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Characterization and Validation of Preclinical Models of KSHV-induced Malignancies to Elucidate Antineoplastic and Antiviral Therapeutic Approaches

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CHARACTERIZATION AND VALIDATION OF PRECLINICAL MODELS OF KSHV-INDUCED MALIGNANCIES TO ELUCIDATE ANTINEOPLASTIC AND ANTIVIRAL THERAPEUTIC APPROACHES

By

Brittany M. Ashlock

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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the requirements for the degree of
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MODELS OF KSHV-INDUCED MALIGNANCIES TO ELUCIDATE
ANTINEOPLASTIC AND ANTIVIRAL THERAPEUTIC APPROACHES

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Introductory Remarks

Kaposi’s sarcoma (KS) and Primary effusion lymphoma (PEL) are two Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8) –induced cancers that are clinically challenging to treat and often have poor prognoses. While these diseases have declined dramatically in the developed world, they are responsible for significant morbidity and mortality in the developing world. Further, current treatments are often associated with considerable toxicity. While KS and PEL are composed of cells harboring KSHV in the latent state, the paracrine hypothesis of tumor development proposes that the tumor is actually being driven by the minority of cells undergoing lytic replication. Therefore, it is believed that potential targets for therapy are the KSHV-infected cells undergoing lytic replication. Lytic replication ensures the production and spread of virions, and allows for the expression of potentially pathogenic genes which have proposed roles in the paracrine neoplasia thought to drive tumorigenesis. The lytic-inductive paradigm for therapy proposes to induce massive lytic reactivation, while concurrently administering a potent antiviral, thereby specifically causing the death of virally infected cells while abrogating the potential increase in viremia, which may lead to unwanted negative clinical sequelae. However, the studying induction of and inhibition of viral lytic replication are generally reliant upon in vitro chemical induction which may not recapitulate the temporally
ordered cascade of events that is thought to occur in vivo. Facile animal models are urgently needed in which to test antineoplastic and antiviral strategies for the development of effective targeted therapies.

Herein, we test the lytic-inductive paradigm in a direct xenotransplant murine model of PEL. We use two clinically approved drugs, one, Vorinostat (suberoylanilide hydroxamic acid, SAHA) is a histone deacetylase inhibitor and a known potent inducer of viral replication. The other, Velcade (Bortezomib, Btz) is an inhibitor of the 26S proteasome and has been shown in other viral models to inhibit viral replication. We found that in PEL inoculated mice, the combination of SAHA and Btz leads to significantly increased survival over mice treated with the single agents. Further, we show that there is massive apoptosis with the combination that correlates with strong lytic induction of KSHV. Importantly, we also show that a potential unwanted side effect of lytic replication, namely increased viremia in vivo, is abrogated by Btz. Indeed, Btz appears to have very potent antiviral effects in the setting of KSHV while the combination of the SAHA and Btz have very potent antitumoral effects. Our results suggest that the lytic-inductive paradigm for treating PEL, and potentially other herpesvirus-induced cancers, may be a viable option in the setting of an immunocompromised host.

To test the lytic-inductive paradigm in the context of KS, a solid tumor, we needed to develop a suitable model. By employing murine bone marrow-derived endothelial-lineage cells (mEC) we have now developed two novel productively infected murine models of KS. We show that the mEC are non-tumorigenic when uninfected, but, upon infection with rKSHV.219, a recombinant replication competent virus, the KSHV-
infected cells efficiently form tumors that pathologically, phenotypically and molecularly resemble Kaposi’s sarcoma. The tumors are composed of LANA positive spindle-cells and that express antigens associated with human KS spindle-cells such as podoplanin and CD31. Further, the virus is able to transcribe a wide array of viral genes representing all stages of the viral replicative cycle culminating with the in vivo production of a herpesvirus-like particle of the correct size and morphology to be rKSHV.219. Given the productive nature of the tumors, these models should provide excellent substrates in which to test the lytic-inductive paradigm for the treatment of KS along with other antineoplastic and antiviral strategies.
This body of work is dedicated to Drs. Locher, Bowman, LiPuma, and Bookman, four key individuals who believed in me, right when I needed it most
I begin by giving gratitude to my Ph.D. mentor, Dr. Enrique Mesri, for providing an environment in which passionate scientific discourse was encouraged and provided a truly unique and thoughtful environment in which to earn a PhD. In addition, I would like to thank Enrique for not only caring about my development as a scientist, but my personal growth as well.

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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<td>BAC36</td>
<td>KSHV genome cloned into a BAC</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CTCL</td>
<td>cutaneous T-cell lymphoma</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>EPC</td>
<td>endothelial progenitor cell</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>HAART</td>
<td>highly active antiretroviral therapy</td>
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<td>HCMV</td>
<td>human cytomegalovirus</td>
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HSV-1  herpes simplex virus-1
Hyg  hygromycin
Ig  immunoglobulin
KS  Kaposi’s sarcoma
KSHV  Kaposi’s sarcoma-associated herpesvirus
LANA  latency associated nuclear antigen
MCD  multicentric Castleman’s disease
mEC  mouse bone marrow-derived cell of endothelial lineage
mECK36  mouse bone marrow-derived cell of endothelial lineage
stably transfected with BAC36
ORF  open reading frame
PEL  primary effusion lymphoma
rKSHV.219/rKSHV  recombinant KSHV
SAHA  suberoylanilide hydroxamic acid
TSA  Trichostatin A
CHAPTER ONE: INTRODUCTION

Human herpesviruses and cancer

The Herpesviridae members are species-specific viruses thought to have coevolved with their hosts for millions of years (Roizman and Baines, 1991). To be denoted as a herpesviridae, a virus must have a specific architecture where the double-stranded DNA (dsDNA) genome is enclosed in an icosahedral nucleocapsid, which is surrounded by tegument. The outermost structure of a herpesvirus is the envelope displaying glycoprotein spikes. All herpesviridae members exist in the host as a life-long latent entity by avoiding host immune response (Roizman and Baines, 1991; Wen and Damania, 2010). The family herpesviridae is further structured into the alpha-, beta- and gamma (γ)-herpesvirinae subfamilies. Only the (γ)-herpesvirinae are associated with neoplastic disease. The (γ)-herpesvirinae are further divided into four genera: Macavirus, Percavirus, Lymphocryptovirus and Rhadinovirus. The lymphocryptovirus, Epstein-Barr virus (EBV), is the third human herpesvirus described and is associated with Burkitt’s lymphoma, Hodgkin’s lymphoma, nasopharyngeal carcinoma, T-cell lymphoma, Natural killer cell lymphoma (Deacon et al., 1993) and post-transplant lymphoma (Raab-Traub, 2002; Young et al., 1989a; Young et al., 1989b). The rhadinovirus, Kaposi’s sarcoma-associated herpesvirus (KSHV;HHV-8) is the eighth, and most recent, human herpesvirus discovered and is associated with three known cancers: the plasmablastic variant of multicentric Castleman’s disease (MCD) (Soulier et al., 1995), primary effusion lymphoma (PEL) (Cesarman et al., 1995a), and Kaposi’s
sarcoma (KS) (Chang et al., 1994). PEL and KS are the two KSHV-induced cancers comprising the major foci for this thesis.

**Kaposi’s sarcoma-associated herpesvirus (KSHV)**

KSHV is thought to have first infected human primates about 60 thousand years ago and has evolved into subtypes that are associated with the pattern of human migration out of Africa (Hayward, 1999). In the United States, KSHV seroprevalence is usually reported to be less than 5%, although it was recently reported that KSHV seroprevalence in children living in South Texas is 26% (Jenson et al., 2000). However, in parts of Africa, such as the Ivory Coast, the seroprevalence of KSHV can come close to 100%. KSHV is an oncogenic (γ)-herpesvirus that primarily infects endothelial and B-lymphoid cells *in vivo*. KSHV, like all herpesviruses, has an armamentarium of gene products that subvert normal cellular pathways such as: innate and adaptive immune responses, cell cycle progression, apoptosis, lymph and vascular angiogenesis (Mesri et al., 2010a).

