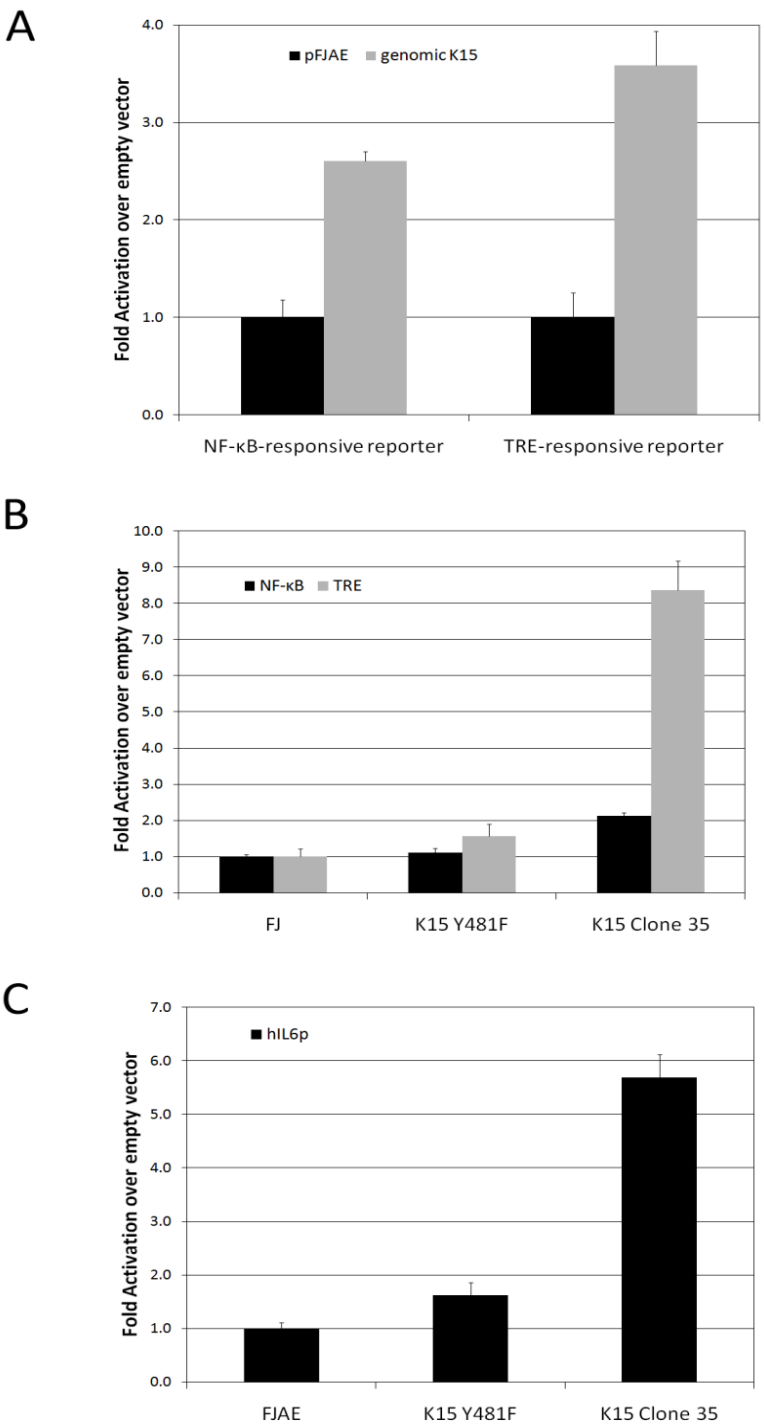

Figure 2.4 Induction of IL-6 by K15 is dependent on K15 signaling. A) Activation of NF-κB and AP-1 by K15 was determined by luciferase assay. BJAB cells expressing K15 (gray bars) or a vector control (black bars) were cotransfected with an NF-κB-responsive luciferase reporter plasmid or an AP-1 responsive luciferase plasmid (TRE-luciferase). Results are presented as fold activation as compared to the vector control. B) An NF-κB-responsive luciferase construct (black bars) or an AP-1 responsive luciferase plasmid (gray bars) was transfected into BJAB cells expressing the wild-type full length K15 Clone 35 cDNA, a full length K15 Y481F mutant, or an FJ vector control. Fold activation over the empty vector is reported for each of the reporter constructs. C) BJAB cells were transfected with the full length human IL-6 promoter luciferase reporter construct and a wild-type full length K15 expression construct (Clone 35), a full length K15 expression construct with a mutation in the putative SH2 binding domain (K15 Y481F), or a vector control. Activation of the promoter was measured by luciferase and is displayed as fold activation over the vector control.

length IL-6 promoter luciferase reporter plasmid. A luciferase assay was performed (Figure 2.4C). K15 Clone 35 induced a 5.7-fold increase in IL-6 promoter luciferase activity as compared to the vector control, whereas the K15 Y481F mutant exhibited a negligible increase in promoter activity (1.6-fold as compared to the vector control).

Determination of the K15-responsive elements in the hIL-6 promoter

To further define the K15-responsive elements in the hIL-6 promoter, we obtained several hIL-6 deletion and point mutant hIL-6 promoter luciferase reporter plasmids (Figure 2.5A) from the Belgian Co-ordinated Collections of Micro-organisms/ LMBP Plasmid and DNA library collections (BCCM/LMBP) (84). BJAB cells expressing K15 were co-transfected with the deletion mutant hIL-6 promoter luciferase constructs. Serum-starved cells were lysed and luciferase activity was determined. No activation of the minimal promoter was evidenced by either K15 or the empty vector control (p50huIL6P-luc+) (Figure 2.5B). In general, K15-mediated IL-6 induction increased as more of the IL-6 promoter was incorporated into the luciferase reporter plasmid. The p110huIL6P-luc+, p234huIL6P-luc+ and p1168huIL6P-luc+ constructs contained 100, 234 and 1168 nucleotides of the IL-6 promoter inserted upstream of the luciferase gene, respectively. Genomic K15 transactivated the p110huIL6P-luc+ construct two-fold, the p234huIL6P-luc+ three-fold, and the p1168huIL6P-luc+ four-fold compared to the empty vector control (Figure 2.5B).

Multiple transcription factors, but most notably NF-κB and AP-1, have been implicated in the upregulation of IL-6 in response to various stimuli (inflammatory cytokines, microbial pathogens, etc.) and in spontaneous tumor formation (13, 19, 37, 54, 58, 64, 84, 86). Importantly, upon infection of epithelial cells, KSHV activates AP-1 transcription factors (90). In B lymphocytes, K15 signaling through tyrosine residue 481 activates AP-1 and NF-κB transcription factors and is also required for K15-mediated human IL-6 promoter activation as shown in Figure 2.4. Since K15 expression in B lymphocytes caused increases in both NF-κB and AP-1 activity, the proximal NF-κB and AP-1 transcription factor binding site mutants of the full length IL-6 promoter luciferase reporter construct were employed to further delineate K15-responsive elements. BJAB cells were co-transfected with a K15 expression construct and an NF-κB or AP-1 mutant hIL-6 promoter luciferase plasmid. Cells were serum-starved and luciferase activity was determined. Human IL-6

promoter activity was diminished to near background levels with the 3' AP-1 mutant but remained unchanged with the NF-κB promoter mutant in cells expressing K15 as compared to the empty vector controls (Figure 2.5C).

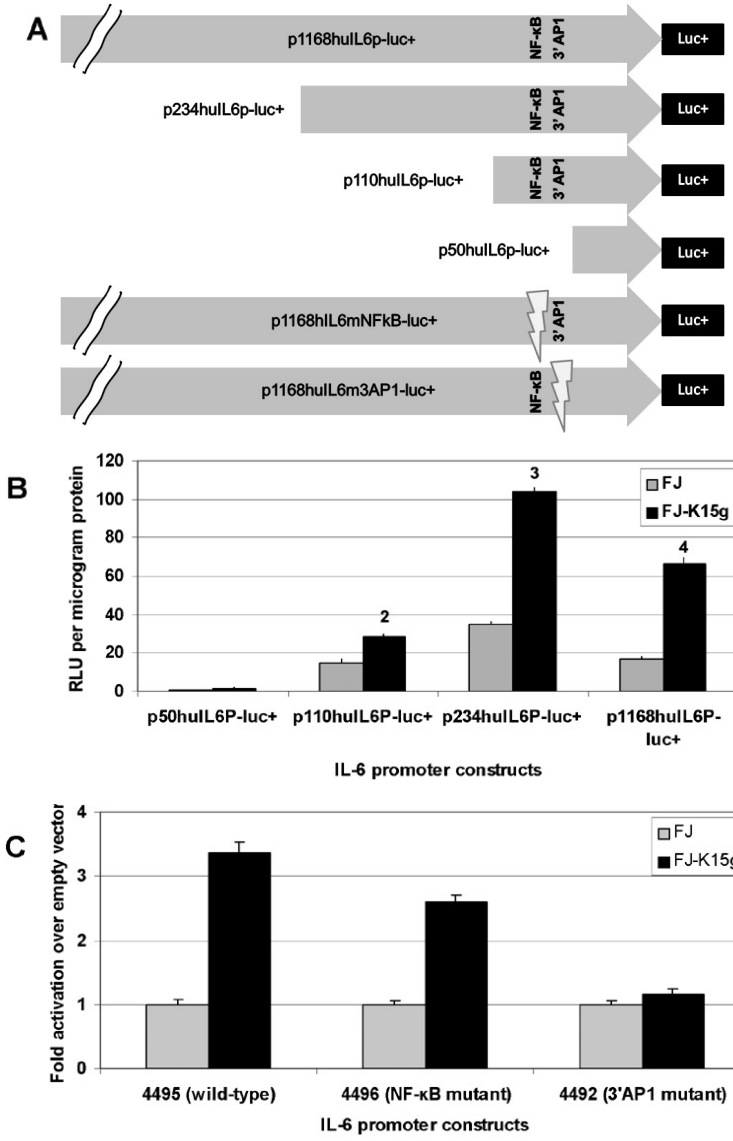

Figure 2.5 Determination of K15-responsive elements in the human IL-6 promoter. A) Deletion and point mutant human IL-6 promoter luciferase constructs were used to determine the K15-responsive elements in the human IL-6 promoter. B) Genomic K15 (black bars) or a vector control (gray bars) was expressed in BJAB cells along with a full length IL-6 luciferase reporter plasmid or deletion constructs containing 234 (p234hulL6p-luc+), 110 bp (p110hulL6p-luc+), or 50bp (p50hulL6p-luc+) of the IL-6 promoter. Results are expressed as RLU per µg protein. Numbers above each pair indicate fold activation over the vector control. C) BJAB cells were transfected with a genomic K15 expression construct (black bars) or a vector control (gray bars) as well as full length IL-6 luciferase reporter plasmids. Wild-type IL-6 promoter constructs and NF-κB or AP-1 mutant IL-6 promoter constructs were tested. Results are expressed as fold activation over a matched vector control.

Investigation of a potential feedback loop with vIL-6

KSHV encodes a viral IL-6 homolog (52). Like K15, vIL-6 is detected in latency, but is upregulated during lytic reactivation (35, 63, 80). It is highly expressed in PEL and has been reported to form an autocrine activation loop with hIL-6 (7, 51, 53, 60, 77). Therefore, activity of the vIL-6 promoter was also assessed in K15-expressing BJAB cells. A K15 expression construct was co-transfected with a vIL-6 promoter luciferase reporter plasmid into BJAB cells. Cells were serum-starved for 24 hours prior to lysis and measurement of luciferase activity. K15 activated the vIL-6 promoter more than five-fold as compared to the vector control (Figure 2.6A).

Next, to demonstrate that K15 enhances vIL-6 expression in naturally infected primary effusion lymphoma cells, a Flag-tagged genomic K15 expression construct or a vector control was transfected into the KSHV-positive PEL cell line, BCP-1. Viral IL-6 protein expression was assessed in cell lysates by immunoblotting with a vIL-6 specific antibody. Introduction of the Flag-tagged genomic K15 construct into BCP-1 cells increased vIL-6 protein expression approximately two-fold in uninduced BCP-1 cells as compared to the vector control (Figure 2.6B).

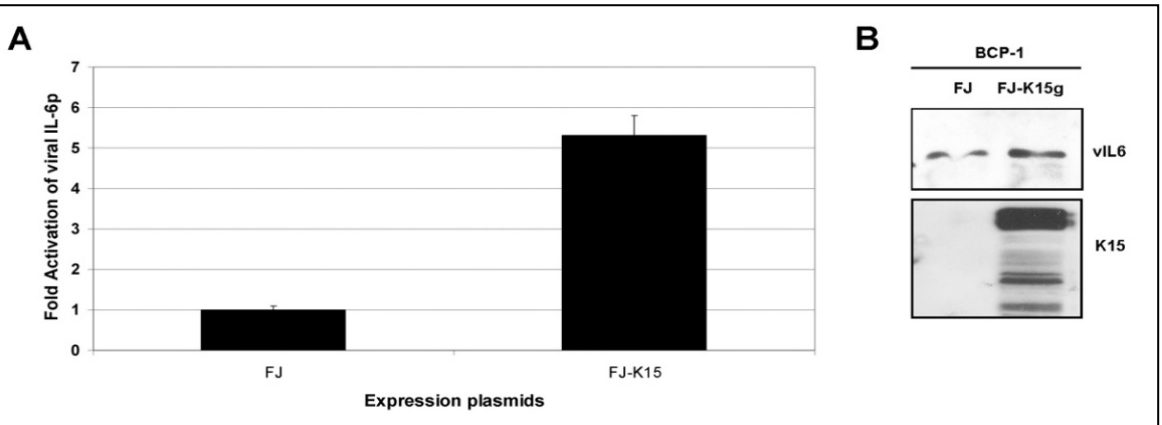

Figure 2.6 K15 expression in B cells induces expression of the viral IL-6 homolog. A) BJAB cells expressing the genomic K15 construct or a vector control were transfected with a viral IL-6 promoter-responsive luciferase plasmid. Luciferase activity is presented as fold activation over a vector control. B) Cells of the primary effusion lymphoma cell line BCP-1 were transfected with a Flag-tagged genomic K15 construct. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and vIL-6 and K15 proteins were detected with anti-vIL-6 antibody followed by HRP-conjugated anti-rabbit IgG or with an HRP-conjugated anti-ECS antibody respectively.

Discussion

The KSHV K15 protein has been detected in primary effusion lymphoma cells and in plasmablasts isolated from patients with multicentric Castleman's disease (74). K15 has been characterized as a latent gene and has been shown to inhibit signaling through the B cell receptor (14, 29, 74). In this chapter we have investigated the impact of K15 expression in B lymphocytes. Earlier studies investigated the function of the individually spliced K15 isoforms in various assays. Here we have created and characterized a genomic K15 expression construct which expresses multiple isoforms in the same cell and may be more physiologically relevant. While all four K15 isoforms could be detected in B lymphocytes transfected with the K15 genomic construct, Clone 35 (49 kDA) and Clone 1 (45 kDa) were expressed at much higher levels than the smaller isoforms Clone 15 (39 kDa) and Clone 20 (35 kDa), indicating that either the larger isoforms are preferentially transcribed or that the smaller isoforms are less stable and are rapidly degraded. Similar to the larger K15 isoforms, the smaller isoforms induced IL-6 transcription and secretion when expressed individually in B lymphocytes, indicating that they are functionally active.

A hallmark of all three KSHV-associated malignancies is the dysregulation of multiple cytokines and growth factors (16, 20, 21, 36, 50, 52, 59, 61, 71, 72, 79). Cytokine and growth factor expression levels can be quickly altered to respond to environmental cues. Stimulus of a cellular receptor poised to detect changes in either the intracellular or the extracellular environment initiates a signaling cascade which culminates in induction or supression of the appropriate cytokines. Given the localization and signaling properties of K15, we hypothesized that K15 signaling may result in changes in the cytokine environment. We used a cytokine antibody array to characterize the changes in cytokine expression resulting from expression of the genomic K15 construct in B lymphocytes. In BJAB cells, K15 induced the expression of GRO, IL-2Ra, IL-6, IL-12 p40, IL-17, I-309, IGFBP-1, leptin,

MCP-1, MCP-2, MCP-3, MIP-3b, MSP-1, NT-4, Osteoprotegrin, Oncostatin M, PDGF-BB, PIGF, TRAIL R4, sTNFRI, sTNFRII, and VEGF-D. IL-6, MCP-1, MIP3α and GRO3 cytokines were similarly induced in epithelial cells (4). However, K15 did not elicit IL1α/β or IL-8 in B cells as in epithelial cells, suggesting that regulation of cytokine expression by K15 may be cell-type specific (4).

IL-6, IL-10, VEGF and Oncostatin M (OSM) are upregulated in PEL and MCD (1, 20, 22, 36, 59). IL-6 and OSM are members of the same cytokine family, initiating signaling cascades through interaction with a specific receptor and the common gp130 subunit (27, 44, 81). Cellular IL-6 binds to the IL6 receptor alpha (IL6-Rα)-gp130 complex to initiate signaling via the JAK-STAT pathway (24, 32, 34, 39, 40, 45, 46, 81, 83). IL-6 and OSM are potent mitogens of KSHV-infected cells (6, 23, 50) and may protect the infected cell from programmed cell death. Of note, expression of IL-6, VEGF and OSM was positively regulated by K15 in the cytokine array analysis. Thus, K15 expression in the context of viral infection may ensure survival of the infected cell by release of cytokines with anti-apoptotic and proliferative end effects.