**KSHV Genome**

Like all herpesviruses, KSHV has a large (170kb) dsDNA genome which is linear when inside the capsid, but circularizes upon infection (Renne et al., 1996). The genome encodes more than 80 open reading frames (ORFs), some of which have been pirated by the virus from the human genome to evade host immune system, deregulate the cell cycle, induce aberrant angiogenesis, and inhibit cellular tumor suppressor pathways (Choi et al., 2001). Similar to the human genome, herpesvirus genomes, and the KSHV genome in particular, is organized into a chromatin structure where the DNA is wrapped
around histones into a nucleosomal structure and is highly regulated by epigenetic modifications such as histone acetylation and DNA methylation among others (Countryman et al., 2009; Pantry and Medveczky, 2009).

**KSHV Replication**

KSHV displays a tightly regulated program of gene expression that can be manifested in two canonical states: latency or lytic replication. Latency is the default state during which subset of viral genes is expressed along with a cadre of microRNAs. Viral proteins expressed during latency including: latency associated nuclear antigen (LANA/ORF73), viral cyclin D homolog (vCyc/ORF72), viral FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein (vFLIP/ORF71), contribute to maintenance of the viral episome, cellular proliferation, and inhibition of apoptosis via activation of NF-κB, respectively (Ganem, 2010a). LANA binds the KSHV genome to the host chromosomes ensuring concurrent host/viral DNA replication and passage to daughter cells during cell division (Kedes 1997, Ballestas 1999, Moore and Change 1998, Cotter and Robertson 1999). Indeed, siRNA or genetic knockdown of the LANA protein resulted in the loss of KSHV episomes from latently infected cells (Godfrey 2005, Ye 2004). LANA also promotes cellular proliferation through degradation of the p53 tumor suppressor by recruiting the EC2S ubiquitin E3 ligase complex, thereby assisting in the ubiquitylation and proteasomal degradation of p53 (Friborg 1999, Radkov 2000, Cai Robertson 2006). Indeed, it has been suggested that reactivating the p53 pathway may prove an effective treatment strategy for PEL (Sarek G 2007). Paul et al., found that at relatively high concentrations, a COX-2 inhibitor can disrupt p53-LANA-1 protein
complexes (Paul et al., 2011) while another group found that at high concentrations, glycyrrhizic acid alters latent gene expression leading to a decrease in LANA mRNA levels and a concomitant increase in vCyc mRNA and protein expression (Curreli et al., 2005). It is well established that vFLIP-induced NF-κB signaling promotes latent PEL growth and survival, while inhibition of NF-κB induces lytic replication (Brown Sun 2003). Although latent proteins are crucial for tumorigenesis, they have proven difficult to target for the treatment of KSHV-induced neoplasias. To date, no clinically approved antivirals have been effective, at clinically approved concentrations, at targeting herpesvirus latency.

While the majority of tumor cells display latent viral infection, KS tumorigenesis is dependent upon viral lytic replication (Grundhoff and Ganem, 2004). Lytic replication is necessary for production and spread of virions within and between the host(s), allow for the production of angiogenic (Bais et al., 1998; Cesaran et al., 2000), inflammatory (Ensoli et al., 1989; Smit et al., 2002; Sozzani et al., 1998; Stine et al., 2000) and mitogenic (Smit et al., 2002) factors and results in cell destruction. Indeed, an increase in replicating virus in the peripheral blood is a strong risk factor for subsequent development of KS (Engels et al., 2003).

Viral lytic gene expression begins with and is totally dependent upon the expression of the viral replication transcriptional activator (RTA) (Sun et al., 1998). The lytic cascade is canonically distributed into temporally defined groups: immediate early (IE), early and late; this reflects the necessary order of production as IE genes transactivate and promote expression of early genes needed for viral DNA replication and subsequent late viral gene expression. Viral genes expressed as late lytic transcripts
encode products needed for DNA packaging (Patel et al., 2008), and structural components needed for capsid and tegument formation (Lu et al., 2004). The lytic replicative cycle then culminates in the release of infectious KSHV particles with ultimate death of the host cell (Figure 1.1).

Figure 1.1. KSHV entry, replication and virion release is an ordered cascade of events culminating in death of the cell. KSHV is an enveloped herpesvirus with an internal capsid structure that surrounds the viral DNA. Upon attachment to an appropriate receptor and entry into the cell, the envelope fuses with the host membrane and the capsid is transported to the nucleus into which the linear viral DNA is released. The viral DNA then circularized into an episome and using LANA, attaches to the host chromosomes to establish latency. When induced into lytic replication, the replicative cycle proceeds through an ordered cascade of events during which the immediate early (I.E.) genes are expressed prior to the delayed early (D.E.) genes. Viral DNA replication occurs after D.E. gene expression and before the late lytic genes are expressed. The late lytic genes are generally structural in nature and upon expression and translation, the mature virion can be assembled and released from the cell. The lytic cycle culminates in death of the cell.
Lytic replication provides many highly selective potentials for antiviral (vPK), targets such as the viral thymidine kinase (vTK), viral phosphotyrosine kinase viral DNA polymerase and viral glycoproteins. As such, KSHV can be chemically induced into lytic replication with histone deacetylase (HDAC) inhibitors: Trichostatin A (TSA), butyrate and Vorinostat (suberoylanilide hydroxamic acid/SAHA) which has been FDA approved for the treatment of cutaneous T-cell lymphoma (CTCL).

**Importance of proteasome activity in herpesvirus replication**

Traditionally, the proteasome has been associated solely with intracellular proteolytic functions, however, more recently, non-proteolytic functions of the proteasome and its subunits have come to light (reviewed in Bhat and Greer, 2011). The 26S proteasome is composed of a 20S proteolytic core capped at both ends by 19S subunits which themselves are composed of 2 subunits which function as ATPases (Kinyamu and Archer, 2007). The 19S ATPase activity of the 19S subunits function in the non-proteolytic roles such as efficient activation of transcription by human RNA polymerase II (Ferdous et al., 2002). Another non-proteolytic role includes chromatin modification as inhibition of the 26S proteasome increases trimethyl histone (H3K4) levels with an increased accumulation of this modification on specific human promoters *in vivo* (Kinyamu and Archer, 2007). Indeed, there is much effort being put forth do elucidate the other non-proteolytic roles of the proteasome.

Accordingly, herpesviruses have evolved multiple strategies to usurp the host ubiquitin-proteasome system (UPS) for use during both latency and lytic replication and therefore interest in exploiting the role of the proteasome for antiviral strategies has
become of great interest as it offers a multitude of antiviral targets. For instance, proteasome inhibition: blocks human cytomegalovirus (HCMV) DNA replication, virion assembly and immunomodulation (Kaspari et al., 2008; Prosch et al., 2003), inhibits influenza virus translocation to the nucleus (Khor et al., 2003), inhibits immediate early and late herpes simplex virus-1 (HSV-1) protein expression (La Frazia et al., 2006), paramyxovirus maturation (Watanabe et al., 2005), rhabdovirus budding (Harty et al., 2001) and, important for HIV-associated viruses such as KSHV, proteasome inhibition interferes with the processing of the Gag polyprotein and ultimately the release of mature human immunodeficiency virus (HIV)-1 and HIV-2 (Schubert et al., 2000). Further, it was found that the proteasome regulates HIV-1 transcription by both proteolytic and non-proteolytic mechanisms (Lassot et al., 2007). Somewhat paradoxically, our group and another have shown that Bortezomib, a 26S proteasome inhibitor, induces latent KSHV into lytic replication (Brown et al., 2005; Sarosiek et al., 2010b).

KSHV-induced AIDS-defining cancers

Kaposi’s sarcoma (KS)

Kaposi’s sarcoma was first described in 1872 as an “idiopathic multiple pigmented sarcoma of the skin” by a Hungarian dermatologist, Moritz Kaposi (Kaposi, 1872). However, it was generally considered a disease of elderly men of the Mediterranean basin until 1981, when KS heralded the beginning of the HIV/AIDS epidemic (Casper, 2011). The cause of KS was finally elucidated in 1994 when Chang and Moore used representational difference analysis and found a herpesvirus-like sequence in the DNA of KS lesions. This previously described herpesvirus, now known
as KSHV, is the confirmed etiologic agent of KS. While it is difficult to confirm all of the classical Koch’s postulates, it is widely accepted that KSHV is the cause of KS based on the following: (i) all KS lesions harbor KSHV DNA, (ii) KSHV infection is localized to the spindle cells, (iii) KSHV seroprevalence is concordant with risk of developing disease, (iv) KSHV infection precedes KS development and is a prospective predictor of increased KS risk (Ganem, 2010b; Newton et al., 2006). However, it must be noted that, while KSHV infection is necessary, it is not sufficient for KS development as only about 1 in 10,000 infected individuals will develop Classical KS (Iscovich et al., 2000). A known co-factor for KS development is HIV infection and AIDS. Compared with the general population in the US, HIV-infected persons have a 3640-fold increased risk of developing KS, and as such KS is considered and AIDS defining cancer (Grulich et al., 2007; Schneider et al., 2008). KS is unlike the majority of cancers in that it is not a clonal outgrowth of an individual cell type. Rather, it often evolves from being a lesion composed of few KSHV-infected endothelial-lineage cells (spindle cells) with many inflammatory cells to a lesion dominated by spindle cells with inflammatory infiltrate and aberrant neoangiogenesis with extravasated red blood cells at more advanced stages (Ganem, 2010b). The spindle cells are poorly differentiated and express markers of many cell lineages: vascular endothelial (CD31, CD34, and Factor VIII), lymphatic endothelial (VEGFR3, lymphatic vessel endothelial hyaluronan 1, D2-40, and podoplanin), dendritic cells (Factor XIII), macrophages (CD68) and smooth muscle cells (α-smooth muscle actin) (Mesri et al., 2010a). Further, most cancers are composed of malignant cells typically displaying signs of transformation such as non-diploid nuclei and a reduced
need for survival enhancing growth factors. Spindle cells, the pathognomonic cell of the KS lesion, do not display these properties; the inflammatory environment is critical to the growth of these cells. Indeed, a model for tumorigenesis has been proposed that progresses in directly the opposite fashion as a traditional cancer. In KS, it is thought that the KSHV-infected spindle cells produce factors that recruit inflammatory cells and stimulate angiogenesis from the very beginning. In contrast, a traditional tumor stimulates immune responses and angiogenesis (the “angiogenic switch”) only after substantial proliferation and establishment (Ganem, 2010b).

**Epidemiology of KS**

Serological studies have shown that KSHV infection if found worldwide with the seroprevalence differing among regions and countries. In northern Europe, North America and Asia, the seroprevalence is less than 10%. In the Mediterranean region seroprevalence ranges from 10-30%. In sub-Saharan Africa seroprevalence ranges from 50-80%. Worldwide, KS is the most common cancer of HIV-infected individuals. Early in the AIDS epidemic, from 1980-1989, US incidence of KS accounted for 14.3% of AIDS-defining cancers (Shiels et al., 2011; Simard et al., 2011). Since the advent of HAART, US incidence has declined, but the standardized incidence ratio among AIDS patients is greater than 3600-fold higher than in the non-HIV infected population (Engels et al., 2006). However, in regions of the world where AIDS incidence is high, but HAART treatment is scarce, such as sub-Saharan Africa, KS is widespread with significant morbidity and mortality (Figure 1.2). Indeed, it is the most commonly
diagnosed cancer in the populations of some sub-Saharan countries such as Uganda (Mosam et al., 2010).
Figure 1.2. **Worldwide prevalence of KS and seroprevalence of KSHV.** (A) The standardized incidence of KS for males varies depending on the region. In the US, Florida, California and New York have the greatest burden. While worldwide, the continent of Africa far surpasses others in males with KS. This is also true for women and children (data not shown). (B) Seroprevalence of KSHV. In northern Europe, Asia and the US, seroprevalence is generally less than 10%. However, in sub-Saharan Africa, the overall seroprevalence is >50%, with some countries, such as the Ivory Coast, reaching 100% in some areas. Figure adapted from Mesri et al., 2010.
Clinical manifestations of KS

There are 4 clinically recognized forms of KS: (i) Classic, an indolent disease typically found in elderly Mediterranean men, (ii) Endemic, a more aggressive form of KS that is endemic to Africa, (iii) Iatrogenic, which develops in the context of immunosuppression associated with organ transplantation, and (iv) AIDS-related KS. Regardless of the type of KS, evolution of the disease is a morphologic continuum pathologically beginning as a so-called patch lesion which are dermal flat lesions composed of inflammatory cells such as B-cells, T-cells and hemosiderin-laden macrophages with subtle neoangiogenesis (Grayson and Pantanowitz, 2008). In the evolution of the dermal KS lesion, the next stage is referred to as a plaque. In the plaque stage, haphazardly arranged spindle cells are seen with a more diffuse vascular infiltrate and abundant inflammatory infiltrate. During this stage, “autolumination”, where an erythrocyte is seen in a clear paranuclear vacuole in the cytoplasm of a spindle-cell can be seen (Grayson and Pantanowitz, 2008). The final and most advanced dermal stage is referred to as nodular. Nodular KS contains abundant erythrocyte-containing slit-like spaces with marked spindle cell proliferation and inflammatory infiltrate (Ganem, 2010b; Grayson and Pantanowitz, 2008). However, KS can also occur in the oral cavity, gastrointestinal tract, liver, lymph nodes and lung. Pulmonary lesions are often associated with significant morbidity and may lead to fatal respiratory compromise.
Figure 1.3. Clinical presentation of KS. (A) Patch stage of KS is considered the early stages, is the least aggressive and is composed of violaceous flat lesions. (B) The next stage is considered the plaque stage with raised nodules and increased aberrant angiogenesis; if left untreated will progress to nodular KS, (C). (D) Depicts KS of the lungs. Once KS is disseminated to the visceral organs it is often fatal. Adapted from: Asuguo et al. Kaposi Sarcoma in Calabar, Southern Nigeria. Oman Med J. 2009 January; 24(1): 33-36., and Dr. M.A. Ansary/Science Photo Library, and NCI public domain images, unknown photographer.

Treatment of KS

Systemic treatment of KS typically involves the administration of one or more chemotherapeutic agents, of which there are 3 that are FDA approved: pegylated liposomal doxorubicin, liposomal daunorubicin and paclitaxel (Sullivan et al., 2009). While response rates vary: 46-59%, 25%, and 62%, respectively, most persons with KS progress within 6-7 months and tumor free survival time decreases with each successive round of therapy (Sullivan et al., 2009). As each of the tumor cells is infected with KSHV, highly specific tumor-targeted therapy should be achievable. Targets for therapy include KSHV lytic replication and host signal transduction pathways utilized by the virus for pathogenicity (Sullivan et al., 2009). During lytic replication the virus expresses multiple targets that are viral specific such as: master lytic viral transactivator (RTA), the viral DNA polymerase, viral thymidine kinase (vTK), viral protein kinase (vPK), among
many more. Indeed in a widely cited clinical study, it was found that by giving intravenous ganciclovir (GCV) to patients with an ocular implant of GCV for the treatment of cytomegalovirus (CMV) retinitis, the subsequent development of KS was inhibited by 93% (Martin et al., 1999). *In vitro* and *in vivo*, the antiviral nucleoside analogues such as: ganciclovir, cidofovir and foscarnet, have varying degrees of efficacy, likely due to the lack of lytically replicating virus (Costagliola and Mary-Krause, 1995; Glesby et al., 1996; Kedes and Ganem, 1997). However, when given in conjunction with a viral lytic inducer, some efficacy has been noted as the nucleoside analogues are converted to their cytotoxic forms by the viral thymidine kinase and/or viral phosphotransferase. These phosphorylated cytotoxic molecules are then incorporated into viral and host DNA, causing termination of elongating DNA. Some evidence exists that incorporating protease inhibitors (PI) into a HAART regimen may be beneficial in treating KS. Laboratory studies have shown that PI are both antiangiogenic and antiviral in the context of KSHV (Sgadari et al., 2002) and an association between increased risk of KS when a patient is switched from a PI-based regime to a non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimen has been noted (Bani-Sadr et al., 2003). However, these drugs warrant further study as retrospective analyses have not found an association between KS diagnosis and HAART regimen (Grabar et al., 2006; Portsmouth et al., 2003).
KS Animal Models