As the role of IL-6 in viral infection is well documented, we investigated the mechanism by which K15 upregulates IL-6 expression. Four cDNA constructs of K15 were assessed individually for their ability to induce IL-6 expression. The four isoforms caused variable increases in IL-6 secretion as determined by ELISA, with IL-6 levels apparently inversely related to the level of K15 expression as determined by Western blot. Increases in IL-6 transcription as determined by RT-PCR and IL-6 promoter luciferase reporter assays did not exhibit the same inverse relationship to the isoform expression levels. This suggests that all four isoforms are similarly capable of signal transduction, but that the isoforms may differentially regulate protein translation or secretion. More likely the variable levels of secreted IL-6 may have been caused by functional impedance and an artifact of overexpression, since 2 µg of K15 Clone 35 cDNA enhanced IL-6 production as compared

to 5 µg, achieving levels similar to those induced by the smaller isoforms when transfected into B lymphocytes (data not shown).

We argued that the genomic K15 construct was likely to be more physiologically relevant than using any individual cDNA construct, since relative expression of the isoforms was likely similar to natural infection and would represent the overall effect of K15 expression in the B lymphocyte. Furthermore, since all four isoforms and the genomic K15 construct exhibited similar effects with respect to IL-6 expression, we used the genomic K15 construct to further investigate the mechanism of regulation. Using a panel of deletion mutants of the IL-6 promoter luciferase reporter plasmids, we determined that the shortest fragment activated by K15 contained an NF-κB and an AP-1 transcription factor binding site. Longer IL-6 promoter fragments further increased activity of the luciferase reporter.

It had previously been reported that NF-κB and AP-1 are important for IL-6 promoter activation by various stimuli (19, 37, 58, 90). We found that K15 can activate both transcription factors in B lymphocytes. However, NF-κB induction was muted in B cells as compared to epithelial cells (3) and may reflect a constitutively high background level of NF-κB activity in BJAB cells (2). In epithelial cells, K15-mediated activity of NF-κB and AP-1 transcription factors was dependent on the tyrosine 481 residue (3, 88). We determined that this residue was also required for NF-κB and AP-1 activity in B lymphocytes, further underscoring the importance of this signaling domain to K15 function.

After determining that K15 was capable of activating NF-κB and AP-1 in B cells, we used a cadre of IL-6 promoter mutants and a signaling incompetent K15 construct to explore the roles of these signaling moieties in K15-mediated IL-6 induction. The K15 Y481F mutant did not induce IL-6 promoter activity, suggesting that the signal transduction cascades initiated by this domain are central to the upregulation of IL-6 by K15. The tyrosine residue of this putative SH2 domain was also required for NF-κB and AP-1 driven promoter activity. However, mutation of the NF-κB site in the IL-6 promoter did not

significantly affect induction of the promoter in response to K15. In contrast, mutation of the 3'AP-1 responsive element in the IL-6 promoter ablated promoter activity. Together these data suggest that K15 initiates signaling cascades in B cells that activate AP-1 transcription factors resulting in the upregulation of cellular IL-6 expression. K15-mediated NF-κB activation may augment but is not required for IL-6 promoter activity. Emphasizing the importance of this finding, KSHV mobilizes AP-1 transcription factors resulting in upregulation of IL-6 expression within six hours after infection of epithelial cells (90).

In addition to stimulating cellular IL-6, KSHV encodes its own viral IL-6 homolog (vIL-6) that is highly expressed in PEL and MCD (7, 52, 63, 77). vIL-6 is primarily a lytic transcript and its expression is very highly induced during viral reactivation, although low levels of this protein are detected during latency (63, 80). We found that K15 increased vIL-6 promoter activity in BJAB cells, indicating that K15 not only induces cellular IL-6, but can also upregulate viral IL-6 transcription. We further demonstrated that introduction of exogenous K15 increases vIL-6 protein expression in the KSHV-positive primary effusion lymphoma BCP-1 cell line. vIL-6 drives PEL cell proliferation similarly to cellular IL-6 (12, 23, 36). Therefore K15 may also indirectly contribute to survival of KSHV-infected cells, by stimulating expression of vIL-6. What is more, vIL-6 interacts directly with the gp130 signal transducer without the need for a specific receptor, in contrast to IL-6 and OSM (51, 53, 60, 87). Since gp130 is ubiquitously expressed, the effects of vIL-6 are potentially more widespread and not restricted to cells expressing specific receptors. Thus, K15-mediated induction of vIL-6 may also serve to stimulate proliferation of uninfected cells in the extracellular milieu.

The high levels of IL-6 expression in KSHV-associated malignancies and the fact that the virus encodes an IL-6 homolog underscore the importance of this cytokine and downstream IL-6 effectors in viral pathogenesis. IL-6 signaling has been shown to result in activation of the JAK-STAT pathway (28, 46). We examined downstream signaling

molecules and observed that activated signal transducer and activator of transcription (STAT) 1 is differentially expressed in BJAB cells transfected with K15. Although IL-6 reportedly activates both STAT1 and STAT3, its proliferative and anti-apoptotic effects are more commonly associated with STAT3, whereas STAT1 is more commonly associated with an interferon-induced antiviral response (15, 33, 67). Thus the increase in activated STAT1 with stable levels of activated STAT3 in K15-expressing cells was perplexing.

Many viruses have devised immune evasion strategies including degradation of STAT1, inhibition of STAT1 activation, and sequestration of STAT1 outside the nucleus (5, 18, 26, 30, 38, 42, 43, 56, 62, 66, 69, 82, 85, 89). We found that in B cells expressing K15, phosphorylated STAT1 did not directly interact with K15 and was present at increased levels in both cytoplasmic and nuclear fractions (unpublished data) arguing against sequestration. Furthermore, K15 expression did not interfere with a Type I interferon response (unpublished data) indicating that STAT1 activity was intact. Thus, K15 does not inhibit classic STAT1 functions and its activation is likely to play a more integral role in KSHV pathogenesis.

Despite the large arsenal of STAT-1 inhibitory mechanisms employed by viruses, induction of and benefit from STAT1 activation have also been reported. In fact, the latent membrane protein (LMP) 1 and SM protein of the closely related Epstein Barr virus also stimulate STAT1 activation (57, 68, 70), but may induce a form of STAT1 that is distinct from the form integral to the antiviral response (48). STAT1 may also serve as a scaffolding protein to orchestrate multiple downstream signaling events (76). Furthermore, the function of STAT1 may be modulated by heterodimerization with the pro-survival and growth stimulatory STAT3 protein (31). In fact, the viral IL-6 homolog of KSHV initiaties JAK-STAT signaling that results in STAT1/STAT3 heterodimerization (51). Therefore, K15's ability to induce STAT1 is likely IL-6 dependent, but may also result from other cytokines, such as OSM, that are induced by K15 (76). Future studies exploring protein-protein interactions

and the DNA-binding properties of STAT1 in B lymphocytes expressing K15 will be necessary to define the functional consequences of IL-6/JAK-STAT signaling in the context of infection.

Acknowledgments

Special thanks to the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMBP) for providing the human IL-6 promoter luciferase plasmids and to Al Baldwin for providing the TRE-luciferase reporter plasmid. This work was supported by NIH grants CA096500 and HL083469 and a Burroughs Welcome Fund grant to BD, as well as the Cancer Cell Biology training grant to TKN. BD is a Leukemia & Lymphoma Society Scholar and Burroughs Welcome Fund Investigator in Infectious Disease.

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Chapter Three

Wild-type KSHV-BJAB cells secrete more IL-6 and exhibit higher cell-free viral loads than KSHVΔK15-BJAB cells

Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) encodes multiple isoforms of the K15 transmembrane protein. Full length K15 is predicted to contain twelve membrane-spanning domains. Smaller isoforms contain fewer membrane domains but also localize to cellular membranes. All isoforms share identical cytoplasmic tails that contain conserved signaling motifs. K15 is expressed in the two B cell malignancies linked to KSHV, including primary effusion lymphoma (PEL) and the lymphoproliferative disorder, multicentric Castleman's disease (MCD). Cytokines are likely to play an important role in disease progression in MCD and in PEL. In particular, interleukin-6 (IL-6) is highly expressed in all KSHV-associated tumors. We previously observed that K15 signaling induces multiple cytokines, most notably IL-6, when expressed ectopically in B lymphocyte cells. In order to determine the consequences of K15 expression in the KSHV-infected cell, we constructed a KSHVΔK15 mutant virus using the KSHV bacterial artificial chromosome (BAC) system. We examined IL-6 expression, proliferation rates and viral loads of cells harboring the WT-KSHV or KSHVΔK15-BAC. We found that IL-6 secretion was significantly higher in cells transfected with the WT-KSHV BAC. Although K15 had no effect on proliferation rate of a transformed B lymphocyte cell line, in the absence of K15 higher cell-free viral loads were achieved, suggesting that K15 may promote latency in B cells.

Introduction

The Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8, has been linked to Kaposi's sarcoma (KS)(9), primary effusion lymphoma (PEL)(7), and the plasmablastic variant of multicentric Castleman's disease (MCD)(52). The virus poses a significant threat to immunocompromised populations, as the KSHV-

associated malignancies are more common in post-transplant patients and patients with AIDS (7, 8, 32, 38, 52). Treatment is typically palliative not curative (reviewed in (25)).

In an otherwise healthy host, KSHV establishes persistent infection via a highly complex and poorly understood process. After primary infection, the virus establishes latency and persists in a quiescent state, with only a few viral genes expressed (22, 48, 54, 64). KS tumors are comprised mainly of KSHV-infected spindle cells of endothelial origin (2, 55, 64), whereas KSHV-infected B lymphocytes are the oncogenic source of PEL and MCD (7, 52). While the majority of infected cells in all three malignancies remain latent, a small percentage of cells undergo spontaneous lytic replication at any given time (64).

Inflammatory cytokines contribute to viral pathogenesis. Infiltrating inflammatory cells secrete numerous cytokines, such as IL-1α, IL-6, Oncostatin M, TNFα, TNFβ, and IFNγ that stimulate spindle cell formation and proliferation in KS (5, 14, 15, 34, 41, 51, 56). Angiogenic cytokines such as VEGF and bFGF play an important role in the highly vascularized KS lesions (12, 45-47). In PEL and MCD, IL-6 and IL-10 are expressed at high levels (13, 16, 23, 39). Additionally, VEGF and Oncostatin M are also produced by PEL cells (1, 13). IL-6 and VEGF were found to be necessary to stimulate the growth of PEL cells injected into SCID mice (1, 17).

In addition to cytokines released from inflammatory cells, KSHV encodes a viral IL-6 homolog (vIL-6) that is expressed at high levels in PEL and MCD (6, 36, 42, 53). vIL-6 can bind directly to the gp130 coreceptor molecule to initiate JAK-STAT signaling, thereby bypassing the need for the IL-6 receptor sub-unit (35, 37, 40, 59). The expression of vIL-6 further enhances IL-6 signaling and drives PEL cell proliferation (10, 23). Furthermore, viral proteins such as K1 and K15 have been shown to induce expression of inflammatory cytokines when expressed ectopically in cell culture. For instance, K1 stimulates VEGF expression in endothelial cells, suggestive of a role in neovascularization (62). Injection of K1-expressing C33A cells into nude mice resulted in highly vascularized lesions(61). The

expression of K15 in HeLa epithelial cells induced expression of IL-8, IL-6, CCL2, CCL20, IL1α/β, and CXCL3 (4). We have seen that K15 expression in B lymphocytes leads to overexpression of numerous cytokines, including GRO, IL-2Ra, IL-6, IL-12 p40, IL-17, I-309, IGFBP-1, leptin, MCP-1, MCP-2, MCP-3, MIP-3b, MSP-1, NT-4, Osteoprotegrin, Oncostatin M, PDGF-BB, PIGF, TRAIL R4, sTNFRI, sTNFRII, and VEGF-D.

The K15 open reading frame (ORF) encodes multiple K15 isoforms, stemming from alternatively spliced transcripts (11, 19, 44). The isoforms encode a variable number of transmembrane domains, but they all maintain identical cytoplasmic tails that contain several conserved signaling motifs, including potential SH2, SH3 and TRAF binding sites (3, 29, 44, 60). The tyrosine 481 residue, which is incorporated into both the SH2 binding domain and the TRAF binding site, is particularly important for K15 signaling functions in epithelial as well as B lymphocyte cells.

In the setting of natural infection, K15 expression has been detected in the two KSHV-associated B cell malignancies—in PEL cells and in plasmablasts of MCD (49). K15 transcripts have been detected in unstimulated PEL cells (11, 19, 44). However, the K15 promoter is responsive to the viral lytic switch protein and K15 transcripts increase upon induction of the lytic cycle by phorbol esters (4, 11, 19, 63). A 23 kDa protein was detected in BCBL-1 PEL cells using an antibody raised to the cytoplasmic tail of the K15 protein (49). A 45 kDa protein consistent in size with the full-length K15 protein was detected in epithelial cells transfected with a KSHV bacterial artificial chromosome containing the entire KSHV genome, and its expression increased upon induction of lytic replication (4).

In this chapter, we describe the construction of a KSHVΔK15 bacterial artificial chromosome (BAC) that was used to determine the function of the K15 protein in the context of the virus. IL-6 concentrations in uninduced B lymphocytes containing the KSHVΔK15-BAC were not measurable but reached 4.5 pg/ml in cells containing the wild-type KSHV-BAC. IL-6 expression and secretion was impaired two-fold in KSHVΔK15-BJAB

cells as compared to the WT KSHV-BJAB cells when the lytic cycle was induced with phorbol 12-myristate 13-acetate (PMA/TPA). BJAB cells transfected with the KSHV-WT and KSHVΔK15 bacterial artificial chromosomes showed similar growth and survival patterns. However, the introduction of the KSHVΔK15 virus into BJAB cells achieved higher cell free viral loads than the introduction of the KSHV-WT virus, suggesting that K15 may regulate lytic reactivation and promote viral latency.

Materials and Methods

Creation of the KSHVΔK15 bacterial artificial chromosome

The wild-type KSHV bacterial artificial chromosome (WT KSHV-BAC) has been previously described(65). Briefly, the WT KSHV-BAC contains the entire viral genome, a green fluorescent protein expression cassette under control of a constitutive promoter, and a hygromycin resistance gene. Therefore, cells harboring the virus fluoresce green and survive hygromycin B selection in mammalian cells. To create the KSHVΔK15-BAC, a kanamycin resistance (Kan^R) gene expression cassette was inserted into the open reading frame (ORF) of the K15 gene by homologous recombination. The Kan^R cassette was amplified from the pKD13 plasmid, and was extended with flanking regions homologous to the K15 ORF. The PCR product was cleaved with DpnI to remove any input template DNA, and then gel purified from a 0.8% agarose gel. For KSHVΔK15#3, the forward primer contained the sequence corresponding to nucleotides 134831-134880 of the KSHV viral genome (Genbank accession number NC_009333), the reverse primer corresponded to nucleotides 136841-136870. For KSHVΔK15#4 the forward primer corresponded to nucleotides 134911-134960 of the viral genome, while the reverse primer corresponded to nucleotides 136751-136800. Finally, for KSHVΔK15#5 the forward primer corresponded to nucleotides 134981-135030, while the reverse primer corresponded to nucleotides 136681-

136730. Homologous recombination was stimulated by growth at 42˚C of the EL350 *E. coli* strain transformed with the WT KSHV-BAC and the respectively cloned Kan^R cassette with K15-homologous flanking regions. Cells were plated on LB-agar plates containing 12.5 µg/ml chloramphenicol and 50 µg/ml kanamycin and grown at 30˚C to select for mutants. To confirm insertion of the kanamycin expression cassette and deletion of the K15 ORF, WT KSHV-BAC and KSHVΔK15-BAC DNA was subjected to restriction enzyme digestion with FmpI, StuI, or MfeI + EcoRI. Digested fragments were resolved by 2% agarose gel electrophoresis and ethidium bromide staining.

Southern Blot

DNA fragments of PstI-digested WT KSHV-BAC and KSHVΔK15-BAC DNA were separated by agarose gel electrophoresis and transferred to nylon membrane by capillary action. Membranes were labeled with a radioactive probe complimentary to the kanamycin gene or to the undeleted 3'-end of the K15 ORF (corresponding to nt134713-135113 of the GenBank accession number NC_009333) and were exposed to a phosphorimaging screen. Detection of the recombined DNA was performed with a phosphorimager.

WT KSHV-BJAB and KSHVΔK15-BJAB stable cell formation

WT KSHV-BAC and KSHVΔK15-BAC DNA was amplified in EL350 *E.coli* cells grown at 30˚C overnight in the presence of 12.5 µg/ml chloramphenicol only (WT KSHV-BAC) or chloramphenicol and 50 µg/ml kanamycin (KSHVΔK15-BAC). BAC DNA was isolated using the PhasePrep BAC DNA kit (Sigma). BJAB cells were transfected with 5 µg of the KSHV wild-type bacterial artificial chromosome or 5 µg of the KSHVΔK15 #3 bacterial artificial chromosome using the human B cell nucleofection kit (Amaxa) and nucleofection program T-16. Following transfection, cells were grown 48h in RPMI-1640 medium supplemented

with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. At 48h post-transfection, growth medium was additionally supplemented with 0.2 mg/ml hygromycin B (Cellgro). Cells were passaged in selection medium for at least two weeks and ≥90% of cells expressed green fluorescent protein, indicating the presence of the virus in those cells.

Cell maintenance

BJAB (KSHV-negative B cell lymphoma) cells (33) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Growth medium and supplements were obtained from Cellgro.