While *in vivo* animal models to study KS pathogenesis have led to major advances in the field, study of KSHV-induced oncogenesis and potential therapeutic regimens have been hindered due to the lack of *in vivo* models that recapitulate the full spectrum of pathogenesis from initial primary viral infection, replication, spread to target organs, and establishment and progression to disease (Chang et al., 2009; Dittmer et al., 1999; Jones et al., 2012b; Picchio et al., 1997; Wu et al., 2006). Parsons et al. were the first to show that murine hosts can support long-term primary infection successfully when NOD/SCID mice were injected with purified KSHV virions and latent and lytic gene expression, target cell infections and virion production were followed. They found that less than 1% of mouse spleen cells were KSHV infected and that of those, KSHV targeted B cells, NK cells, macrophages and dendritic cells. NOD/SCID-hu mice were then employed to study antibody response *in vivo* and to test the utility of GCV administration along with the KSHV inoculation. In 3 out of 13 animals, human antibodies against KSHV LANA were detected in the serum and GCV reversibly halted the production of KSHV DNA (Parsons et al., 2006). However, none of the animals were found to have KSHV-associated disease. Our lab recently developed an *in vitro/in vivo* model, termed mECK36 (Figure 1.4). Here, bone marrow-derived endothelial lineage cells were transfected with BAC36, a bacterial artificial chromosome harboring the full KSHV genome (Zhou et al., 2002a). When subcutaneously injected into immunodeficient mice, KS-like tumors developed within weeks. However, a major limitation of the mECK36 model is its inability to complete a full replicative cycle as determined by lack of infectious virion production. Lytic replication is crucial to viral
maintenance within, and spread between, hosts. Lytic replication is also crucial for development of disease. Therefore, it may be that a model capable of completing the full lytic cycle, rather than an abortive lytic one, would be more representative of KS (Chang et al., 2009; Mesri et al., 2010a).

Figure 1.4. mECK36 induce vascularized spindle cell sarcomas in immunocompromised mice. (A) Mouse with subcutaneous mECK36 tumor. (B) Dissected mECK36 tumor. (C) Tumor growth in nude mice injected with mECK36 cells (closed circles) or mEC-V (open squares). (D) Histological section of a mECK36 tumor stained with H&E. (E) Image of a spindle cell sarcoma foci. (F) Image of a spindle cell tumor in the lungs stained with H&E. Scale bar = 50µM. Adapted from Mutlu et al., 2007.

Most recently, Chang et al succeeded in creating a marmoset model of KS where primary KSHV infection led to the development of a KS-like lesion in one of the animals. While being a major achievement, the development of the lesions is occasional, making
the model somewhat unreliable for large scale experimentation of KSHV pathogenesis (Chang et al., 2009).

With these models in mind, a large part of this thesis consists of improving upon these previous models to create a small animal model of KS that more reliably recapitulates the disease, is reproducible, and easily accessible for the evaluation of antiviral and antitumor strategies to treat the disease.

**Primary Effusion Lymphoma (PEL)**

PEL was first described in the context of HIV-infected persons in 1989 (Knowles et al., 1989). While PEL may develop in elderly immunosuppressed HIV-negative individuals, it more commonly affects HIV-positive patients, and like KS, PEL is an AIDS-defining illness (Schneider et al., 2008). PEL is an aggressive subtype of non-Hodgkin lymphoma (NHL) with a median survival of less than 6 months which typically presents as B-cell lymphoproliferative effusions in the serous body cavities without a contiguous tumor mass (Carbone et al., 2009). PEL cells are characterized by immunoblastic protein expression such as: CD30, CD39 and IRF4, and plasma cell protein expression like: BLIMP1 and CD138. However, they lack expression of the typical B-cell markers: CD19, Oct2, Pax5, and surface immunoglobulins (Ig). But PEL is clearly derived from the B-cell lineage as indicated by the presence of Ig gene rearrangements and more specifically, is of a B-cell that has transitioned through the germinal center, as determined by the presence of somatic hypermutation in the rearranged Ig variable regions (Ballon et al., 2011).
While PEL are consistently infected with KSHV, Epstein- Barr virus (EBV) is present in approximately 80% of cases (Simonelli et al., 2003). It is KSHV that drives the tumor as KSHV expresses multiple genes: LANA, vCyc, vFLIP, vIRF3/LANA2, and 11 microRNAs, that provide proliferative and antiapoptotic survival signals while EBV only expresses EBNA1 in PEL (Carbone et al., 2009). Indeed, shRNA and siRNA silencing experiments showed that targeting KSHV vFLIP expression induced greater apoptosis in PEL cell lines (Guasparri et al., 2004). Further, chemically disrupting LANA association with host proteins p53 and mdm2 leads to apoptosis in PEL lines while LANA2 has been reported to be an inhibitor of apoptosis (Esteban et al., 2003; Sarek et al., 2007). Finally, vCyc and some of the KSHV microRNAs have been shown to regulate multiple proteins involved in cell cycle regulation and apoptosis (Jarviluoma et al., 2006; Ojala et al., 1999).

**Treatment of PEL**

In HIV-infected individuals, initiation of HAART therapy can induce PEL regression and may result in a good prognosis (Hocqueloux et al., 2001). PEL is usually treated with the standard cyclophosphamide, vincristine, doxorubicin, prednisone (CHOP) therapy in combination with antiviral therapy with remissions in only 43% of patients and a dismal outcome of 6 months survival (Simonelli et al., 2003). Two other chemotherapeutic regimes have been reported to be superior to CHOP for treating HIV-associated lymphomas. The first is a continuous infusion of cyclophosphamide, doxorubicin, etoposide (CDE) and the second involves a continuous infusion of cyclophosphamide, doxorubicin, etoposide, vincristine and prednisone (EPOCH) (Little
et al., 2003; Sparano et al., 2004). Although high dose chemotherapy was previously associated with an increase of morbidity due to opportunistic infections, it was most recently suggested that PEL patients may benefit from more intense regimens (already shown to be clinically beneficial in HIV negative lymphoma patients) as long as HAART is also implemented (Carbone et al., 2009). Preliminary studies have also shown durable lymphoma remission by treating with a combination of high dose chemotherapy along with autologous peripheral blood stem cells (Gabarre et al., 2004; Krishnan and Forman, 2010; Krishnan et al., 2005). Other strategies proposed for PEL treatment include activating TNF-related apoptosis-inducing ligand (TRIAL)-mediated apoptosis by concurrent administration of IFN-alpha and AZT (Toomey et al., 2001), and by inhibiting NFκB activity (Keller et al., 2000). Another strategy may be to treat by selectively inhibiting COX-2 function which has been shown to induce proliferation arrest, activate the p53/p21 tumor suppressor pathway by disrupting LANA-1/p53 complexes and induce cell death in PEL in vitro (Paul et al., 2011).

PEL animal models

Although, these virally-induced cancers should be ideal for highly targeted treatment strategies, the rarity of occurrence and lack of suitable animal models of PEL have hindered the development of effective therapeutic regimens and execution of large scale clinical trials. Most studies are performed on PEL cell lines that have been adapted to grow in culture. It is well known that culture adapted cells do not fully recapitulate the physiology of the in vivo disease and KSHV transcriptional and protein expression changes have been reported (Mutlu et al., 2007; Staudt et al., 2004). In 1995, the first
PEL model described was using the KS-1 cell line, a KSHV+/EBV- PEL derived from an HIV-negative patient that was inoculated into triple immunodeficient mice (Said et al., 1996). Within 3 weeks of inoculation the mice developed massive ascites with cells that harbored KSHV-like capsids of 110nm in diameter. Shortly afterwards, Picchio et al., utilized the inoculation of the body cavity based lymphoma-1 (BCBL-1) line into SCID mice (Picchio et al., 1997). Angiogenic solid tumors developed, but the group was unable to rescue infectious virus from co-implanted human PBMC, which was one of the main goals of the study (Picchio et al., 1997). These models, derived from cells that were inherently culture-adapted, may not allow for recapitulation of the true pathophysiology of disease. We previously published a model of PEL (UM-PEL-1) using a direct xenotransplant from an HIV-negative male to NOD/SCID mice (Sarosiek et al., 2010b). A diagnosis of PEL was made from a pleural tap of the patient on the basis of atypical lymphoid cells staining positive for CD45, CD30, CD38, CD138, HLA-DR, LANA, and the EBV encoded RNA, EBER, while the cells stained negative for CD3, CD19, CD20, and CD79a and the B-cell transcription factor PAX5. This staining pattern was accompanied by a monoclonal VH4-34 IgG heavy chain that was 83% homologous to the germ line and a “complex” karyotype of: 51, XY, dup(1)(q22q31), +4, +5, +7, +19, add(21)(p13) (Sarosiek et al., 2010b).
Inducing lytic KSHV replication for therapeutic benefit (lytic-inductive paradigm/virus-activated cytotoxic therapy)