RT-PCR

Following 24h serum starvation and PMA/TPA treatment, RNA was isolated from KSHV-BJAB stable cells using an RNeasy (Qiagen) RNA isolation kit. 1µg RNA was reverse transcribed with the Reverse Transcription System (Promega). PCR was performed with the following primer pairs: β-actinF 5'- GGCATCGTGATGGACTCCG-3' and β-actinR 5'-GCTG GAAGGTGGACAGCGA-3', K15 SeqF2 5'-GCTGTGTTGATGACAAACATGCTGG-3' and K15 SeqR2 5'- GACTTAATCCTGCAGCGGTGG-3', or IL6F 5'-GGTACATCCTCGACGGCA TCTC-3' and IL6R 5'-GTTGGGTCAGGGGTGGTTATTG-3', or ORF75flF 5'-ATGGCCTACG ACGTCACTG-3' and ORF75flR 5'-TTAGTGGTGGTCGTTGATCTTCT-3'. Products were resolved by agarose gel electrophoresis and visualized with ethidium bromide staining.

Detection of K15 expression in KSHV-BJAB cells

KSHV-BJAB cells were treated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA/TPA) or a vehicle control for 24h. Cells were lysed in RIPA buffer, subjected to SDS-PAGE and

transferred to nitrocellulose membranes for detection of K15 proteins with a polyclonal antibody (UNC221) raised in rabbits immunized with a K15 C-terminal peptide.

IL-6 ELISA

WT KSHV-BJAB and KSHVΔK15#3-BJAB stable cells were treated with PMA/TPA and serum starved for 24h. Conditioned growth medium was collected from the cells and centrifuged to remove cellular debris. Secreted IL-6 was detected using a typical sandwich ELISA (eBioscience) as per manufacturer's instructions.

KSHV-BJAB growth curve

WT KSHV-BJAB and KSHVΔK15#3-BJAB stable cells were maintained in culture for 20+ passages. ≥90% of cells continued to express GFP, indicating the viral genome was maintained in the majority of cells in each population. 48h prior to beginning the growth curve experiment, both cell lines were seeded at 3×10^5 cells/ml. On day 0, cells were recounted and seeded at 5×10^4 cells/ml in 3 ml RPMI-1640 medium supplemented with 2% fetal bovine serum (RPMI-2%). On days 3 and 5, culture volumes were increased to 6 ml and 18.5 ml with RPMI-2% in order to keep the cells in log growth phase. Each day, live and dead cells were counted by the trypan blue exclusion method.

Real-Time QPCR viral load assay

Total DNA (viral and cellular) was isolated from WT KSHV-BJAB and KSHVΔK15#3-BJAB cells using the Genomic DNA wizard kit (Promega) as per manufacturer's instructions. Conditioned medium was also collected. Equal volumes of resuspended total DNA or conditioned medium from each of the KSHV-BJAB cell cultures was used as template for real-time QPCR viral load assays. The real-time QPCR viral load assay detects viral

genomes using primers that anneal to the K49 ORF. For cell-associated viral loads, primers annealing to the cellular GAPDH gene were used to determine the number of viral genomes per cellular genome. Standard curves were derived from serially diluted K49 and GAPDH expression plasmids with known copy number.

Results

Creation of the KSHVΔK15 bacterial artificial chromosome

The wild-type KSHV bacterial artificial chromosome (BAC) (65) is easily manipulated and therefore is a useful tool to investigate viral gene function in the context of viral infection. We created three KSHVΔK15-BACs to determine the relevance of the K15-mediated IL-6 induction in the context of viral infection. A Kanamycin resistance expression cassette (Kan^R) was PCR amplified from the pKD13 plasmid using primers complimentary to the Kan^R cassette at the 3' ends and complimentary to the K15 ORF at the 5' ends of each primer, so that at either end the amplified Kan^R cassette is homologous to the region of K15 that will be deleted. The PCR product was then introduced into EL350 *E. coli* cells containing the KSHV wild-type BAC (WT KSHV-BAC). Homologous recombination was stimulated by growth of the bacteria at 42˚C. Recombinants were selected on agar containing kanamycin (Figure 3.1A).

To confirm deletion of the K15 ORF, KSHVΔK15-BAC or WT KSHV-BAC DNA were subjected to restriction enzyme digestion with the following enzymes or enzyme pair: FspI, StuI, or MfeI + EcoRI. Digested DNA was resolved by agarose gel electrophoresis and ethidium bromide staining (Figure 3.1B). A new FspI restriction enzyme site was introduced into the KSHVΔK15-BACs, such that a 13 kb band in the WT KSHV-BAC was replaced by 10.5 kb and 1804 bp fragments in KSHVΔK15#3-BAC, by 10.6 kb and 1884 bp fragments in KSHVΔK15#4-BAC, and by 10.7 kb and 1954 bp fragments in KSHVΔK15#5-BAC.

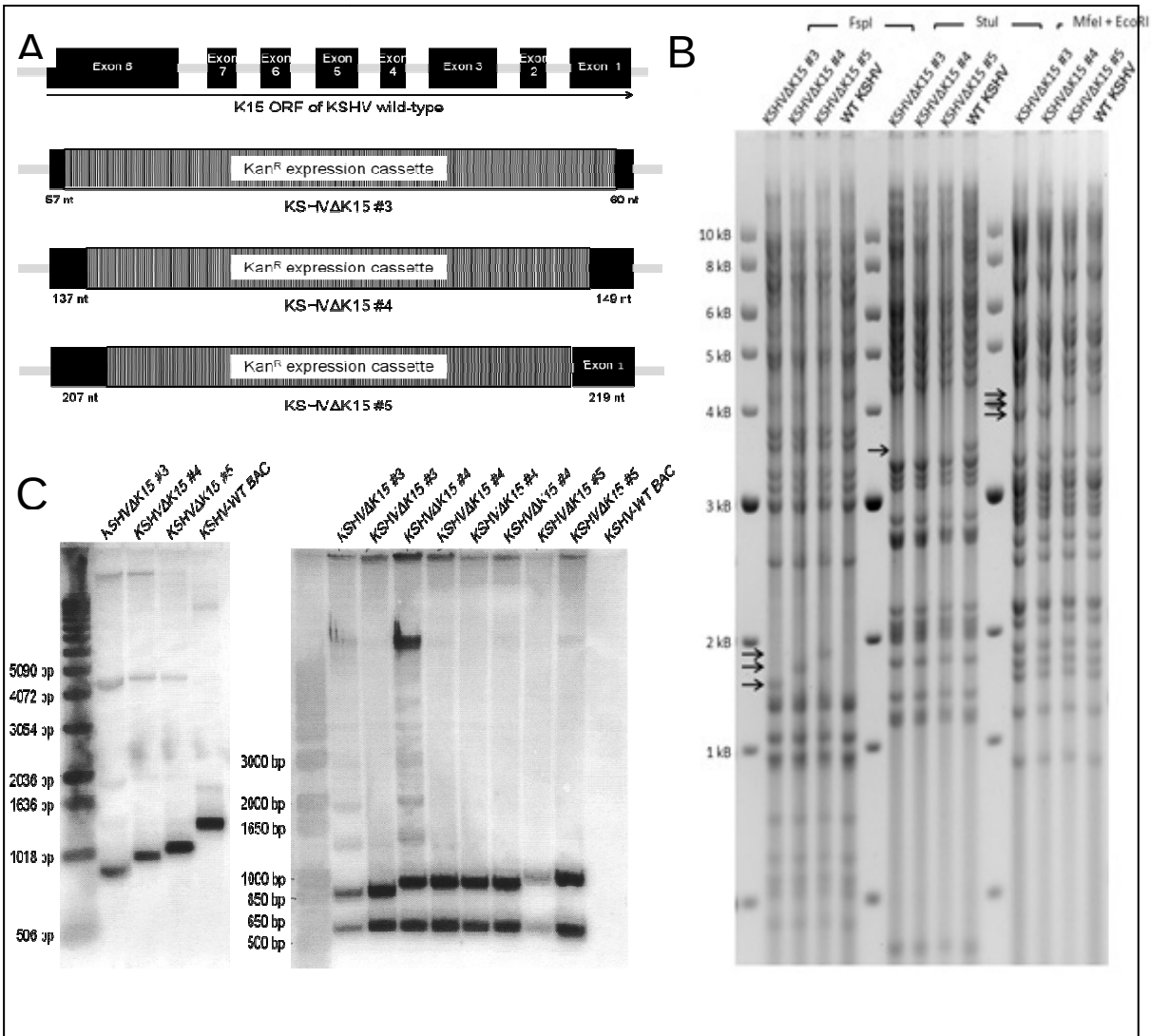
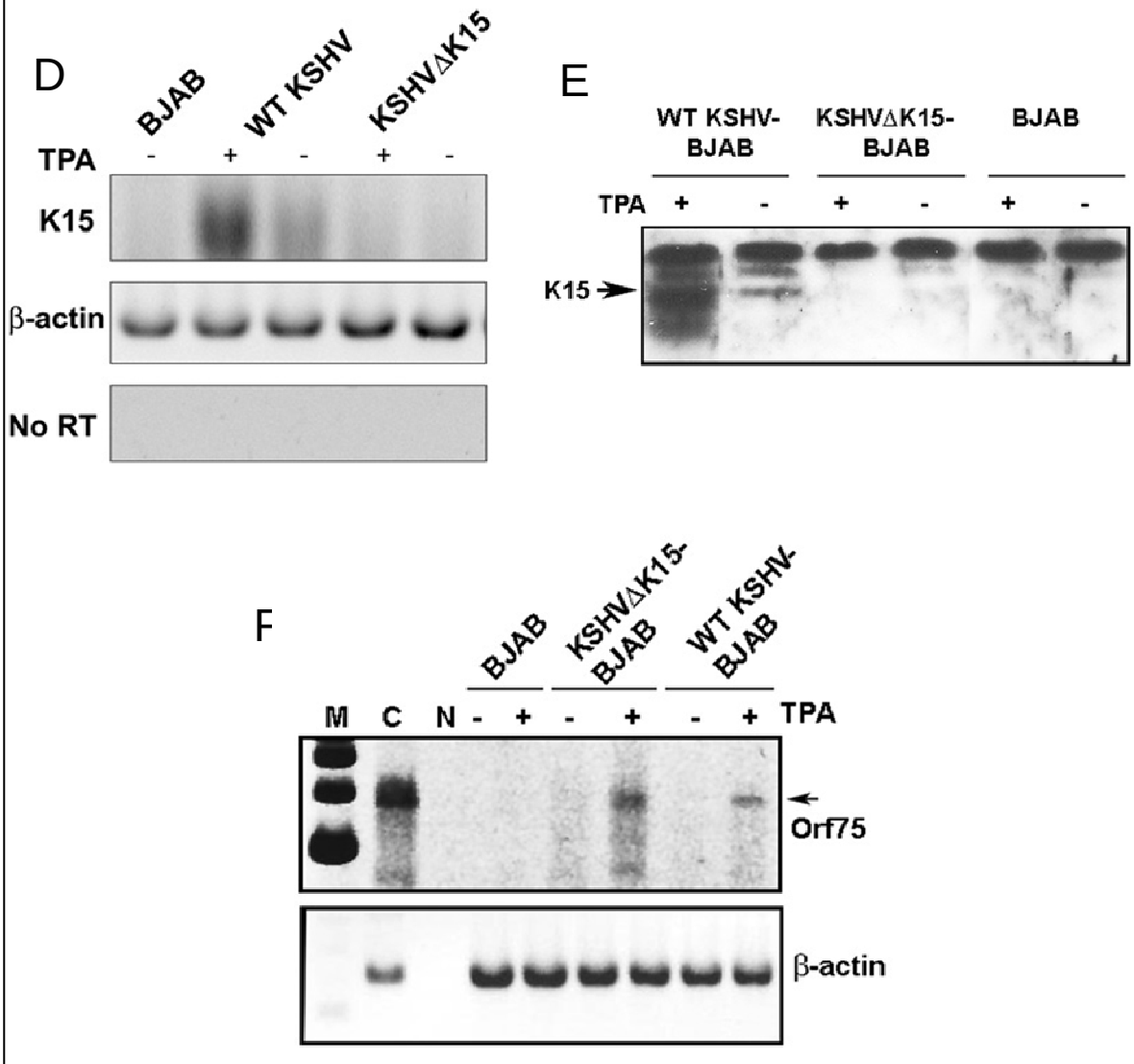


Figure 3.1 Deletion of the K15 open reading frame from the KSHV genome. A) The K15 open reading frame (ORF) was deleted by homologous recombination from the KSHV genome in the context of a bacterial artificial chromosome. Kanamycin resistance cassettes were designed to recombine with three different regions of the K15 ORF. KSHVΔK15#3 was designed to have all but 117 nt of the 2 kB K15 ORF deleted. KSHVΔK15#4 has all but 286 nt deleted, while KSHVΔK15#5 retains the first exon and 207 nt of exon 8. B) WT KSHV or KSHVΔK15 bacterial artificial chromosomes (BAC) were digested with FspI, StuI, or MfeI + EcoRI and digested fragments were resolved by agarose gel electrophoresis with ethidium bromide staining. Restriction enzyme sites were gained or lost in the recombination event. Changes in digested DNA fragment sizes are designated by arrows. C) Deletion of the K15 ORF and insertion of the kanamycin resistance cassette was confirmed by southern blot of PstI-digested WT KSHV or KSHVΔK15-BAC DNA. For detection of K15, a probe complimentary to the 3' end of the K15 ORF was designed so that a 1.3 kb band was detected in the WT KSHV-BAC lane, whereas bands of 864 bp, 944 bp and 1014 bp were detected in the KSHVΔK15 #3, #4, and #5 lanes respectively. Bands complimentary to the Kanamycin probe were only detected in the KSHVΔK15 lanes and not in the WT KSHV lane, as expected. Generation of the KSHVΔK15-BAC and Northern blot analysis were performed by Cyprian Rosetto in the Pari lab at the University of Nevada.

Figure 3.1 (continued) D, E) K15 expression was investigated at the transcriptional and translational levels in KSHV-BJAB cells either uninduced or induced to undergo lytic reactivation with phorbol 12-myristate 13-acetate (PMA or TPA). D) RNA was isolated from WT KSHV or KSHVΔK15-BJAB cells and reverse transcribed. PCR was performed with primer sets that amplify K15 or β-actin. RNA isolated from uninfected BJAB cells was included as a negative control. E) Lysates from WT KSHV or KSHVΔK15-BJAB cells were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Endogenous K15 expression was detected using an antibody raised to a C-terminal K15 peptide. Lysates from uninfected BJAB cells were included as a negative control. F) The K15 ORF may overlap with the ORF75 promoter. To investigate if deletion of the K15 ORF affected expression of the lytic ORF75, RNA was isolated from WT KSHV or KSHVΔK15-BJAB cells treated with or without PMA. RNA was reverse transcribed into cDNA. PCR was performed with a primer pair designed to amplify the full-length transcript. RNA isolated from BJAB cells was used as a negative control. N = no template control, C = genomic DNA isolated from BCBL-1 cells, as a positive control, M = size marker.



The smaller fragments are identified with arrows in Figure 3.1B. Insertion of the Kan^R expression cassette removed a StuI site from the KSHVΔK15-BACs, resulting in the loss of 3.6 and 6.0 kb fragments in StuI-digested WT KSHV-BAC. These fragments were replaced by 8.9, 9.1 and 9.3 kb fragments in the KSHVΔK15-BACs #3, #4 and #5 respectively. Loss of the 6.0 kb fragment from the mutant KSHV-BACs is highlighted in Figure 3.1B. Finally, digestion of the KSHV-BACs with MfeI + EcoRI resulted in a 4283 bp fragment in the WT KSHV-BAC, a 4002 bp fragment in the KSHVΔK15#3-BAC, a 3960 bp fragment in the KSHVΔK15#4-BAC, and a 4100 bp fragment in the KSHVΔK15#5-BAC, as depicted in Figure 3.1B.

Finally, to confirm insertion of the Kan^R expression cassette in the K15 ORF, the KSHVΔK15-BAC DNA and WT KSHV-BAC DNA were digested with restriction enzyme PstI, subjected to agarose gel electrophoresis and transferred by capillary action to nylon membrane. Membranes were probed with a radiolabeled probe complimentary to an undeleted region of the K15 ORF or a radiolabeled probe complimentary to the kanamycin resistance gene (Figure 3.1C). As expected with the probe complimentary to the K15 ORF, a 1295 bp band was detected in the WT KSHV-BAC, whereas a band of 864 bp was detected in the KSHVΔK15#3-BAC, a band of 944 bp in the KSHVΔK15#4-BAC, and a band of 1014 bp in the KSHVΔK15#5-BAC. No radiolabeled bands were detected in the PstI-digested WT KSHV-BAC with a probe complimentary to the Kan^R cassette. A 587 bp band was common to all KSHVΔK15-BACs. In addition, the Kan^R probe also detected an 864 bp band in KSHVΔK15#3-BAC, a 944 bp band in the KSHVΔK15#4-BAC, and a 1014 bp band in the KSHVΔK15#5-BAC (Figure 3.1C).

The WT KSHV and KSHVΔK15#3 BAC DNA (from here on referred to as KSHVΔK15-BAC) were then introduced into BJAB cells. The KSHV-BJAB cells were cultured in the presence of hygromycin selection for at least two weeks to obtain a population of cells that stably maintained the viral genome, as evidenced by > 95% green

fluorescent protein (GFP) expression. RNA was isolated from the uninduced stable cell populations and was subjected to RT-PCR for the viral K15 gene and a cellular β-actin gene. Viral K15 transcripts were detected in the WT KSHV-BJAB cells, but not in the KSHVΔK15-BJAB cells, whereas β-actin transcripts were amplified from all cultures but not a no template control (Figure 3.1D). Consistent with previous reports, K15 transcription increased upon reactivation of lytic replication by phorbol 12-myristate 13-acetate (PMA/TPA) in the WT KSHV-BJAB cells.