While both latent and lytically infected cells are being considered as targets for antiviral therapy, most antivirals that have proven efficacious generally target KSHV DNA replication occurring during the lytic replicative cycle (Casper et al., 2008; Casper et al., 2004; Martin et al., 1999). Induction of lytic replication has been shown by numerous epidemiologic studies to be a crucial step in KS progression and detection of virus in the peripheral blood is associated with an increased risk of disease progression (Gao et al., 1996a; Gao et al., 1996b; Whitby et al., 1995). However, a major limitation to the use of antivirals targeting the lytic cycle is that the majority of the KSHV-infected cells in a KS lesion are latent. A proposed mechanism for enhancing the efficaciousness of these antiviral therapies is to induce the virus into lytic replication (lytic-inductive paradigm). The lytic-inductive paradigm for therapy proposes to induce massive lytic reactivation, while concurrently administering a potent antiviral, thereby specifically causing the death of virally infected cells while abrogating the potential increase in viremia, which may lead to unwanted consequences in the form of worsening disease (Klass et al., 2005; Klass and Offermann, 2005; Westphal et al., 2000). Antivirals that have been proposed generally include nucleoside analogues: cidofovir (CDV), ganciclovir (GCV), valganciclovir, phosphonoformic acid/forcarnet, and azidothymidine/zidovudine (AZT) (Krug et al., 2004; Medveczky et al., 1997; Uldrick et al., 2011). The virally encoded thymidine kinase (vTK/ORF21) phosphorylates AZT and to some extent GCV, while ORF36, which encodes a phosphotransferase (vPK), phosphorylates GCV, an acyclic nucleoside, into a toxic triphosphate moiety. The
The triphosphate moiety will then be incorporated into the newly synthesized viral DNA along with the cellular DNA, leading to premature DNA synthesis termination and cellular apoptosis (Tomicic et al., 2002). Lytic induction can be achieved by multiple means such as hypoxia (Davis et al., 2007), DNA methylation, histone deacetylation, proteasome inhibition (Fu et al., 2008; Sarosiek et al., 2010a) and is crucial for the antivirals to have an effect as the viral kinases are only expressed during lytic replication. The choice of lytic inductive method is crucial as not all cells will be induced into lytic replication at any given time. Major questions that arise are: Why are some cells refractory to lytic induction? Can they be induced at a later timepoint? These are crucial questions important for the eradication of the reservoir as leaving only one infected cell can lead to a repopulation of virally infected cells. A very strong inducer of KSHV lytic replication is SAHA, a pan-HDAC inhibitor. HDAC inhibition allows for acetylation of core histones leading to relaxation of viral DNA structure allowing for binding of host transcription factors and activation of viral lytic transcription. Recent studies have shown that, both in the context of HIV and EBV, SAHA, a clinically approved pan-histone deacetylase inhibitor is a highly effective viral lytic-cycle inducer (Contreras et al., 2009; Hui and Chiang, 2010). SAHA decreases the electrostatic interaction between the epsilon-amino groups of lysine residues and their interactions with the negatively charged DNA backbone, thus allowing access of transcription factors to the target genes, such as the replication and transcription activator (RTA), the master lytic transactivator of KSHV. Importantly, SAHA does not alter transcriptional patterns in a global manner, rather distinct patterns of transcriptional changes occur in genes that belong to families such as oncogenes/tumor suppressors, cytokine-induced survival pathways,
proteasome/ubiquitin function and cell cycle mediators. While SAHA has not shown pre-clinical promise in non-herpesvirus associated solid tumors (Robey et al., 2011), it is currently being evaluated in the context of HIV (Clinical trial NCT01319383) (Contreras et al., 2009; Matalon et al., 2011; Saleh et al., 2011).

Multiple studies have shown efficacy by inducing EBV lytic replication with concomitant use of antiviral nucleoside analogues (Daibata et al., 2005; Faller et al., 2001; Mentzer et al., 1998; Moore et al., 2001). Using butyrate to stimulate EBV-infected Burkitt’s lymphoma line into lytic replication, and GCV as an antiviral agent, Ghosh et al. found that by pulsing the cells for 6 hours at 24 hour intervals, the uninduced cells remained susceptible to further cycles of combination treatment (Ghosh et al., 2007). Further, the combination of butyrate and ganciclovir has shown to be of benefit in patients with EBV-driven lymphomas (Perrine et al., 2007). Recently, a group showed that lytic induction induced by hypoxia enhanced the cytototoxicity of GCV and AZT in KSHV-infected cells (Davis et al., 2007). A pilot study of lytic-inductive therapy was undertaken in another KSHV-induced disease, multicentric Castleman’s disease (MCD). MCD is known for the propensity to be much more lytic in vivo than KS or PEL. Patients diagnosed with this highly lytic tumor were given high dose AZT (30mg/kg/day) in combination with valganciclovir. While dosing regimens need to be optimized, 86% of the patients attained major clinical responses with 50% attaining major biochemical benefit, suggesting that targeting lytic replication may be beneficial in KSHV-induced cancers (Uldrick et al., 2011).
CHAPTER TWO: EMPLOYING THE LYtic INDUCTIVE PARADIGM AS AN ANTINEOPlASTIC AND AntIVIRAL STRATEGY IN A MURINE MODEL OF PEL

Background

We recently established a direct xenograft model of PEL (UM-PEL-1) where freshly isolated PEL cells derived from the peritoneal cavity of a PEL-bearing patient were injected directly into NOD/SCID mice, resulting in progressive and reproducible tumor growth similar to the human PEL (Sarosiek et al., 2010b). We found that Bortezomib was an effective lytic inducer of KSHV in vivo and led to increased survival of tumor bearing mice over doxorubicin treatment (Sarosiek et al., 2010b). Further, Btz has been found to increase the survival of SCID mice inoculated with lymphoblastoid cell lines in type III latency (Zou et al., 2007).

As herpesviruses are dependent on the proteasome for replication, transcription, and mature viral production, induction of lytic replication with concomitant inhibition of the proteasome may provide a highly targeted strategy for eradicating KSHV infected cells without leading to increased viremia. Consequently, we hypothesized that lytic induction therapy, combining Btz with SAHA, may act synergistically to increase survival in a NOD/SCID-PEL direct xenograft model without increasing viral load.

To improve the monotherapy and to develop effective anti-PEL regimen we evaluated the efficacy of SAHA/Btz combination in PEL cell lines and UM-PEL-1 xenograft model. This study revealed that Btz/SAHA combination induces the apoptosis to a greater extent than Btz or SAHA alone and extends the survival of tumor bearing mice by inhibiting tumor progression. Moreover, our findings suggested that in KSHV
Btz interferes with KSHV latency and that in combination with SAHA, induces lytic reactivation while concurrently inhibiting late-lytic gene expression. Importantly, Btz also interfered with the full KSHV lytic replication resulting in inhibition of infectious virus production, indicating that Btz functions as both inducer and inhibitor of KSHV replication in vivo. We believe that this is the first comprehensive report identifying the potential of the lytic inductive paradigm for herpesvirus treatment implying “antiviral” effect by bortezomib in combination with a potent herpesvirus lytic inducer such as SAHA implying potentially protective and clinically beneficial activity in immunocompromised patients suffering from PEL and other γ-herpesvirus-induced malignancies.