Endogenous K15 protein expression was also detected in WT KSHV-BJAB but not KSHVΔK15-BJAB cells by western blot with an antibody raised against the C-terminus of the K15 protein (Figure 3.1E). Upon PMA/TPA induction, increased K15 RNA (Fig. 3.1D) and protein expression (Fig. 3.1E) was detected in WT KSHV-BJAB cells but remained undetectable in the KSHVΔK15-BJAB cells and the untransfected BJAB cells.

In order to ensure that the deletion of the K15 ORF did not affect the adjacent ORF75 gene, we examined ORF75 transcription in the WT KSHV- BJAB cells. The WT KSHV-BJAB and KSHVΔK15-BJAB stable cells were either left uninduced or induced with PMA/TPA for 24h. RNA was isolated from both cell lines and RT-PCR was performed with a primer set designed to amplify the full-length ORF75 transcript or β-actin as a control (Figure 3.1F). The lytic ORF75 was not expressed either in uninduced WT KSHV-BJAB or uninduced KSHVΔK15-BJAB cells, but was expressed by both cell types upon reactivation of lytic viral replication by addition of PMA/TPA to the growth medium.

K15 promotes IL-6 secretion in the context of viral infection

In order to determine if K15 affects IL-6 expression in the context of viral infection, we treated WT KSHV-BJAB stable cells or KSHVΔK15-BJAB stable cells with PMA/TPA or a vehicle control to determine if K15 exerts an effect on hIL-6 expression during viral

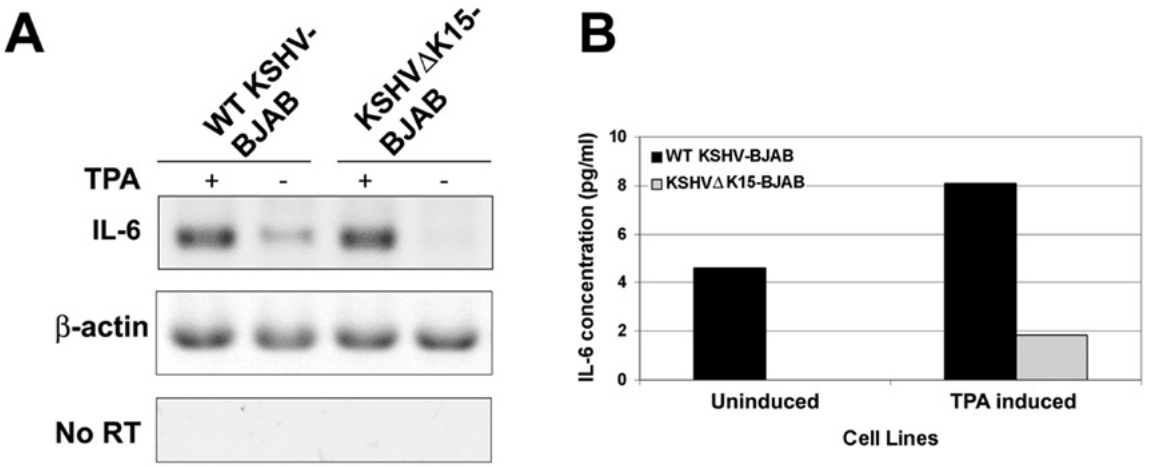


Figure 3.2 BJAB cells harboring the wild-type KSHV genome exhibit higher IL-6 levels than BJAB cells harboring a KSHV genome with the K15 open reading frame deleted. Induction of IL-6 expression was examined at the level of transcription (A) and translation (B) in KSHV-BJAB cells. A) Transcription of IL-6 was measured by RT-PCR using cDNA reverse transcribed from RNA isolated from WT KSHV or KSHVΔK15-BJAB cells either untreated or treated with PMA/TPA. B) IL-6 secretion was measured in conditioned medium from BJAB stable cells harboring either the WT KSHV- BAC or the KSHVΔK15-BAC. Cells were either treated with PMA/TPA or left uninduced.

reactivation and during viral latency. After 24h, we collected conditioned medium from the cells and performed RT-PCR to detect IL-6 transcripts (Figure 3.2A) and an ELISA to detect IL-6 cytokine secretion (Figure 3.2B). The IL-6 RNA levels in the uninduced WT KSHV-BJAB cells were 50% higher than in the uninduced KSHVΔK15-BJAB cells. Upon PMA/TPA reactivation, similar amounts of IL-6 transcripts were obtained in both cell lines (Figure 3.2A). Small amounts of cellular IL-6 (4.6 pg/ml) were secreted by uninduced WT KSHV-BJAB cells, while the PMA/TPA induced cells secreted 8.1 pg/ml IL-6. IL-6 secretion from uninduced KSHVΔK15-BJAB cells was below the limit of detection for the assay. Following PMA/TPA treatment, IL-6 levels in culture medium from KSHVΔK15-BJAB cells did increase to detectable levels (2 pg/ml), but secretion was still impaired as compared to the wild-type KSHV-BJAB cells (Figure 3.2B). Thus, K15 promotes hIL-6 secretion when ectopically expressed in B lymphoctes as well as in the context of viral infection.

K15 does not affect cellular proliferation or survival of KSHV-BJAB cells

Since WT KSHV-BJAB cells secrete more IL-6 than KSHVΔK15-BJAB cells, we hypothesized that WT KSHV-BJAB cells might have a growth advantage as compared to cells that did not express K15. 5×10^4 WT KSHV-BJAB and KSHVΔK15-BJAB cells were seeded at equal density in RPMI-1640 medium supplemented with 2% fetal bovine serum. The cells were monitored daily during log growth phase. Live and dead cells were counted using the trypan blue exclusion method to identify any difference in cell growth and/or death. WT KSHV-BJAB and KSHVΔK15-BJAB cells had similar growth and death characteristics. While the WT KSHV-BJAB cell population doubled 5.9 times (±0.2 times) during the course of the 8 day experiment, the KSHVΔK15-BJAB cell population doubled 5.4 times (±0.1 times) (Figure 3.3A). Furthermore, both WT KSHV-BJAB and KSHVΔK15-BJAB cells remained largely viable (Figure 3.3B).

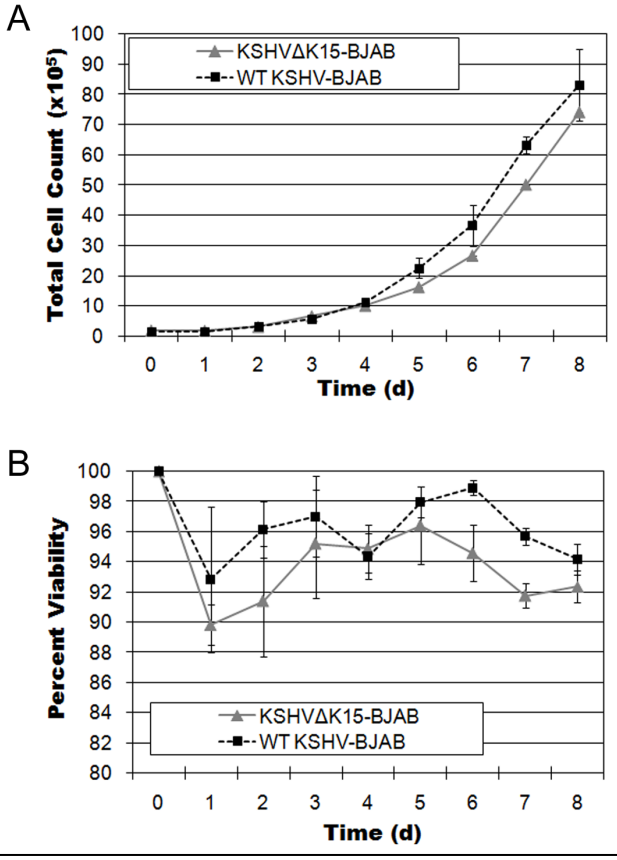


Figure 3.3 K15 does not affect cell growth or survival of BJAB cells. Stable cell populations of WT KSHV-BJAB and KSHVΔK15-BJAB cells were seeded at equal density on Day 0. Cultures were monitored daily by trypan blue exclusion to measure A) cell growth and B) cell death. A) WT KSHV-BJAB cells did not have a significant growth advantage over KSHVΔK15-BJAB cells. B) KSHVΔK15-BJAB cells may have been slightly more prone to cell death. However, both cultures remained mostly viable during the course of the experiment.

K15 expression may promote latency in KSHV-BJAB cells

To determine if K15 affects viral DNA replication during latency, WT KSHV-BJAB and KSHVΔK15-BJAB cell cultures were seeded at equal density as in the cell growth experiments. Cell cultures were allowed to grow for 48h. Then, total DNA including both host chromosomal and viral genomic DNA was isolated from the cells to determine cell-associated viral load. Medium was also collected from the cultures to determine cell-free viral load. In each case, viral load was determined by real-time quantitative PCR (QPCR) with primers that amplify the viral K49 ORF. Viral and cellular gene copy numbers were determined by comparing cycle threshold values to those derived from serial dilution of a K49 or GAPDH expression plasmid of known copy number. To determine the cell-associated viral load, viral copy numbers were normalized to GAPDH. Although cell-associated viral loads were identical (Figure 3.4A), cell-free viral load was significantly higher (5-fold) in KSHVΔK15 cells as compared to WT KSHV-BJAB cells (Figure 3.4B). This suggests that a higher percentage of KSHVΔK15-BJAB cells are undergoing spontaneous lytic reactivation as compared to WT KSHV-BJAB cells.

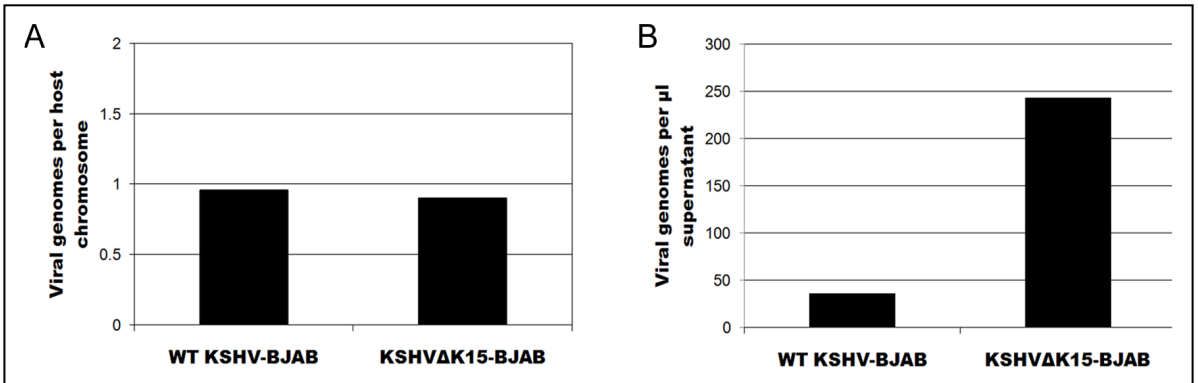


Figure 3.4 K15 may promote latency in KSHV-BJAB cells. A) Total DNA including viral genomic DNA and cellular chromosomal DNA was isolated from WT KSHV-BJAB and KSHVΔK15-BJAB cells. Real-time QPCR was performed using primers that amplify viral ORF K49 and cellular GAPDH to determine the cell-associated viral load. Equivalent numbers of cell-associated viral genomes were detected in both populations. B) QPCR was also performed using the K49 primer set to assess the presence of viral genomes in conditioned medium of WT KSHV-BJAB and KSHVΔK15-BJAB cells. Cell-free viral loads were five-fold greater in the KSHVΔK15-BJAB cells, suggesting that K15 may promote latency in B lymphocytes.

Discussion

The KSHV K15 protein has been detected in primary effusion lymphoma cells and in plasmablasts isolated from patients with multicentric Castleman's disease (49). K15 has been characterized as a latent gene, although K15 expression does increase upon induction of lytic replication (4, 11, 19, 49, 63). In this chapter we have investigated the role of K15 in the viral life cycle. We are the first to report the construction of a KSHV bacterial artificial chromosome with the K15 open reading frame (ORF) deleted by homologous recombination. The K15 ORF was replaced by a kanamycin resistance gene expression cassette in the mutant KSHVΔK15-BAC. Restriction enzyme digestion and Northern blot analysis suggested that there were no other large deletions or insertions introduced during the homologous recombination event. Introduction of the wild-type and mutant constructs into cell lines allows the investigation of K15's function by reverse genetics. An added advantage to the KSHV-BAC system is that the KSHV-BAC also contains a green fluorescent protein (GFP) expression cassette to monitor viral infection (65).

Stable BJAB B lymphocyte cell lines were created containing the wild-type KSHV-BAC or the KSHVΔK15-BAC. After selection with hygromycin B, at least 95% of cells in each culture expressed GFP. K15 mRNA and protein were detected in WT KSHV-BJAB cells but not in KSHVΔK15-BJAB cells. mRNA and protein levels increased in the WT KSHV-BJAB cells upon induction of lytic replication, consistent with previous reports that expression of K15 is induced by the viral lytic switch protein, RTA. Substitution of the K15 ORF by the Kan^R cassette did not affect expression of the adjacent ORF75 gene product, which was detected in both cell cultures after induction of lytic replication. These results were reasonably reassuring that deletion of the K15 ORF by homolgous recombination did not introduce any unintended alterations in the viral genome or interfere with adjacent viral gene transcription. Thus, the BAC system was ideal for characterization of K15's function.

Since we had previously determined that ectopic expression of K15 in B lymphocytes induces multiple cellular cytokines and most notably IL-6, we first measured IL-6 expression in WT KSHV-BJAB and KSHVΔK15-BJAB cells. The importance of the IL-6 signaling pathway in viral pathogenesis is underscored by the high levels of cellular and viral IL-6 expression in KSHV-associated malignancies (1, 13, 23, 36, 39, 41). Our study indicates that K15 can induce cellular IL-6 expression during KSHV latency in B cells. During viral latency and PMA/TPA-induced viral reactivation, IL-6 secretion was significantly increased in the wild-type KSHV-BJAB cells (4.6 and 8.1 pg/ml respectively) as compared to the KSHVΔK15-BJAB cells where IL-6 was absent in uninduced cells and barely detectable following induction with PMA/TPA. The concentration of IL-6 secreted by wild-type KSHV-BJAB cells is similar to IL-6 secretion by BCBL-1 cells (50), from which the KSHV-bacterial artificial chromosome was derived (65). Low-level IL-6 expression was detected in KSHVΔK15-BJAB cells only after PMA/TPA stimulation. Lytic viral proteins have also been shown to induce cellular IL-6 expression and may account for this low-level of expression following induction of viral lytic replication. Alternatively, the IL-6 promoter has TPA-response elements (TREs). PMA/TPA activates AP-1 transcription factors that initiate transcription after binding to TREs in the promoters of target genes, such as IL-6. In aggregate, these results suggest that the KSHVΔK15-BJAB cells are capable of producing IL-6, but that the absence of K15 significantly impairs IL-6 induction in KSHV-positive cells. Thus, K15 may contribute to the induction of IL-6 in KSHV-associated malignancies. Furthermore, since IL-6 has been shown to promote cell survival, it follows that K15 may indirectly stimulate proliferation and survival of the infected cell by upregulating the IL-6 signaling pathway.

Cellular IL-6 binds to the IL6 receptor alpha (IL6-Rα)-gp130 complex to initiate signaling via the JAK-STAT pathway with anti-apoptotic and proliferative end effects (18, 20, 21, 27, 28, 30, 31, 57, 58). Therefore, we queried whether induction of IL-6 expression by

the latent K15 protein might stimulate cell growth and division and protect the infected cell from programmed cell death. In order to shed light on the role of K15 in cell growth and survival, we examined cell proliferation and cell death in our KSHV-BJAB stable cell lines. We noticed minimal differences in cell growth characteristics and in cell death. In retrospect, this finding is not surprising since the BJAB cell line was created by adaptation of an EBV-negative Burkitt's lymphoma to tissue culture, hence the cell line is already transformed (33). Moreover, no appreciable levels of IL-6 are detected in uninfected BJAB cell cultures even after PMA/TPA induction (24) (and Nun and Damania, unpublished data), suggesting that this B cell line is no longer dependent on IL-6 for proliferation. This is further supported by the observation that IL-6 did not stimulate BJAB proliferation in reduced serum conditions (26). In spite of this, IL-6 transcript and protein levels can be detected after introduction of exogenous proteins into BJAB cells (24), demonstrating that the IL-6 gene is not deleted in BJAB cells but is highly regulated. Regardless, to determine if K15-mediated IL-6 secretion provides a survival advantage to infected B cells, the role of K15 in survival of primary EBV-negative B cells should be assessed after introduction of the WT KSHV-BAC or KSHVΔK15-BAC.

During viral latency only a small number of genes are expressed. Thus the functions of those genes are presumed to be important for maintenance of the latent viral genome, for cell survival and evasion from the immune system, and/or for viral oncogenesis. Although we were unable to define the role of K15 in cell growth and survival, the KSHV-BJAB system did allow us to address a corollary change in viral life cycle. We used real-time quantitative PCR to measure latent intracellular and extracellular viral loads in BJAB cells harboring the wild type or mutant K15 viral genomes. Both WT KSHV- and KSHVΔK15-BJAB cells carried approximately one viral genome per cellular chromosome prior to induction with PMA/TPA as determined by real-time QPCR. Despite that both cell lines contained similar viral genome copies per cell, the KSHVΔK15-BJAB cells had 5-fold higher

viral genome copies in the supernatant as compared to the WT KSHV-BJAB cells. This suggests that K15 expression might limit spontaneous viral reactivation in the latent cell population. We might have expected that intracellular viral genome copy number would also be increased in spontaneously reactivated cell cultures. However, small differences in percentage of cells undergoing lytic reactivation may mask this effect, while cell-free virus accumulates in the medium. Additionally, lytic reactivation culminates in death of the infected cell. Based on the cell proliferation assay, there was a very slight difference in cell death in the KSHVΔK15-BJAB cells as compared to the WT KSHV-BJAB cells. Viral genomes may have been released into the supernatant medium upon cell death accounting for the large increase in extracellular viral genomes, since we did not differentiate genomes incorporated into virions from naked viral genomes in the cell-free supernatant.

Although we did not specifically examine the role of K15 in the lytic cycle, it has been reported by many groups that K15 expression increases during lytic replication in response to promoter activation by the viral lytic switch protein, regulator and transcription activator (RTA) (4, 11, 19, 48, 63). Indeed, we have shown here that K15 expression increases following PMA/TPA induction in our KSHV-BJAB model system. The highest levels of K15 expression are seen 24h after PMA/TPA induction and persist beyond 48h (43, 48). Perhaps K15 expression following lytic induction favors a return to viral latency and evasion of the immune response. Future studies should address a possible role of K15 in repression of lytic replication and/or virion release following chemical induction in B cells. A potential protective role for K15 via repression of lytic replication should also be examined in *in vivo* studies. It would also be interesting to investigate a potential effect of IL-6 secretion on viral genome replication and the viral life cycle beyond cell survival and proliferation.

The studies included in this chapter indicate that K15 serves to regulate the cytokine environment in infected B cells and plays an important role in the viral life cycle. Future studies will undoubtedly confirm the importance of K15 in modulating the host cell

environment. We propose that K15 may alter the cell environment in such a way that supports viral latency and limits lytic replication, thus favoring viral persistence with minimal detection by the host organism.

Acknowledgments

Creation of the KSHVΔK15-BAC by homologous recombination (Figure 3.1A) and Northern blot analysis (Figure 3.1C) were performed by Cyprian Rosetto of Greg Pari's laboratory at the Unviersity of Nevada. This work was supported by NIH grants CA096500 and HL083469 and a Burroughs Welcome Fund grant to BD, as well as the Cancer Cell Biology training grant to TKN. BD is a Leukemia & Lymphoma Society Scholar and Burroughs Welcome Fund Investigator in Infectious Disease.

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Chapter Four

Development of a fluorescence-based assay to screen antiviral drugs against Kaposi's sarcoma-associated herpesvirus

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Molecular Cancer Therapeutics 2007 August; **6**(8): 2360-2370.

Abstract

Tumors associated with Kaposi's sarcoma-associated herpesvirus (KSHV) infection include Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease. Virtually all of the tumor cells in these cancers are latently infected and dependent on the virus for survival. Latent viral proteins maintain the viral genome and are required for tumorigenesis. Current prevention and treatment strategies are limited, because they fail to specifically target the latent form of the virus, which can persist for the lifetime of the host. Thus, targeting latent viral proteins may prove to be an important therapeutic modality for existing tumors as well as in tumor prevention by reducing latent virus load. Here, we describe a novel fluorescence-based screening assay to monitor the maintenance of the KSHV genome in B lymphocyte cell lines and to identify compounds that induce its loss, resulting in tumor cell death.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8, is a member of the gammaherpesvirus family, distinguished by the ability of its members to transform host cells. KSHV has been linked to multiple types of cancer, including all forms of Kaposi's sarcoma (KS) (1), primary effusion lymphoma (PEL) (5) and the plasmablastic variant of multicentric Castleman's disease (MCD) (40). These cancers are more prevalent in immunodeficient populations, such as transplant patients and HIV-infected individuals (18, 28, 38, 40). In fact, KS is the most frequent AIDS-associated cancer in the US and world-wide (27).

Following primary infection, KSHV establishes latent infection in the host cell, with only a small population of cells undergoing spontaneous lytic reactivation (31, 41). During latency, a limited number of viral proteins are expressed, including the latency-associated

nuclear antigen (LANA), vFLIP, vCyclin, kaposin and K15 (36, 37). Each viral latent protein plays an important role in viral pathogenesis and KSHV-associated tumorigenesis. LANA plays a pivotal role in maintenance and segregation of the viral genome during latency (2), and thus is also essential for cell survival (8). Maintenance of the viral genome is absolutely dependent on the LANA protein, which tethers the latent viral episome to the host cell chromosome, ensuring that the viral genome is replicated with the host genome and is not diluted out of the expanding population of latently infected cells (9, 16). If the viral episome is lost, LANA and the other latent viral oncogenes are no longer expressed, and the tumor cell dies.

Currently there is neither a cure nor a therapeutic vaccine for KSHV infection. Highly active antiretroviral therapy has reduced the incidence of KSHV-associated tumors in the HIV-positive population, yet KS remains the most prevalent AIDS-associated neoplasm, even in individuals on long-term therapy (12). Ganciclovir, which specifically inhibits lytic viral replication, has reduced the incidence of KSHV-related tumors in transplant recipients (reviewed in (21)). However, treatment for pre-existing KSHV-associated malignancies relies on interferon-alpha administration and systemic chemotherapeutic regimens, developed for non-virus-associated cancers, that target DNA replication of all dividing cells (reviewed in (46)). Although clinical trials are assessing new treatment options, a cure remains elusive largely due to the lack of compounds that specifically target latent proteins, which allow the virus to persist throughout the host's lifetime. Recently, Curreli et al. reported that high concentrations of glycyrrhizic acid, originally isolated from licorice (*Glycyrrhiza glabra)*, could downregulate the expression of LANA *in vitro* (11).

Nature continues to be a valuable source for new anti-microbial and anti-cancer pharmaceuticals (22, 39). From 1984-1995, over 65% of new drugs in these medical fields were derived directly from natural sources or were synthesized, but modeled after a natural product lead compound (10, 29). Two anticancer chemotherapeutics derived from natural

products, Taxol (44) (paclitaxel) and FDA approved analogs of camptothecin (43) (irinotecan and topotecan), together represent at least one third of the world-wide market for anti-neoplastic agents (30). Indeed, natural products represent a rich reservoir of chemically diverse compounds with biological relevance in many disease states (4).

This report describes the development of a fluorescence-based assay to screen for samples that inhibit latent KSHV persistence. The assay identifies samples that interfere with viral genome maintenance during latency irrespective of the specific biochemical mechanism, thus multiple targets are screened for simultaneously. In order to accomplish this, a KSHV-GFP recombinant virus (47) was introduced into a KSHV-negative B lymphocyte cell line (BJAB) to create a KSHV-BJAB cell line. KSHV-BJAB cells were chosen for two reasons. First, fluorescence and hence, viral genome maintenance, could be monitored over time in live KSHV-BJAB cell cultures. Samples that interfere with viral genome maintenance could be identified by measuring an accelerated reduction in fluorescence with respect to a vehicle control since the recombinant viral genome is expelled from the dividing cell population. Second, since the BJAB cell line does not require KSHV infection for its survival but can support long-term latent viral persistence (6), using KSHV-BJAB cells uniquely allowed the distinction between samples that caused loss of the latent viral episome and those that were generally toxic to the host cell. This was essential, because naturally infected PEL cell lines, such as BCBL-1 (34), require viral infection to survive, and therefore do not allow a distinction between specific anti-viral and broadly cytotoxic compounds, since compounds that induce loss of the virus also lead to cell death. As broadly cytotoxic compounds are often associated with multiple side effects resulting from non-selective toxicity, the utility of a live cell screen should improve the identification of compounds that may exhibit selective antiviral activity *in vivo*. In this report, samples that proved efficacious in the KSHV-BJAB cell line were also tested in the naturally infected BCBL-1 cell line to confirm that loss of the virus corresponded with PEL cell death.

In addition to the application described here, this assay can be adapted for use with other viruses, such as Epstein-Barr virus, that exist episomally in the host cell. It can be used to screen various collections (ranging from pure compounds to crude extracts) for samples that interfere with viral persistence. In this report, we used this assay to screen a small library of plant extracts and identified two that selectively induced loss of KSHV virus from infected cells.

Materials and Methods

Cells and media.

The KSHV-negative BJAB lymphoid cell line and the KSHV-positive BCBL-1 cell line were obtained from ATCC. BJAB cells were maintained in RPMI-1640 medium supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. BCBL-1 cells were maintained in the same medium additionally supplemented with 1mM $NaHCO_3$ (Gibco) and 0.05 mM 2-mercaptoethanol (Sigma). The BJAB-derived cell line, KSHV-BJAB, was created by nucleofection of KSHV-negative BJAB cells with the KSHV bacterial artificial chromosome containing a hygromycin antibiotic resistance marker and the green fluorescent protein expression cassette (47). Cells containing the KSHV genome were selected for a minimum of two weeks in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2 mg/ml hygromycin B. Unless otherwise noted, growth media and supplements were obtained from Cellgro.

Plant collection and processing.

Plant samples were collected in Manus Island, Papua New Guinea, in 2003 under a UIC-UPNG Memorandum of Agreement 2003-2008 and approval of the PNG BioNET/Department of Environment and Conservation dated May 21, 2003. A set of

voucher herbarium specimens has been deposited at both the Herbarium of the University of Papua New Guinea, Port Moresby and the John G. Searle Herbarium, Field Museum, Chicago. Taxonomic identifications were performed by one of the authors (PP) and confirmed by staff of the Lae Herbarium, Papua New Guinea and by one of the authors (DDS). The collection and processing strategies for these understudied plant specimens from tropical rainforests has been recently reviewed (20). Briefly, a pilot sample (~20 g, dry weight) was extracted with 9:1 MeOH:H_2O. The resultant extract was defatted with hexanes, and the residual material was partitioned between 4:1:5 $CHCl_3$:MeOH:H_2O. Importantly, the organic-soluble fraction was washed with 1% NaCl to remove tannins (42), which are known to interfere with some biological assays. The de-tannified organic fraction of each sample was tested for biological activity.

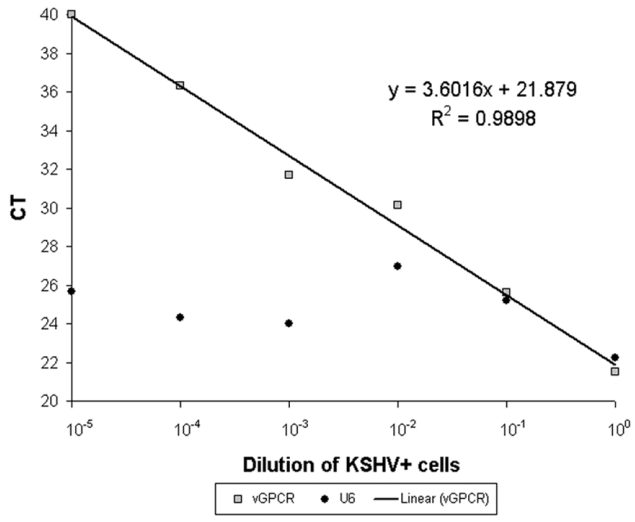
Fluorescence assay.

100,000 cells of each B lymphocyte cell line were suspended in RPMI-1640 medium (Cellgro) supplemented with 2% fetal bovine serum (Cellgro), 100 U/mL penicillin and 100 µg/mL streptomycin (Cellgro) and were placed in subsequent wells of a 24-well plate. Hygromycin selection was withdrawn from KSHV-BJAB cells to prevent competition with screened samples. In the case of plant samples, the de-tannifed organic fraction or an equal volume of dimethyl sulfoxide (DMSO) was added to the growth medium at a final protein concentration of 2 µg/ml. The pure compound and positive control, glycyrrhizic acid (Sigma) dissolved in 5% EtOH at a pH of 7.2, was diluted to 2 mM (1680µg/ml), 3 mM (2520µg/ml), 4 mM (3360µg/ml), and 6 mM (5040µg/ml) in RPMI-1640 (Cellgro) supplemented with 2% fetal bovine serum (Cellgro), 100 U/mL penicillin and 100 µg/mL streptomycin (Cellgro). Half of the culture medium was siphoned from each well and replenished with fresh medium and identical concentrations of glycyrrhizic acid or plant

sample (2 µg/mL) twice each week without passaging the cells. Mean fluorescence of the live cultures, incubated with test or control samples, was measured using a Fluostar fluorimeter every two to three days. Optimal parameters for detection were excitation wavelength 485 nm, emission wavelength of 510 nm and orbital well scanning.

Real Time QPCR.

Total DNA (including cellular genomic and viral DNA) was isolated from cells after five weeks incubation with test samples or vehicle controls using the Promega Wizard Genomic DNA Kit. Real Time QPCR was performed with primers specific for the cellular U6 gene and the viral vGPCR gene as previously described (13). Using this method to analyze BCBL-1 cells serially diluted in a suspension of uninfected BJAB cells, we were able to detect as few as 1 infected cell in 10,000 (Supplementary Figure 4.1) with 95% efficiency. In the BJAB and BCBL-1 cells, the cytotoxicity of the plant samples was assessed using the equation $1.9^{-\Delta CT(U6)}$ to normalize the data to the DMSO control. Results were expressed as % viability. In KSHV-BJAB cells, selective inhibition of the virus was assessed by first normalizing the cellular and viral data to the DMSO control, then determining the ratio, or selectivity index $SI = 1.9^{[\Delta CT(U6)-\Delta CT(vGPCR)]}$, of viral toxicity versus cellular toxicity.



Supplementary Figure 4.1 Determination of the efficiency of the real time QPCR assay and limit of detection. Naturally infected BCBL-1 cells were serially diluted into a suspension of uninfected BJAB cells. Real time QPCR was performed using viral (vGPCR) and cellular (U6) primer sets. The cycle threshold for the viral primer set was plotted against the dilution. The slope of the line was 3.6, corresponding to 95% efficiency based on the equation 10(1/m), where m is the slope. Finally, the viral QPCR yielded a detectable signal with 1 infected cell in 10,000 but not for 1 infected cell in 100,000, thus establishing our limit of detection at 1 infected cell in 10,000.

Immunofluorescence.

After one week, approximately 100,000 BCBL-1 cells cultured with plant extract or a DMSO vehicle control were spotted on slides. Cells were fixed and permeabilized in pre-cooled acetone at -20°C for 15 min. Slides were washed in PBS. Normal goat serum (10% in PBS) was used to block non-specific antibody binding. The slides were incubated for 1 h at room temperature with anti-KSHV ORF-73 (LANA) rat monoclonal antibody (1:100, Advanced Biotechnologies). Slides were washed twice in PBS, then incubated for 30 minutes at room temperature with anti-rat TRITC-conjugated IgG (1:100, Sigma). Slides were washed twice in PBS and once in distilled water and allowed to dry. Vectashield was applied to preserve fluorescence.

Results

Development of the screening assay.

The KSHV-BJAB cell line was established by introducing the complete KSHV genome in the context of a bacterial artificial chromosome (KSHV-BAC) (47) into uninfected B lymphocytes (BJAB). The KSHV-BAC contains both a mammalian hygromycin antibiotic resistance marker and the green fluorescent protein (GFP) expression cassette. Transfection of BAC viral DNA was previously shown to result in fully replication-competent virus and circumvented any receptor or post-entry blocks that may limit the efficiency of natural infection of B cells with KSHV (6, 23, 47). KSHV-BJAB cells were selected in 0.2 mg/ml hygromycin B in order to achieve a stable KSHV-positive cell line.

KSHV-BJAB cells harboring the KSHV-BAC express GFP, thus providing a means to screen for samples that interfere with latent viral genome maintenance. Inhibition of viral genome maintenance results in loss of fluorescence as the viral episome is lost from the dividing cell population. Since KSHV episome loss occurs at cell division (17), multiple cell

division cycles must take place before a significant loss of fluorescence can be observed. The design as a multi-cycle assay also increased the sensitivity and allowed us to prioritize potential lead samples based on their effectiveness.

To screen for samples with activity against latent KSHV infection, hygromycin selection was removed from the KSHV-BJAB cells to prevent competition with the screened samples. Cells were incubated with test and control samples for five weeks. Culture medium and samples were replenished twice each week without passaging the cells. Primary screening of samples was achieved by measuring the mean fluorescence of the GFP+ KSHV-BJAB cells every two to three days (Figure 4.1A). Mean fluorescence was plotted over time to identify samples that accelerated loss of fluorescence in the KSHV-BJAB cell line with respect to a vehicle control. The uninfected BJAB cell line served as an additional control against broad-spectrum cytotoxic effects of the tested samples. A naturally infected KSHV-positive PEL cell line, BCBL-1 (34), was also included to confirm the antiviral effects of lead samples by real-time QPCR and indirect immunofluorescence assays for LANA (Figure 4.1A).