**The combination of Btz and SAHA blocks proliferation and induces cell cycle arrest and apoptosis in PEL cells.**

Given our previous observations that Btz induces KSHV lytic replication and confers a survival advantage in PEL-bearing mice, we tested the Btz/SAHA combination for PEL treatment. We hypothesized that, if the outcome of KSHV lytic infection is apoptosis, the combination of these drugs would induce greater apoptosis and thus confer a longer survival advantage for mice bearing PEL tumors. To test this hypothesis we tested the effects of Btz and SAHA in PEL cell proliferation, cell cycle distribution and survival (Figure 2.1 and 2.2). Cells were treated with different concentrations of Btz, SAHA or their combination (Btz/SAHA) for up to 72h and analyzed by an MTT assay. PEL cell lines exhibited time dependent decrease in cell proliferation with the maximal effect achieved with the combination of drugs as compared to individual agents
(p<0.0001, Figure 2.2A). A more profound inhibition of cell proliferation was observed at the higher doses of 10nM Btz and 0.75µM SAHA (Figure 2.1A). Cell cycle profiling of UM-PEL-1c demonstrated a significant increase in percentage of G0 cells (from 0.55 ± 0.39% in control to 41.14 ± 3.86%, p=0.009) treated with the combination of 10nM Btz and 0.75µM SAHA (Figure 2.1B). This effect was less pronounced at lower doses (5nM Btz and 0.5µM SAHA; data not shown). Cell viability measured by YO-PRO-1 and PI staining revealed that Btz/SAHA combination induced higher levels of apoptosis in UM-PEL-1c, BC-1 and BC-3 compared to single drugs in dose dependent manner (Figure 2.1C and 2.2B). Btz and SAHA at 5nM and 0.5µM respectively, induced ~30% of apoptosis in BC1, BC3 and UM-PEL-1, but the 5nM Btz/0.5µM SAHA combination induced apoptosis in ~60% of the cells (Figure 2.2C). By increasing the doses of Btz and SAHA to 10nM and 0.75µM respectively, the drug combination induced apoptosis in more than 80% of UM-PEL-1c (Figure 2.1C). Overall, these findings demonstrate that the combination of Btz/SAHA is more efficient in inhibiting cell proliferation, inducing cell cycle arrest and apoptosis of PEL cells compared to either drug alone.
Figure 2.1 The combination of Btz with SAHA inhibits proliferation, induces cell cycle arrest and triggers apoptosis of UM-PEL-1 cells. In vitro cultured UM-PEL-1c were treated with 10nM Btz, 0.75µM SAHA or 10nM Btz/0.75µM SAHA for indicated time points. (A) The fold change in cell proliferation as assessed by MTT assay at 0, 24, 48 and 72h post-treatment. (B) Analysis of the percentage of cells in different phases of the cell cycle (G0, G1, S and G2/M) at 24h after treatment with the indicated drugs. Cells were stained with PI to measure DNA content and analyzed for cell cycle distribution by flow cytometry. (C) The percentage of apoptosis measured by flow cytometric analysis of YO-PRO-1/PI stained cells after 24h treatment. Experiments depicted in A-C were repeated thrice independently in triplicate. The representative data from one experiment is shown. The error bars represent standard error of the mean.
Figure 2.2. Combination of Btz/SAHA induces proliferative arrest and apoptosis in various PEL lines *in vitro*. For panels A and C, *in vitro* cultured UM-PEL-1c, BC-1 and BC-3 cells were treated with 5nM Btz, 0.5µM SAHA or 5nM Btz/0.5µM SAHA. (A) Cell proliferation was determined at indicated time points by MTS assay. (B) UM-PEL-1c, BC-1 and BC-3 cells were treated with concentrations of SAHA (ranging from 0 to 2µM) with or without Btz (0, 5nM, 10nM) for 24h then stained for YO-PRO/PI to quantify apoptosis by flow cytometry. (C) Apoptosis was quantified by YO-PRO PI staining at 24h post treatment. Experiments were repeated at least three times and error bar indicates SEM.
The combination of Btz/SAHA synergistically induces KSHV lytic replication in PEL cells.

To determine the effect of Btz/SAHA combination on KSHV lytic induction in UM-PEL-1c, genes representing all stages of the viral replicative cycle were analyzed by qRT-PCR at 24h post-treatment. Compared to individual treatment with Btz or SAHA, Btz/SAHA combination induced an additive or synergistic upregulation of the immediate early (IE) genes (RTA and ORF45) and early genes (ORF21/vTK, ORF36/vPK) (Figure 2.3A). While both Btz and SAHA induced viral G-protein coupled receptor (ORF74/vGPCR) expression at both the mRNA and protein levels as measured by qRT-PCR and immunofluorescence assay (IFA), respectively, Btz/SAHA was more efficient in upregulating this early lytic viral gene (Figures 2.3A and B). Moreover, vGPCR staining indicated a very high ratio of lytic reactivation for the SAHA/Btz combination that correlates with its apoptotic activity depicted in Figure 2.1. The late lytic gene K8.1 mRNA was 30-fold induced in SAHA only treated cells, but remain unaffected in the Btz treated cells (Figure 2.3C). Furthermore, concurrent exposure to Btz abrogated the SAHA-induced K8.1 upregulation by ~60% (Figure 2.3C). This K8.1 transcriptional pattern was mimicked at the protein level with a decrease in the percentage of K8.1 expressing cells following Btz treatment (Figure 2.3D). This effect appeared to be locus-specific, since the IE gene K8 exhibited similar pattern of mRNA expression as K8.1 (Figure 2.3A). Overall our data indicate that Btz and SAHA synergize to induce KSHV lytic replication with selective repression of some IE and late lytic genes correlating with the high rates of PEL cell cytotoxicity.
Figure 2.3. Btz and SAHA synergize to induce KSHV lytic replication while concurrently inhibiting expression of selective lytic genes in UM-PEL-1c. For all panels, UM-PEL-1c cells were treated in vitro with 10nM Btz, 0.5µM SAHA or 10nM Btz/0.5µM SAHA for 24h. Total RNA was harvested for qRT-PCR analysis of viral mRNA expression (A, C). Cells were cytopun, fixed and immunofluorescence was performed for viral proteins (B, D). (A) qRT-PCR analysis reveal that Btz and SAHA induce the expression of immediate early and early lytic viral mRNAs with the exception of K8, which was inhibited by Btz. (B) The vGPCR protein was immunostained to determine the extent of viral reactivation. Widespread reactivation is seen in the Btz/SAHA combination treated cells. (C) K8.1, a late lytic viral gene, was inhibited by Btz at the transcript level. (D) Left panel: Immunostaining images for K8.1 protein confirm the inhibition of K8.1 expression by Btz. K8.1 expression was normalized to DAPI to account for potential differences in cell number. Right panel: Shows the relative increase in K8.1 positive cells after SAHA alone, and their decrease after adding Btz. Results are representative of 3 individual experiments. Error bars represent the SEM.
The combination of Btz/SAHA induces marked apoptosis in PEL xenografts and enhances survival of tumor bearing NOD/SCID mice.