Validation of the screening assay

To validate the assay, a known inhibitor of KSHV latency, glycyrrhizic acid (GA), was tested. At 3 mM, GA has been shown to downregulate expression of the latency-associated nuclear antigen (LANA) of KSHV (11). KSHV-BJAB cells were incubated with 2-6 mM GA or an ethanol control (final ethanol concentration 0.6%). Culture medium was replaced twice each week with fresh medium plus GA without passaging the cells. In the ethanol control or 2-4 mM GA-treated KSHV-BJAB cultures, fluorescence increased from day 1 to day 7 as KSHV-BJAB cells proliferated. After day 7, fluorescence leveled off in the ethanol control cultures, whereas cells incubated with GA showed a dose-dependent decrease in mean

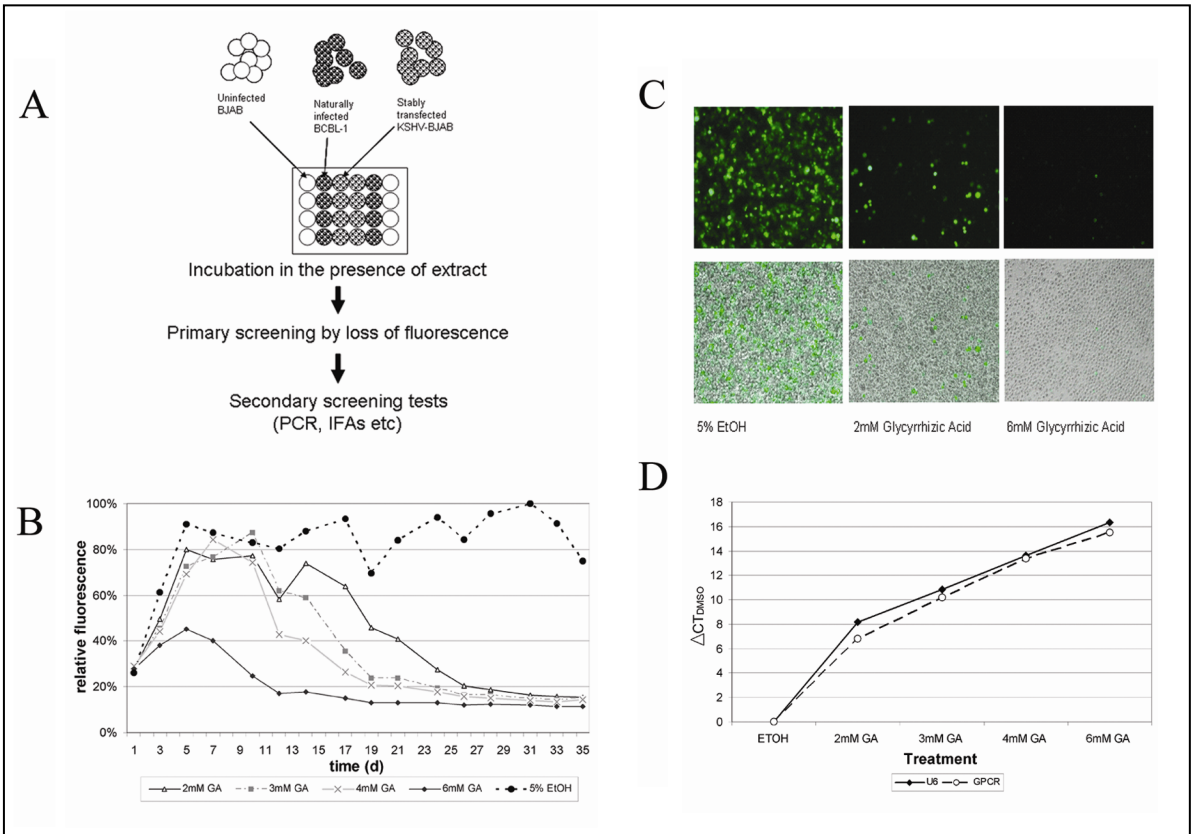


Figure 4.1 Validation of the assay. A) Cells from three B lymphocyte cell lines (BJAB, KSHV-BJAB, BCBL-1) are incubated in RPMI media containing test or control samples. Samples are initially screened for loss of fluorescence in the KSHV-BJAB cells, suggestive of loss of virus. To verify loss of virus from the cells, additional assays are performed including real time PCR viral load assays and immunofluorescence assays for LANA. B) Incubation of KSHV-BJAB cells with a known inhibitor of KSHV latency, glycyrrhizic acid, leads to a dose-dependent decrease in mean fluorescence as compared to an ethanol control. C) Digital images of the KSHV-BJAB cells incubated with glycyrrhizic acid (2 mM and 6 mM) or an ethanol control. The top image of each pair represents the fluorescence. The bottom image of each pair represents the merged fluorescent and bright field images. D) Real time QPCR using primer sets to amplify viral (vGPCR) and cellular (U6) regions of DNA isolated from BCBL-1 cells incubated with glycyrrhizic acid or an ethanol control. Dose-dependent increases in the cycle threshold for viral (open circles) and cellular (black diamonds) template as compared to the EtOH control were evident.

fluorescence (Figure 4.1B). At 6 mM, GA inhibited the initial proliferative burst of KSHV-BJAB cells, most likely due to broad cytotoxicity. By day 18, the majority of KSHV-BJAB cells incubated with 2 mM GA no longer exhibited green fluorescence, but remained viable (Figure 4.1C, middle panel), in contrast to the gross cytotoxicity observed at 6 mM GA (Figure 4.1C, right panel). On day 18, uninfected BJAB cells also remained viable at 2 mM GA, but lost viability at 6 mM GA (data not shown).

The antiviral effect of GA was verified by viral load assays. Total cellular and viral genomic DNA was isolated from BCBL-1 cells or KSHV-BJAB cells incubated with GA or an ethanol control for seven days. As expected, the cycle threshold for both the cellular (U6) and viral (vGPCR) primer sets increased in a dose-dependent manner indicating a simultaneous reduction of both viral and cellular DNA (Figure 4.1D). This was expected as GA interfered with viral latency, leading to loss of viral DNA and, since the KSHV genome is required for BCBL-1 cell survival, a loss of cellular DNA as well. GA selectively inhibited the virus in KSHV-BJAB cells, which do not depend on the virus for survival, with reductions in viral load on average 17 times greater than reductions in cellular DNA (data not shown). These results served to validate our assay. However, cumulative cytotoxic effects became evident in all GA cultures, including the uninfected BJAB cultures, after 18 days of treatment (data not shown). Therefore, despite its initial selective antiviral effect, GA is unlikely to be a good drug candidate given its cumulative broad cytotoxicity. Moreover, the relatively high concentration required for a positive response *in vitro* could be difficult to achieve *in vivo*.

Identification of antiviral plant extracts

Having validated the assay, a screening set of 81 plant extracts was tested for anti-KSHV activity. All extracts were dissolved in dimethyl sulfoxide (DMSO) and were tested at a final concentration of 2 μg/ml in RPMI. An equal volume of DMSO served as a vehicle control. Culture medium was replaced twice each week with fresh medium plus plant extract, without passaging the cells. Mean fluorescence measurements from KSHV-BJAB cultures were taken every 2-3 days. Fluorescence from KSHV-BJAB cells treated with DMSO plateaued within one week as the cells achieved equilibrium. Sixty-two extracts exhibited insignificant changes in fluorescence of KSHV-BJAB cultures with respect to the DMSO control. Eight extracts marginally reduced fluorescence, while six extracts

Supplementary Table 4.1 Summary of results

Plant Extract	Loss of Fluorescence	ΔCT_{DMSO} for Viral Primer Set: KSHV-BJAB	ΔCT_{DMSO} for Viral Primer Set: BCBL-1	Indirect Immunofluorescence Assay (Anti-LANA Staining)
A05807	No	+2.2	-0.9	Speckled nuclear staining similar to DMSO control
A05810	Yes	+4.0	+4.2	Reduced levels of LANA staining
A05814	Yes†	+14.8†	+11.7†	ND
A05830	Yes	+2.8	+0.8	ND
A05831	Yes	+13.1	+2.3	Near background levels of staining
A05853	Yes	+9.6	+11.5	Near background levels of staining
A05854	Yes††	ND	ND	ND
A05898	Yes	+2.7	-0.7	ND
A05901	Yes	+13.4	-0.1	Fewer cells with speckled nuclear staining

† delayed (cumulative) cytotoxicity observed, †† acute cytotoxicity observed, ND no data

consistently decreased fluorescence by at least 50% of the DMSO control without apparent cytotoxicity (Supplementary Table 4.1). Four extracts exhibited acute and one delayed (cumulative) cytotoxic effects irrespective of the cell's infection status.

Extracts that decreased fluorescence

Figure 4.2 displays the mean fluorescence graphs of six extracts (A05810, A05830, A05831, A05853, A05898 and A05901) that consistently decreased fluorescence by ≥50% of the DMSO control in four separate trials. Two distinct trends were observed within this group of six extracts. Fluorescence of KSHV-BJAB cells incubated with extracts A05810, A05830, and A05898 (Figure 4.2A) peaked within 7 days, then decreased steadily until achieving a new plateau level of fluorescence at least 50% less than the DMSO control, but still above background. Fluorescence from KSHV-BJAB cells incubated with extracts A05831, A05853 and A05901 (Figure 4.2B) peaked within 7 days, then steadily declined throughout the experiment.

Figure 4.3 displays digital images of KSHV-BJAB cells after 20 days incubation with the six aforementioned extracts and the DMSO control. Roughly equivalent numbers of cells were present in each field. However, the number of GFP+ cells was reduced ≥50% by each of the extracts as compared to the DMSO control. No visually apparent cytotoxic effects of these extracts were evident at day 20. Furthermore, these extracts were

assessed at day 14 in uninfected BJAB cells (Supp Figure 4.2A) and in three unrelated cancer cell lines (Supplementary Figure 4.2B) and were found to have limited cytotoxicity in these assays. These six extracts that repeatedly decreased fluorescence of KSHV-BJAB cells may interfere with episomal maintenance and were chosen for further analyses.

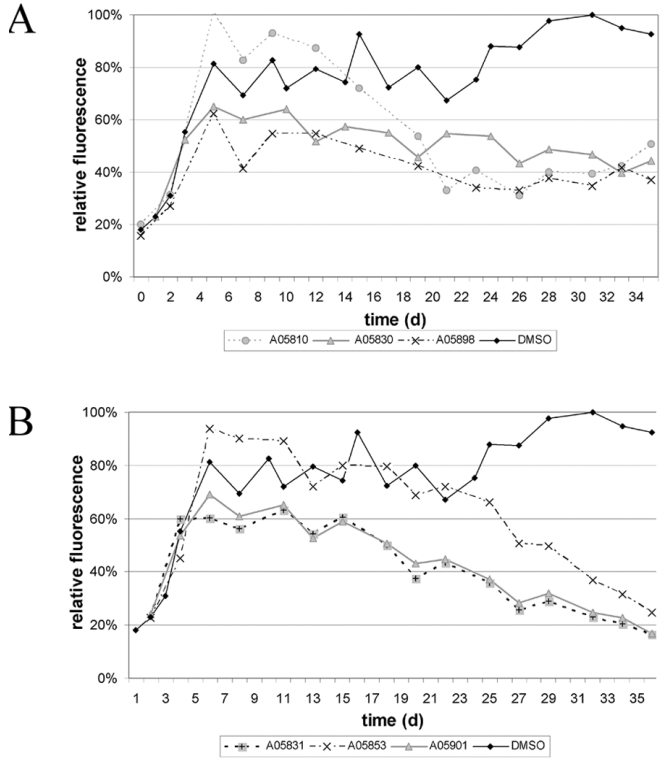


Figure 4.2 Initial screening for antiviral activity is based on loss of fluorescence. Mean fluorescence of live B lymphocyte cultures incubated with potential antiviral compounds is measured every two to three days for five weeks. A) Extracts A05810 (grey circles), A05830 (gray triangles) and A05898 (black crosses) caused accelerated loss of mean fluorescence in KSHV-BJAB cultures as compared to the DMSO control (black diamonds), eventually establishing a new plateau level of fluorescence ≥50% less than the DMSO control. B) After incubation with extracts A05831 (grey boxes), A05853 (black crosses) and A05901 (grey triangles) fluorescence declined steadily and without leveling off, suggesting continuous reduction of the viral load.

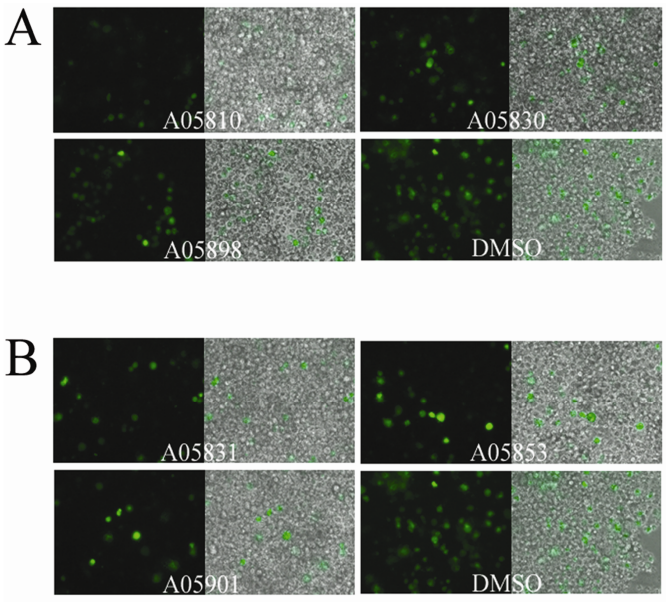
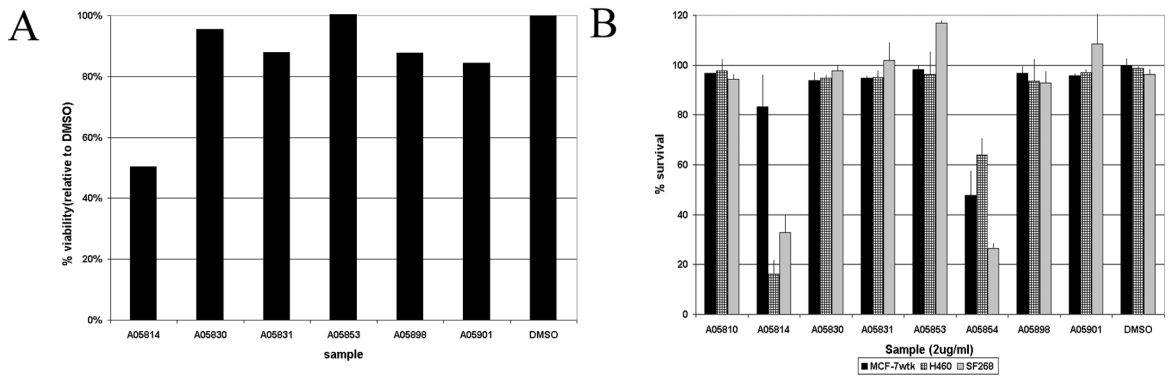


Figure 4.3 Microscopic evaluation of KSHV-BJAB cells incubated with potentially antiviral plant extracts. Digital images of KSHV-BJAB cells incubated with representative plant extracts that exhibited an accelerated loss of fluorescence as compared to a DMSO control, suggesting loss of the viral genome. The left image in each sub-panel represents the fluorescence. The right image in each sub-panel represents the merged fluorescent and bright field images. A) Extracts A05810, A05830 and A05898 which achieved a new plateau level of fluorescence, as compared to DMSO. B) Digital images of KSHV-BJAB cells incubated with extracts A05831, A05853 and A05901 which steadily reduced fluorescence as compared to the DMSO control.



Supplementary Figure 4.2 Assessment of the toxicity of plant extracts. A) Uninfected BJAB cells were incubated with plant extracts (2µg/ml) for 14 days, with growth medium and extract replacement twice each week. Cell viability was assessed by the trypan blue exclusion method, where cells not incorporating the dye are scored as viable. Results are normalized to the DMSO control. B) Three unrelated cancer cell lines (MCF7-wtk, H460 and SF268) were incubated with plant extracts (2µg/ml) for three days. Cells were fixed and stained with a sulforhodamine B dye. Toxicity was assessed by detecting the amount of incorporated sulforhodamine B dye, correlating to the overall biomass. Figure 4.2B was generated by Yuka Nakanishi.

Cytotoxic extracts

In contrast to the extracts that showed a consistent decrease in fluorescence without visually apparent cytotoxicity, four extracts, as represented by extract A05854 in Figure 4.4, were acutely and uniformly cytotoxic to all three B lymphocyte cell lines tested regardless of their infection status. Additionally, one extract A05814 was found to have cumulative non-specific cytotoxic effects. As before, fluorescence from DMSO-treated KSHV-BJAB cells increased sharply as cells proliferated, whereas the fluorescence of KSHV-BJAB cells incubated with the acutely cytotoxic extract A05854 failed to increase at all and quickly achieved baseline levels (Figure 4.4A). Extract A05814 initially permitted cell proliferation concomitant with escalating fluorescence measurements (Figure 4.4A). However, by day 14, fluorescence had plummeted to near-background levels. Whereas the cytotoxicity of extract A05854 was unmistakable (Figure 4.4B) at day 20, with only few apoptotic cells present, many more cells were present in the A05814 culture (Figure 4.4B) with apoptotic changes just becoming visually apparent at this time point. Key to the distinction and

subsequent exclusion of these cytotoxic extracts from further analyses was their non-selective toxicity to uninfected BJAB and infected BCBL-1 cells alike (data not shown). Thus, inclusion of uninfected BJAB cultures in this assay distinguishes generally cytotoxic samples from those that are selectively active against KSHV-infected cells and allows us to separate specific antiviral activity in our samples from acute and delayed cytotoxic activity.

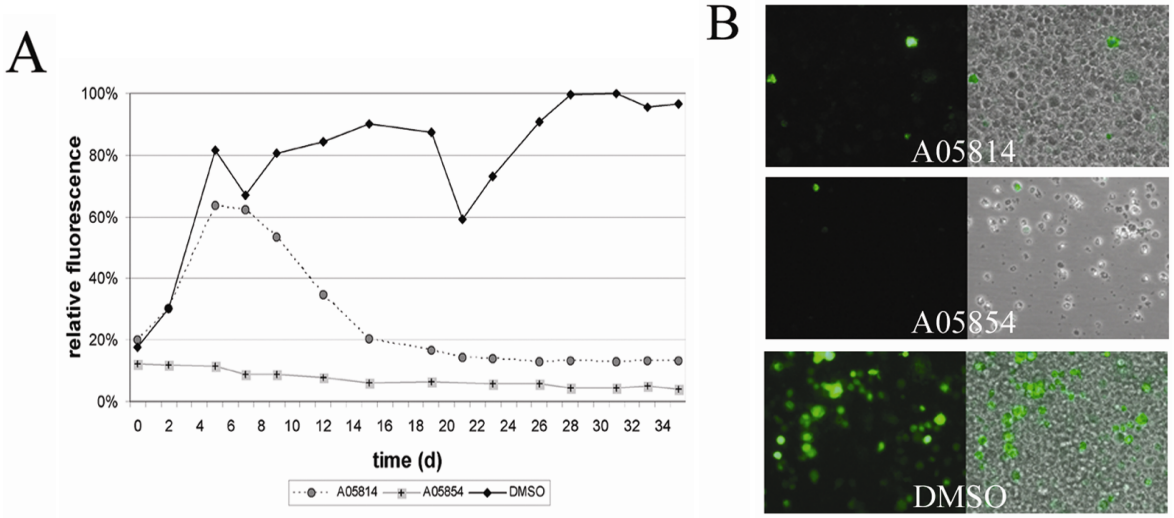


Figure 4.4 Five extracts exhibit cytotoxicity to all three B lymphocyte cell lines. A) Mean fluorescence graph representative of acute and delayed cytotoxic samples, A05854 (circles) and A05814 (squares) respectively. B) Digital images of KSHV-BJAB cells incubated with acutely cytotoxic sample A05854 or delayed cytotoxic sample A05814. The images on the left represents the fluorescence, on the right the merged fluorescent and bright field images.

Confirmation of antiviral activity by real time quantitative PCR.

The six extracts (A05810, A05830, A05831, A05853, A05898, A05901 from Figures 4.2 and 4.3) that decreased fluorescence with respect to the DMSO control in the initial fluorescence-based screen were selected for further study. After incubation with test or control samples for five weeks, viral genomic and host chromosomal DNA was isolated from BJAB, KSHV-BJAB and BCBL-1 cells. Each culture began with an identical number of cells and was not split during the course of the experiment. The isolated DNA was resuspended in the same volume of buffer, and an equal volume of the isolated DNA was used as

template for real time quantitative polymerase chain reactions (QPCR) with viral (vGPCR) and cellular (U6) primer sets. Thus, any increases in cycle threshold (CT) for the cellular primer set were likely due to cytotoxic effects of the extract, while changes in the viral CT were due to changes in the cell-associated viral load. After normalizing the raw data to the DMSO control, either toxicity in BJAB and BCBL-1 cultures or selectivity in KSHV-BJAB cultures was ascertained.

As expected, the non-template control yielded no signal with either primer set after 40 cycles. Likewise the cycle threshold (CT) for the viral primer set in uninfected BJAB cells was 40 indicating the absence of viral DNA, whereas the cellular primers (directed against the U6 gene) gave a consistent signal (mean CT = 22 ± 1, across the six extracts) similar to the DMSO control (CT = 21 ± 1). Although BJAB cultures remained at least 80% viable at day 14 as measured by trypan blue exclusion (Supplementary Figure 4.2A), cytotoxic effects became apparent by QPCR at 5 weeks (Figure 4.5A). BJAB cells treated with extracts A05830 and A05831 remained viable (109% and 89% viable respectively, as compared to the DMSO control). Extracts A05810 and A05898 reduced viability by ~50%, while extracts A05853 and A05901 reduced viability by greater than 80% compared to the DMSO control.

Next, naturally infected BCBL-1 B lymphoma cells were evaluated. Each cell contains approximately 70 copies of the KSHV episome (25) and its maintenance is vital for BCBL-1 survival. If all viral genomes are lost, the cell dies. Given the interdependence of viral genome maintenance and cell survival in BCBL-1 cells, we analyzed only cytotoxicity of the extracts in BCBL-1 cells compared to the DMSO control (Figure 4.5B). Two of the six extracts—A05831 and A05853—were toxic to BCBL-1 cells, reducing viability to 46% and 2% respectively. These extracts exhibited expectable increases in both viral and cellular CT compared to DMSO (Supplementary Table 4.1).

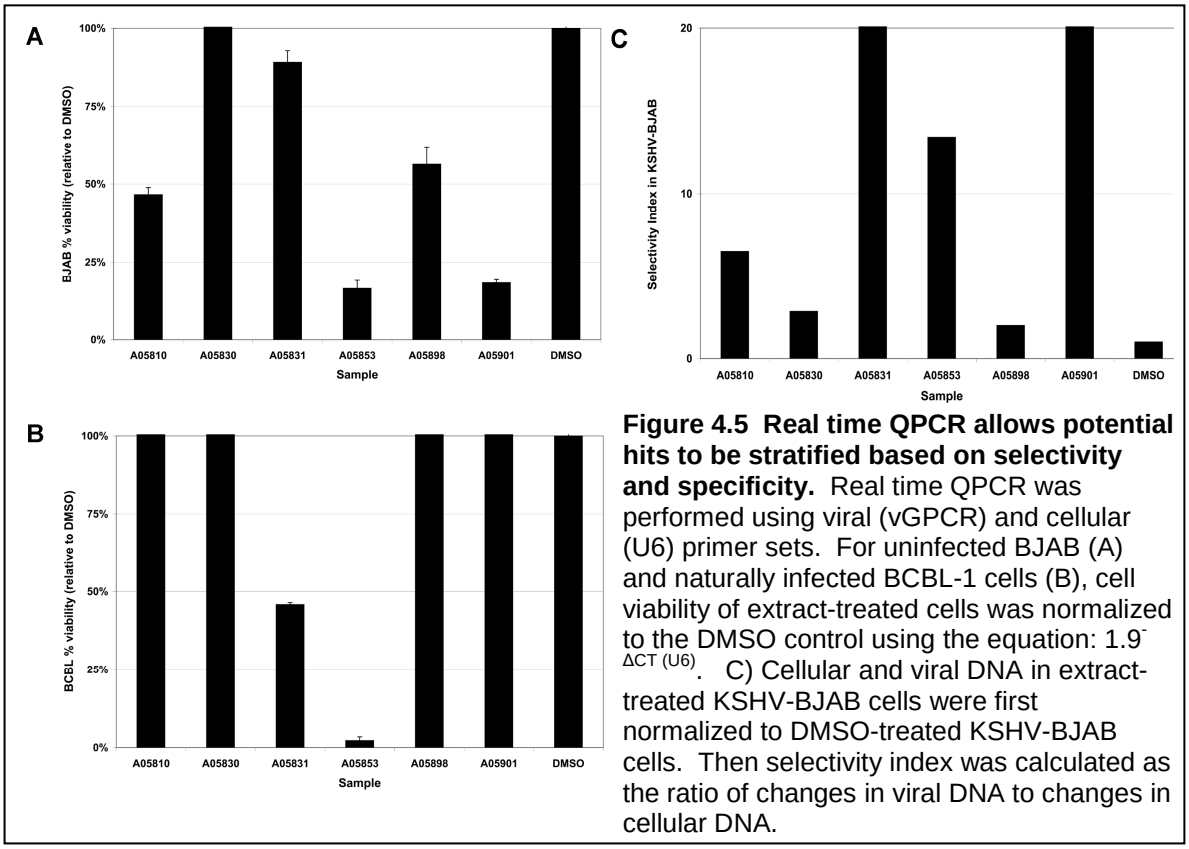


Figure 4.5 Real time QPCR allows potential hits to be stratified based on selectivity and specificity. Real time QPCR was performed using viral (vGPCR) and cellular (U6) primer sets. For uninfected BJAB (A) and naturally infected BCBL-1 cells (B), cell viability of extract-treated cells was normalized to the DMSO control using the equation: $1.9^{-\Delta CT\ (U6)}$. C) Cellular and viral DNA in extract-treated KSHV-BJAB cells were first normalized to DMSO-treated KSHV-BJAB cells. Then selectivity index was calculated as the ratio of changes in viral DNA to changes in cellular DNA.

Finally, selectivity of each extract was assessed in KSHV-BJAB cells (Figure 4.5C), representing the key innovation of this report since non-specific cytotoxic effects can be uncoupled from changes in viral load as KSHV-BJAB cells do not require the virus for survival. Here, the KSHV-specific primers (directed against the vGPCR gene) detected drastic changes in KSHV viral DNA resulting in CT values ranging from 28 ± 1 to 39 ± 1 in extract-treated cells and 25 ± 1 in DMSO-treated cells, while the cellular DNA (U6 gene) remained largely unchanged (mean CT = 22 ± 1 across six extracts, CT = 20 ± 1 in DMSO-treated cells). Changes in cellular and viral genomic DNA were first individually normalized to DMSO-treated cells. The selectivity index of each extract was then determined by the ratio of viral DNA reduction to host DNA reduction i.e. cytotoxic effects. Three extracts—A05831, A05853 and A05901—were highly selective as indicated by selectivity indexes

greater than 10, indicating that extract-mediated reductions in viral DNA were 232, 13 and 152 times greater than reductions in KSHV-BJAB cellular DNA, respectively.

Taken together the information gleaned from each QPCR experiment was used to prioritize samples by the selectivity of their antiviral activity and specificity of their cytotoxic effects. Specifically, extracts that exhibit (i) high selectivity (selectivity index >10 in KSHV-BJAB cells), (ii) large reductions in BCBL-1 viability (>50%), and (iii) minimal cytotoxic effects in BJAB cells would receive higher priority in subsequent analyses. Of the three highly selective extracts we identified, only extracts A05831 and A05853 were toxic to BCBL-1 cells, resulting in 54% and 98% reductions in BCBL-1 viability respectively. While extract A05831 was mildly toxic to uninfected BJAB cells (11% reduction in cell viability), A05853 was significantly toxic (84% reduction in cell viability) and may require further refinement to achieve priority status. Nonetheless, the fluorescence-based screening assay and the QPCR data suggest that extract A05831 and A05853 selectively interfere with viral genome maintenance and were chosen for further study as described below. However, it should be noted that, of all the extracts tested, extract A05831 was most selective for viral genome loss with minimal cytotoxicity in BJAB cells.

Assessing LANA expression by immunofluorescence.

We used an indirect immunofluorescence assay against KSHV latency-associated nuclear antigen (LANA) as a first step toward elucidating the antiviral mechanism(s) behind extracts A05831 and A05853. BCBL-1 cells were incubated with plant extracts for seven days, then stained with an antibody directed against LANA (anti-KSHV ORF 73) followed by fluorophore-conjugated anti-idiotypic immunoglobulins. Cells incubated with either the DMSO control (Figure 4.6A) or extract A05807 (Figure 4.6B) that showed no antiviral effect in the screening assays, displayed characteristic speckled nuclear staining. In BCBL-1 cells

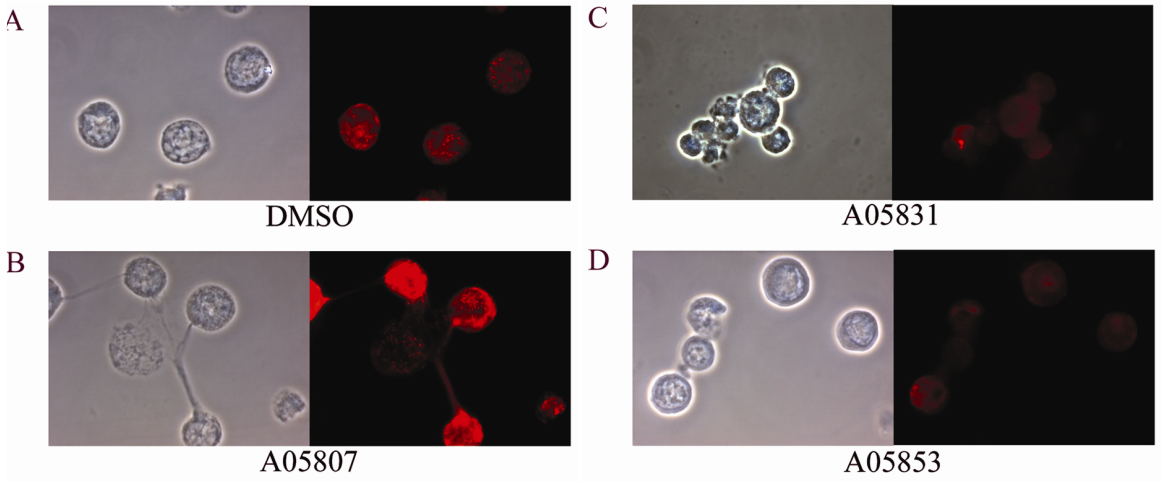


Figure 4.6 Indirect immunofluorescence assays for LANA investigate possible mechanisms of interference with viral genome maintenance. BCBL-1 cells incubated with plant extract or a DMSO control were spotted on slides and fixed with pre-cooled acetone. Incubations with anti-KSHV ORF73 (LANA) monoclonal antibody followed by TRITC-conjugated anti-rat IgG were used to detect the presence of LANA. Images on the left of each panel represent the bright field image. Images on the right represent the fluorescent staining of the KSHV LANA. A) DMSO control B) Extract A05807 C) Extract A05831 D) Extract A05853

that were incubated with extract A05831 or A05853 (Figure 4.6C and 4.6D respectively), anti-LANA immunofluorescent staining was decreased to near background levels, indicating that loss of viral genome correlated with loss of LANA expression.

Discussion

Eradicating latently infected cells represents the ultimate goal in therapy of KSHV-associated malignancies, where loss of the viral episome expectedly leads to tumor cell death. Currently, treatment of KS, PEL and MCD typically includes chemotherapeutic agents that target all replicating cells, failing to distinguish between virally infected and uninfected cells. Such regimens are associated with severe side effects, including myelotoxicity and pancytopenia, which can become life-threatening in an already immunocompromised population. Since KSHV infection remains in a latent state in the majority of infected tumor cells, drugs that target latent viral proteins may be more effective

than current regimens at both preventing and treating disease and may have an added benefit of fewer side effects.

This report describes an assay designed to identify samples that induce viral episome loss, irrespective of the specific mechanism, and without generalized cytotoxicity. The design hinges on a two-step screen. The first step identifies samples that cause loss of the latent virus in a cell line (KSHV-BJAB) that does not depend on the virus for viability. The second step validates those hits in a cell line (BCBL-1) that does depend on the virus for survival.

The initial screening step employs a B lymphocyte cell line (KSHV-BJAB) carrying the KSHV-BAC and expressing green fluorescent protein. KSHV-BJAB cells are incubated in medium containing test samples and are monitored for loss of fluorescence (i.e. loss of the viral episome). Seven percent of 81 screened plant extracts consistently reduced fluorescence ≥ 50% in KSHV-BJAB cells as compared to a DMSO control. An additional 6% of the extracts were cytotoxic to all B lymphocyte cell lines tested, regardless of their KSHV infection status. Eighty-seven percent had no significant effect (data not shown).

Since non-specific promoter silencing could diminish fluorescence in KSHV-BJAB cells, virus-specific effects were verified by real time QPCR for the viral genome. Using primer sets for both a viral (vGPCR) and a cellular (U6) gene and DNA templates isolated from BJAB, KSHV-BJAB or a PEL cell line, BCBL-1, after incubation with the test samples, cytotoxicity and selectivity of each extract were assessed. Six extracts, identified as potential hits (≥ 50% reduction of fluorescence) in the first screening step, were tested. Extracts A05831 and A05853 demonstrated selective activity against latent virus, as the viral episome was lost from a model infection (KSHV-BJAB) at least 10 times more efficiently than host chromosomal DNA (corresponding to cell death). Furthermore, both extracts resulted in at least a 50% reduction in cell viability from naturally-infected tumor cells (BCBL-1), as compared to a DMSO control. Extract A05831 receives higher priority for

further study since it is relatively non-toxic in uninfected BJAB cells, while extract A05853 may prove too non-selectively toxic in its unrefined state.

In principle, compounds that interfere with viral genome maintenance may target cellular or viral proteins required to maintain latency. Samples exerting their effects by targeting viral proteins are preferred, since a specific antiviral effect may be less toxic to other highly replicating cells and, presumably, would have fewer side effects than currently available chemotherapeutics. KSHV latency-associated nuclear antigen (LANA) is a likely viral target for antiviral samples, since it is essential for KSHV genome maintenance. In a complex with multiple cellular proteins (3, 14, 15, 24, 26, 32, 33), LANA tethers the viral genome to the cellular chromosome, ensuring that the two are replicated coincidentally and segregated equally to each daughter cell. Samples that target LANA may accomplish their antiviral effect by one or more means, including: (i) transcriptional downregulation, (ii) degradation or post-translational modification, (iii) sequestration outside the nucleus, (iv) interference with binding to the host chromosome or the viral genome, or (v) similarly targeting cellular proteins that complex with LANA. Additionally, other viral latent proteins may also be involved in viral genome maintenance and may also be targeted.

To explore the mechanism(s) by which extracts A05831 and A05853 propel episomal loss, a LANA immunofluorescence assay was performed on BCBL-1 cells incubated with the two lead extracts, identified by the initial fluorescence-based screening step and verified by QPCR. Both extracts resulted in near background levels of LANA immunofluorescent staining. Thus, these plant extracts may contain one or more compounds that downregulate transcription of LANA, accelerate its degradation, or cause post-translational modifications that render it undetectable by this antibody. Therefore, extracts A05831 and A05853 are prime candidates for further experimentation. Since each extract likely contains hundreds of compounds, future studies will employ a bioactivity-directed fractionation strategy to purify and identify the antiviral constituent(s).