The *in vitro* antiproliferative and cytotoxic activities of Btz/SAHA in PEL cell lines suggested its therapeutic potential *in vivo*. Therefore, we evaluated the effects of Btz/SAHA in our direct xenograft PEL model. Four groups of NOD/SCID mice (n=5), were inoculated i.p. with UM-PEL-1 cells and treated i.p. with Btz (0.3mg/kg twice weekly), SAHA (60mg/kg daily), Btz/SAHA combination, or DMSO (50µl daily) for 3 weeks, starting on day 3 post-injection. All regimens were well tolerated. Ascites cells collected from lymphoma bearing mice at day 7 were positive for CD30 staining (97.23%, Figure 2.4A), suggesting that majority of cells in the ascites are indeed UM-PEL-1. Treatment with SAHA alone showed comparable efficacy to Btz, extending the overall survival compared to control mice (median survival of 23, 33 and 15 days for SAHA, Btz and DMSO control, respectively; Figure 2.4A). However, the Btz/SAHA combination led to a further significant increase in the overall survival as compared to single drugs or DMSO control groups (median survival of 57 days; p<0.0001; Figure 2.5B). There was increased apoptosis of UM-PEL-1 cells obtained from mice treated for 24h with single dose of Btz/SAHA combination (47 ± 3.78%) and Btz (37 ± 4.35%) compared to SAHA (19 ± 1.52%) and DMSO control (12.86 ± 3.1) as assessed by YO-PRO-1/PI staining (Figure 2.4D). This was further confirmed by TUNEL assay, which demonstrated that most cells in the combination treated mice exhibited signs of DNA fragmentation and were committed to apoptosis (Figure 2.4C).
Figure 2.4. The combination of Btz and SAHA extends the survival of PEL-bearing mice inducing PEL xenograft cell apoptosis. (A) Flow cytometry histogram indicates positive staining for CD30 of the UM-PEL-1 cells harvested from mice. Grey histogram represents staining with the CD30 antibody and white histogram represents isotype control. (B) Kaplan-Meier survival curve of PEL xenograft mice treated with different combinations of drugs. Four groups of NOD/SCID mice (n=5 mice/group) were injected intraperitoneally (i.p.) with $25 \times 10^6$ UM-PEL-1 cells. Starting from day 3, mice were treated for 3 weeks with DMSO (Control), Btz (0.3 mg/kg body weight twice weekly), SAHA (60mg/kg body weight daily), and with the combination (Btz/SAHA). Results are representative of two independent experiments. For panels C and D, UM-PEL-1 bearing mice (n=3) were treated separately with a single dose of either DMSO, Btz, SAHA or combination of Btz/SAHA for 24h, sacrificed and UM-PEL-1 cells were harvested from peritoneal effusions. (C) Immunofluorescence image of TUNEL staining (green, middle panel) of UM-PEL-1 cells harvested from peritoneal effusions. Nuclei are stained with DAPI (blue, left panel) and merged image of DAPI and TUNEL are shown in right panel. TUNEL positivity is indicative of fragmented DNA, a characteristic of apoptotic cells. TUNEL stain performed by Luis Diaz of Dr. Juan Carlos Ramos lab. (D) Depicts in vivo apoptosis of UM-PEL-1 cells obtained by YO-PRO-1/PI staining followed by flow cytometry. Each circle represents one mouse with the line drawn at the mean percentage apoptosis.
Bortezomib-induced apoptosis is accompanied by the accumulation of p53, p21, Bax, and the activation of caspase-8 in vivo.

To investigate the antitumor mechanisms of Btz/SAHA in UM-PEL-1, we examined the expression of the tumor suppressor protein p53 and its downstream targets p21 and Bax, an inhibitor of cell cycle progression and mediator of apoptosis, respectively. There was a strong induction of p53, p21 and Bax proteins in the Btz and Btz/SAHA-treated mice, while SAHA alone led only to a slight increase in p53 and Bax levels (Figure 2.5A). The increased expression of p53 exclusively occurred at the protein level while its mRNA levels were decreased (Figure 2.5A and C), suggesting that the Btz-mediated inhibition of the 26S proteasome resulted in the accumulation of p53 protein. In contrast, Btz/SAHA combination therapy markedly increased both mRNA and protein levels of the p21 (Figure 2.5C). Since the activation of the caspase cascade is a pivotal step in apoptosis, we next examined the expression of caspases by immunoblotting. Btz alone or in combination with SAHA induced caspase-8 cleavage indicative of the activation of extrinsic apoptotic pathway, which is considered to be death receptor-mediated and p53 independent (Fulda and Debatin, 2006) (Figure 2.5A).
Figure 2.5. Btz/SAHA induces expression of p53, p21 and Bax, histone 3 acetylation and down regulates c-Myc in PEL xenografts. UM-PEL-1 bearing mice (n=3 mice/group) were treated with a single dose of Btz, SAHA, and the combination of Btz/SAHA (C=DMSO control, B=Btz, S=SAHA, and BS=Btz/SAHA). At 2h and 24h after treatment UM-PEL-1 cells were harvested from mice ascites and used for whole cell lysate preparation followed by western blot analysis with the indicated antibodies. mRNA was also extracted and used for qRT-PCR studies. (A) Immunoblotting of indicated proteins from cells treated for 24h with specified treatment. β-actin and GAPDH were used for loading control. (B) Immunoblotting for acetylated histone 3 (H3) subunit after 2h (top panel) and 24h (bottom panel) treatment. (C) qRT-PCR analysis of indicated genes at 24h after treatment. Results are representative of 2 independent experiments, each performed in triplicates. Error bars represent standard error of the mean.
**Btz and SAHA increase the levels of acetylated histones in PEL in vivo.**

SAHA is a hydroxamic acid that reversibly inhibits class I and class II HDACs, resulting in the acetylation of histones and other cellular proteins (Richon et al., 1998). Recent reports indicated that proteasome inhibitors can also cause hyperacetylation of histones that may contribute to tumor cell apoptosis by both their accumulation and transcriptional repression of HDACs (Kikuchi et al., 2010; Miller et al., 2009; Sato et al., 2011). Accordingly, we analyzed the effect of SAHA and Btz on the acetylation of histone subunit 3 (H3) in PEL xenografts. As expected, SAHA caused increased levels of acetylated H3 as early as 2h after treatment (Figure 2.5B, top panels). This effect was transient and reversible since SAHA-induced H3 acetylation diminished by 24h (Figure 2.5B, bottom panels). Noticeably, Btz progressively increased the expression of total and acetylated H3 from 2 to 24hrs (Figures 2.5B). These results suggest that the efficacy of Btz in combination with SAHA might be partly explained by its ability to decrease proteosomal degradation of hyperacetylated histones. Neither Btz nor SAHA affected the expression of other important cell cycle regulators, including: p16, p27, and Rb proteins (Figure 2.6).
A proposed mechanism of Btz-mediated cell killing is induction of ER stress from the accumulation of non-degraded proteins leading to the activation of the unfolded protein response (UPR) (Obeng et al., 2006). To evaluate the effect of Btz on the UPR in UM-PEL-1 xenografts *in vivo*, we measured the expression of proteins known to be upregulated by the UPR (CHOP, GRP-78, GRP-94, ATF6, total and phospho- eIF2α) by immunoblotting. Btz alone and in combination with SAHA led to increased CHOP, however no significant changes in the expression of other proteins including phospho-eIF2α were observed (Figure 2.7).
Figure 2.7. Although Btz induces CHOP expression, the combination of Btz/SAHA does not induce terminal UPR pathway in UM-PEL-1 xenografts. Tumor bearing mice (n=3 mice/group) were treated with single dose of DMSO (control), SAHA, Btz or combination (Btz/SAHA) for 24h (C=DMSO control, B= Btz, S=SAHA, and BS=Btz/SAHA). (A) Whole cell lysates prepared from peritoneal effusions were used for immunoblotting of indicated proteins. GAPDH served as loading control. Results are representative of two different experiments.

**Btz treatment induces NF-κB activity and downregulates c-myc in PEL in vivo.**

Constitutive NF-κB activity is a critical pro-survival mechanism for PEL and inhibiting NF-κB function induces PEL cells apoptosis (Keller et al., 2006; Keller et al., 2000). Therefore, we analyzed the effect of Btz/SAHA on NF-κB activity in vivo using nuclear extracts from murine PEL xenografts by electrophoretic mobility shift assay (EMSA). Btz induced IκB-α phosphorylation and increased NF-κB (p50/p65) DNA binding at 24h as compared to control and SAHA alone treated mice (Figure 2.8A and B). Btz-mediated NF-κB activation was further augmented in the presence of SAHA (Figure 2.8A).
Figure 2.8. Btz and SAHA induce NF-κB activity in vivo in PEL xenografts. UM-PEL-1-bearing mice were treated with a single dose of Btz, SAHA, or combination of Btz/SAHA (C=DMSO control, B=Btz, S=SAHA, and BS=Btz/SAHA). Peritoneal cells from ascites were harvested at 24h. (A) Nuclear extracts prepared from ascites of tumor bearing mice were subjected to EMSAs using NF-κB–specific consensus oligonucleotides probes to capture NF-κB complexes. Oct-1 was used as normalizing control. (B) Western blots of specified proteins from whole cell lysate of UM-PEL-1 ascites collected at 24h after specified treatments. β-actin served as a loading control. Results are representative of 2 independent experiments, each performed in triplicates.