Loss of fluorescence mediated by culturing KSHV-BJAB cells with antiviral samples is dependent on two things: loss of the KSHV-BAC, which contains the GFP gene and degradation of GFP that is made prior to loss of the gene. GFP has a reportedly long half life, ranging from 26 to 80 hours in eukaryotic cells (7, 19, 35), which contributed to the length of the initial screen, which was further extended to five weeks in order to assess cumulative cytotoxicity. Analysis of the data, however, showed that the most potent inhibitors already displayed a significant effect by day 20 and the second screening step allowed for earlier detection of cumulative cytotoxic effects.

In modern medicine many highly effective therapeutic agents, such as camptothecin and Taxol/paclitaxel, were first isolated from plant extracts and have revealed novel targets and mechanisms for antitumor drug action. While certain herbal extracts were recently found to reactivate KSHV (45), the novel assay described in this report identifies plants as a rich source for antiviral compounds that may cure KSHV infection by interfering with latent viral episome maintenance. Although a relatively small sample set was tested, the results are representative of the discovery potential for samples with therapeutic promise, as an overall hit rate of 2% is consistent with other natural product screens, where hit rates typically range from 0.5 to 5%, regardless of the biological target. Indeed, small molecule libraries derived from plant or other natural product sources may prove to be repositories for antiviral agents with varied targets and activities against a breadth of currently incurable viral infections.

Acknowledgements

We thank A. Douglas Kinghorn, of the Ohio State University, for access to the plant samples, which were collected with the consent of the Papua New Guinea Government's Department of Environment and Conservation, the Papua New Guinea Institute of

Biodiversity, and the local people of Manus Province. We thank Yuka Nakanishi for supplemental cytotoxicity assays (Supplementary Figure 4.2B) and sample preparation.

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Chapter Five

General Conclusions

Conclusions

Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) are three neoplastic diseases associated with infection of malignant cells by the Kaposi's sarcoma-associated herpesvirus (KSHV) (14, 15, 59, 74). In each neoplasia, the virus persists in a latent state in the majority of infected endothelial or B lymphocyte cells. During latency, only a few viral genes are expressed that are responsible for maintaining the viral genome, stimulating cell survival, preventing apoptosis, and evading the host immune response (41, 65, 71, 72). Currently, there are no known antiviral agents that specifically target the latent phase of the herpesvirus life cycle. Antiviral agents that disrupt lytic replication may limit viral spread, but their effects do not extend to a cure (38). Thus, therapies used to treat the three KSHV-associated malignancies rely on radiation therapy and conventional chemotherapeutic agents. These strategies target all proliferating cells and thus are associated with severe side effects. With this in mind, the main goals of this dissertation project were 1) to investigate the role of the viral K15 protein as a potential future target for new antiviral therapies in KSHV-infected B lymphocytes, and 2) to identify new antiviral agents with activity against latent viral infection.

Antiviral agents that disrupt viral latency may provide the elusive cure for KSHV infection and, therefore, also KSHV-associated malignancies by eradicating the oncogenic stimulus. The antiviral "wonder drug" would specifically target cells infected with KSHV, and would thus be nontoxic to uninfected cells. Ideally, the drug would selectively inhibit viral gene products that are expressed during latency, either by steric inhibition, by selectively stimulating viral protein degradation, or by modification of the viral protein to an inactive form. In addition to targeting viral proteins, interference with signal transduction pathways that are specifically deregulated in cancer cells has proven to be an effective

strategy for some types of cancer. For instance, in chronic myelogenous leukemia, the constitutively active kinase resulting from bcr-abl fusion has been successfully targeted by imatinib and dasatinib, achieving high remission rates (24, 53). Likewise, understanding the signaling properties of viral proteins could help identify new dysregulated cellular targets for treatment of KSHV-associated diseases.

Two methods have been commonly used in drug discovery. Classically, drugs have been discovered by screening known compounds for a desired effect without regard to the chemical composition of the drug or the target (6). In Chapter 4 of this thesis we describe an unbiased screening assay that we devised to identify antiviral drugs that are toxic to KSHV-infected cells. More recently, drug discovery has relied on tailor-made chemical or biological inhibitors, designed with a specific "druggable" target already in mind (6). A druggable target is one that is likely to be inhibited by an orally bioavailable pharmaceutical compound or by a biological inhibitor, such as an antibody (36, 70). Although defining a target's druggability may be difficult, properties to consider include the tissue distribution and subcellular localization of the target. Proteins located at the cell membrane, e.g. epidermal growth factor receptor, or proteins involved in signal transduction, such as cellular kinases, have proven to be good druggable targets (36). Considering the limited subset of viral proteins expressed during KSHV latency, we propose the viral K15 protein as a promising new target.

K15 is expressed in B lymphocytes and epithelial cells latently infected with KSHV (9, 19, 31, 73). It is a membrane protein located at the cell surface, and thus would be more easily accessible to a small molecule or biological inhibitor than an intracellular viral protein. Furthermore, embedded in the cytoplasmic tail of all K15 isoforms are multiple highly conserved signaling motifs (8, 31). K15 reportedly activates various signal transduction pathways, including MAPK and NF-κB pathways, in epithelial and endothelial

cells (8). In chapter 2, we show that MAPK and NF-κB pathways are also activated by K15 in B lymphocytes.

We further analyzed the signaling functions of K15 in B lymphocytes when expressed ectopically or in the context of viral infection. We hypothesized that dysregulation of MAPK and/or NF-κB pathways by K15 may lead to changes in the cytokine environment. We found that K15 induces the expression of GRO, IL-2Ra, IL-6, IL-12 p40, IL-17, I-309, IGFBP-1, leptin, MCP-1, MCP-2, MCP-3, MIP-3b, MSP-1, NT-4, Osteoprotegrin, Oncostatin M, PDGF-BB, PIGF, TRAIL R4, sTNFRI, sTNFRII, and VEGF-D in B lymphocytes by at least two-fold as compared to a vector control. Similarly, IL-6, MCP-1, MIP3α and GRO3, IL-1α/β and IL-8 are upregulated by K15 in epithelial cells (9).

Interleukin-6 (IL-6) is a pleiotropic cytokine, which plays a role in wound healing (28, 52), stimulates cell proliferation (3, 39, 40, 55, 58), and prevents apoptosis (5, 16, 17) via its downstream effector molecules. It is also involved in B cell maturation and differentiation (4, 7, 44, 75). Upon binding to the IL-6 receptor (IL-6R), the IL-6/IL-6R complex interacts with the gp130 coreceptor molecule to initiate JAK-STAT signaling (50, 76). JAK kinases associated with the cytoplasmic tail of the gp130 coreceptor molecule are autophosphorylated following complex formation. The JAK kinases in turn phosphorylate STAT molecules which homo- or heterodimerize and move into the nucleus to induce transcription of downstream effector genes (50).

IL-6 expression is elevated in many disease states, including almost all major types of cancer (advanced stage breast, colorectal, lung, prostate and kidney cancers as well as hematological cancers such as lymphoma and multiple myeloma) (1, 2, 20-22, 25, 51, 60, 77, 79, 84, 85, 88). The role IL-6 plays in cancer formation is not entirely understood. There is no solid evidence that IL-6 alone has transformation potential. However, some evidence suggests that IL-6 may play a role in initiation of B cell tumors. Overexpression of IL-6 in mice results in a high incidence of plasmacytoma formation (33-35, 78), reinforcing

the growth stimulatory effect of IL-6 in B cells. In fact, IL-6 has a growth stimulatory effect in most types of cancers and KSHV-associated malignancies (especially PEL and MCD) are no exception (23, 27, 42, 63). In breast cancer and melanoma, elevated IL-6 levels are associated with growth inhibition at early stages, but are associated with more aggressive disease and poor outcomes at later stages (47-49, 62, 88). Thus, deregulated IL-6 may also function in tumor maintenance and metastasis by enhancing proliferation, blocking apoptosis, and promoting neovascularization and metastasis.

Although IL-6-specific antibodies did not show an effect *in vitro,* IL-6 neutralizing antibodies administered to mice injected with KSHV-positive PEL cells caused retardation or regression of tumor development (27). Further emphasizing the role of IL-6 in KSHV-associated disease, KSHV encodes an IL-6 homolog (vIL-6) that can engage the gp130 coreceptor to initiate JAK-STAT signaling, even in the absence of the IL-6R (56, 57, 64, 80). vIL-6 is highly expressed in MCD where a larger fraction of cells support lytic replication, and to a lesser extent in PEL and KS where the majority of cells remain latent. In summary, these findings suggest that cells latently infected with KSHV express the IL-6 receptor and respond normally to cellular IL-6 stimulation, but that cells undergoing lytic replication are less responsive to cellular IL-6 stimulation and require increased stimulation with the viral IL-6 homolog. Alternatively, viral IL-6 may act in paracrine to stimulate proliferation of neighboring cells that do not normally express the IL-6 receptor.

Given the importance of the IL-6 signal transduction pathway in viral pathogenesis and tumorigenesis, we studied the mechanisms of K15-mediated upregulation of IL-6 and found that K15 not only induces expression of cellular IL-6, but also of viral IL-6. Most notably, activation of AP-1 transcription factors by K15 induces cellular IL-6 expression when K15 is expressed ectopically and in the context of viral infection. Additionally, K15 also induced expression of the viral IL-6 homolog when introduced into KSHV-positive PEL cells.

In chapter 3, we go on to show that JAK-STAT signaling, the downstream signaling pathway common to many of the cytokines induced by K15, is also deregulated in B lymphocytes expressing K15. Increased levels of multiple cytokines and chemokines in KSHV-associated malignancies, as well as the acquisition of multiple cytokine and chemokine homologs by the virus, highlight the importance of cytokine signaling, and specifically JAK-STAT signaling, in viral tumorigenesis. The signal transducer and activator of transcription 1 (STAT1) protein is hyperphosphorylated at the activating tyrosine residue 701 in B lymphocytes expressing K15. Activation of STAT1 by four K15 isoforms mirrors their ability to induce cellular IL-6. Yet, STAT1 is typically activated by antiviral interferon responses leading to induction of pro-apoptotic downstream effector gene expression (11, 26, 37, 67). In addition, STAT1 has been proposed to have tumor suppressor activity, since STAT1 knockout mice incur tumors more frequently in response to carcinogens than mice with unimpaired STAT1 function (43). Although activation of STAT1 by IL-6 has been reported (50), the terminal effects of IL-6 activation are more consistent with activation of the STAT3 molecule (29). Thus, concurrent activation of STAT1 and IL-6 by K15 seemed perplexing and that observation instigated the experiments described in chapter 3.

Given the characteristic role of STAT1 in antiviral responses induced by interferon, it is not surprising that many viruses have evolved inhibitory mechanisms—targeting STAT1 for degradation, blocking transactivation or sequestering STAT1 outside the nucleus (12, 32, 69, 87). We determined that STAT1 did not bind directly to K15 in coimmunoprecipitation experiments. Levels of hyperphosphorylated STAT1 were increased both in cytoplasmic and nuclear fractions in B lymphocytes expressing K15, suggesting that K15 does not stimulate STAT1 sequestration. Furthermore, no differences were seen in expression of interferon-regulated genes in B cells treated with type I interferon as compared to control cells. Therefore, STAT1 did not seem to be directly

inhibited by K15. In the future, the role of STAT1 will need to be assessed in the context of viral infection, where other viral gene products could serve to regulate STAT1 activity.

Although the downstream effects of IL-6 and STAT1 seem contradictory, like IL-6 it is not uncommon that STAT1 is activated in cancer. In fact, STAT1 is upregulated in some of the same cancers that are associated with high IL-6 levels, including breast and multiple myeloma (13, 81). Furthermore, STAT1 is upregulated in cancers associated with Epstein-Barr virus, a gammaherpesvirus closely related to KSHV (18, 61, 82). The latent membrane protein 1 (LMP1) of EBV shares functional homology with K15. Both membrane proteins interact with TRAFs to signal through NF-κB and activate multiple MAPK pathways (10). In addition, *in vitro* studies of LMP1 function have revealed that LMP1 upregulates STAT1 expression in B lymphomcytes by an NF-κB-dependent mechanism (68). STAT1 activity is increased in cells expressing LMP1 and in co-cultured cells not expressing LMP1, suggesting an indirect mechanism of regulation (30, 68). Therefore, STAT1 activation may be a conserved function of herpesvirus membrane proteins that is important for viral pathogenesis or oncogenesis.

To assess the role of K15 in a model infection, we created a KSHVΔK15 recombinant virus in the context of a bacterial artificial chromosome. Introduction of the wild type (WT) KSHV and KSHVΔK15 viruses into BJAB cells did not evidence any differences in cell proliferation or apoptosis, despite the fact that WT KSHV-BJAB cells secreted more IL-6 than KSHVΔK15 cells. When we assessed viral load in these stable cell lines we noted that the differences in cell-associated viral load were small, in contrast to stark differences in cell-free viral load. Cell-free viral load was five-fold greater in KSHVΔK15-BJAB cells as compared to WT KSHV-BJAB cells, and the difference was magnified upon induction of lytic replication with PMA. Taken together these results suggest that K15 promotes latency by restricting lytic replication. It may be that K15-induced IL-6 expression and STAT1 activation promote cell survival by simultaneously

stimulating cell proliferation and restricting lytic replication of the virus. Although the role of STAT1 was not specifically addressed in the viral load experiments discussed in chapter 3, we could envision a scenario where K15-mediated activation of STAT1 serves to limit viral replication and promote latency, while IL-6 stimulates cell proliferation. It must be noted that BJAB cells are already transformed and are no longer dependent on IL-6. Therefore, we did not detect differences in cell proliferation and only slight differences in cell survival. These studies should be repeated in primary EBV/KSHV-negative B lymphocytes following introduction of the two viruses to confirm this hypothesis.

The functions we have ascribed K15 in this thesis support the notion that K15 may be a good candidate for drug therapy. If K15 promotes cell proliferation via IL-6 signaling, then inhibition of K15 signaling could lead to impaired cell survival. If K15 limits lytic replication and expression of the full complement of viral gene products, then inhibition of K15 signaling or targeted degradation of K15, could force the virus out of antigenic hiding. Used in combination with antiviral agents that block lytic replication, the virus could be eliminated altogether (45). Similar strategies of purging the latent reservoir are being investigated for other viruses including the human immunodeficiency virus and EBV (46, 54, 66, 86).

In chapter 4, we took advantage of the WT KSHV-BJAB cells in a screening assay to identify antiviral agents, regardless of their antiviral mechanism, with activity against latent KSHV infection. KSHV-BJAB cells co-incubated with test samples are monitored for loss of fluorescence over time, which is a surrogate for loss of the viral episome since the green fluorescent protein (GFP) has been inserted into the viral genome (89). Loss of viral DNA is then confirmed by real time quantitative PCR. Toxicity is also monitored in uninfected BJAB cells and a naturally infected PEL cell line. Advantages of this system include: 1) presence of GFP, which affords a means to screen for loss of the viral episome, 2) KSHV-BJAB cells do not depend on the virus for survival, so selective inhibition can be

confirmed in the single cell line, 3) an unbiased approach does not predetermine the mechanism of antiviral action, and 4) cumulative toxicity can be assessed. The major disadvantage of the system is the relatively long half-life of GFP which lengthens the assay, since the loss in fluorescence is dependent on degradation of GFP after the latent viral episome has been lost from the dividing cell population.

Using the screening assay we developed, we identified two potential hits out of 81 that we screened. Both “hits” were plant extracts and likely contained more than one hundred compounds. The extracts were composed of the detannified organic fraction of the root, stem, bark or leaf of understudied plants found in Papua New Guinea. One extract achieved three ideal characteristics for a new antiviral agent, as it was relatively nontoxic to uninfected cells, toxic to naturally infected cells that depend on the virus for survival, and exhibited highly selective viral inhibition in KSHV-BJAB cells. The second potential hit demonstrated only two of those ideals and was markedly toxic to uninfected cells. Perhaps further purification will minimize the toxic effects and hone in on the therapeutic effects present in that extract. Although these results still need to be narrowed by bioactivity assays following repeated fractionation of the extracts to pure compounds, it supports the utility of this type of screen in future drug discoveries. Our study demonstrates the pharmaceutical potential of herbal remedies and indigenous medicinal plants, while a parallel study demonstrated the potential for herbal remedies, or “oncoweeds”, to promote KSHV replication (83).

A deeper understanding of the contributions of each viral protein to viral pathogenesis and tumorigenesis may provide insight into new therapeutic strategies and promote targeted drug design. The work we have done to characterize the signaling properties of the K15 protein of KSHV in B lymphocytes has identified K15 as a potential target for future therapies, as K15 activates the IL-6/JAK-STAT pathway to promote cell proliferation and survival while simulatenously limiting viral genome replication. In addition

to these efforts, we also took an unbiased approach to identification of potential therapeutic compounds that target viral latency in general. Of 82 samples screened, two exhibited specific viral inhibition and cytotoxicity in our *in vitro* assays utilizing latently infected cells. Although much work will be required to confirm the activity of the antiviral extracts we identified in chapter 4 and to elucidate their mechanism of action, we are hopeful that one might progress into the clinics and prove efficacious against the latent virus. Ultimately, it is our hope that this thesis will contribute to the field of KSHV research and advance the endeavors of many researchers seeking that elusive cure for viral infection.

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