To further explore the effect of Btz and SAHA on NF-κB activity, we analyzed expression of key NF-κB target genes: cellular inhibitor of apoptosis protein (cIAP2), cellular FLICE inhibitory protein (cFLIP), interferon regulatory factor 4 (IRF4), Bcl-2, Bcl-X_L, and c-myc by immunoblotting. Btz and Btz/SAHA-treated groups showed induction of cFLIP and abrogation of c-myc, whereas remaining proteins were unaltered (Figure 2.9). The abrogated expression of c-myc by Btz at both mRNA and protein levels was consistent with our previous in vitro observations (Sarosiek et al., 2010b) and suggest that this effect is not mediated by the NF-κB in this context. Overall, our
observations suggest that the antiapoptotic effect of the Btz/SAHA combination is not mediated by suppression of NF-κB signaling in PEL.

![Western Blot Image]

**Figure 2.9.** Treatment of Btz in UM-PEL-1 xenografts does not affect NF-κB target genes except cFLIP. UM-PEL-1 bearing mice were treated with a single dose of Btz, SAHA, or the combination of Btz/SAHA (C=DMSO control, B=Btz, S=SAHA, and BS=Btz/SAHA). Each group contained 3 mice, sacrificed at 24h post treatment. Whole cell extract was used to carry out the Western blot analysis of indicated genes. GAPDH served as the loading control.

**Btz inhibits KSHV late lytic gene expression in PEL xenografts.**

Since the Btz/SAHA combination induced reactivation of KSHV in UM-PEL-1c in culture (Figure 2.3), we next examined this effect in vivo in UM-PEL-1 xenografts. While the transcription of latent genes was minimally affected by any treatment (Figure 2.11A), KSHV lytic reactivation was potently induced by the SAHA and/or Btz/SAHA
combination in a gene specific manner, beginning with the immediate-early (IE) lytic switch RTA (Figure 2.10B). SAHA-induced IE transcripts (K8/K-bZip and ORF45) were inhibited by concurrent Btz treatment (Figure 2.10C). Indeed, with the exception of LANA, RTA, and ORF21/vTK, the majority of SAHA-induced KSHV gene transcripts were almost uniformly inhibited in vivo by addition of Btz (Figure 2.10).

Importantly, in contrast to the in vitro data showing disparate regulation of some late lytic genes, all late lytic genes examined in the in vivo UM-PEL-1 xenografts were consistently induced with SAHA, but inhibited by addition of Btz (Figure 2.10D).

The consistent finding of the inhibition of all the tested late lytic viral transcripts suggested that Btz treatment, although effective in inducing lytic reactivation, was at the same time leading to a block in KSHV replicative cycle. To test this possibility we examined the production of KSHV virus following SAHA and/or Btz treatment by measuring the amount of encapsidated extracellular viral DNA. In the presence of the lytic inducer SAHA, there was a 40-fold increase of encapsidated viral DNA as compared with untreated control (Figure 2.10E). This was in sharp contrast with Btz treatments that showed no increase in virion DNA in Btz only treated animals and totally abrogated SAHA induced virion production in animals treated with Btz/SAHA combination. Although PEL is driven by KSHV, this PEL line is also EBV infected and we wanted to evaluate the effect of these drugs on EBV transcription. Interestingly, while there was no effect on EBV lytic reactivation after 24h in vivo treatment, Btz reduced the transcription of the latent genes, EBNA1 and LMP1 (Figure 2.11 A-D). Given that EBV latency program is what gives EBV its transformative potential, these findings warrant further studies into the potential for using Btz in EBV-induced malignancies as well.
Figure 2.10. Btz/SAHA potently induce KSHV lytic reactivation in vivo while Btz inhibits the expression of key genes required to complete replicative cycle resulting in inhibition of virus production. For all the panels, tumor bearing mice were treated for 24h with indicated drugs and UM-PEL-1 cells were harvested from peritoneal effusions for qRT-PCR analysis. Line at 1 on the y-axis represents DMSO treated control mice to which the experimental mice were normalized. Each circle represents one mouse with the line drawn at the mean fold induction. mRNA expression of KSHV: (A) latent, (B) immediate early lytic, (C) early lytic, and (D) late lytic gene expression. Results are representative of 2 independent experiments. (E) Tumor bearing mice were treated with a single dose of Btz, SAHA, or combination of Btz/SAHA for 72h. Peritoneal effusions were harvested for virion quantification. The graph depicts the number of encapsidated viral DNA copies normalized to volume of ascites recovered from a representative mouse. The error bars are the SEM of quintuplicate wells.
Figure 2.11. qRT-PCR analysis of representative EBV gene expression. For all panels, UM-PEL-1 tumor bearing mice (n=3 mice/group) were treated with a single dose of Btz, SAHA, or the combination of Btz/SAHA. At 24h after treatment, peritoneal effusions were harvested, total RNA was isolated and qRT-PCR was performed. (A-D) mRNA expression of latent, immediate early, delayed early lytic and late lytic EBV genes. The line at 1 on the y-axis represents DMSO treated control mice to which the experimental mice were normalized. Each circle represents one mouse with the line drawn at the mean fold induction. Results are representative of 2 independent experiments.
Btz treatment blocks KSHV infectious virion production by UM-PEL-1c.

Our in vivo results showed that the Btz/SAHA antitumor effect correlated with apoptosis and viral lytic induction occurring in the absence of late lytic transcription. The fact that lytic gene inhibition correlated with a lack of extracellular encapsidated viral DNA increase suggested that infectious viral production was blocked. This would be a desirable outcome in the clinical setting in treating PEL in the context of immunosuppression and AIDS. To assess the antiviral potential of these treatments, we examined the effect of Btz/SAHA on KSHV DNA replication and virus production in cultured UM-PEL-1c. UM-PEL-1c were stimulated with Btz, SAHA, or Btz/SAHA and the intracellular KSHV DNA load was measured at 72h by qPCR, and the infectivity of the supernatants was assessed by infection of 293 cells and LANA-based KSHV detection. UM-PEL-1c treated with either Btz alone or Btz/SAHA contained significantly higher intracellular viral DNA levels compared to control or cells treated with SAHA only (Figure 2.12A). However, virus production decreased in both Btz alone and Btz/SAHA-treated UM-PEL-1 cells compared to SAHA-treated cells (Figure 2.12B and C). Consistent with the results of Fig 2.10, we found that Btz abrogated the SAHA-induced production of infectious virions. Overall, these results demonstrate that while both Btz and SAHA reactivate KSHV, Btz blocks mature virion production.
Figure 2.12. Btz induces accumulation of viral DNA with concomitant inhibition of infectious virion production in vitro. (A) Cultured UM-PEL-1c were treated for 72h with 10nM Btz, 0.5µM SAHA or a combination of the 10nM Btz/0.5µM SAHA. Total intracellular DNA was isolated and 10ng was used for qPCR viral load determination. (B-C) Cell free supernatant from PBS (Control), Btz, SAHA or Btz/SAHA-treated cells was applied to uninfected HEK293 cells. 48h later, the cells were fixed and stained for LANA. For graphing purposes, one non-doublet LANA positive 293 was defined as one infectious unit. Immunofluorescence of a representative field for each condition is shown. Results are representative of 2 independent experiments.
Discussion

An attractive option for treating PEL and other γ-herpesvirus induced cancers is targeting endogenous latent viruses with drugs that reactivate their lytic replication, thereby eradicating virally-infected reservoirs. In this study, using a direct xenograft PEL model, we demonstrate that the combination of the antineoplastic agents, Btz and SAHA, synergize to induce KSHV lytic replication while leading to extensive apoptosis and a significant survival advantage in PEL-bearing mice. Importantly, this potent killing effect occurred in the absence of production of infectious KSHV.

Considering that PEL is latently infected with KSHV, the antitumorigenic effect observed by the combined use of Btz and SAHA might be in part accomplished by their ability to target latency and induce KSHV lytic replication. Viral lytic induction per se is known to cause G0/G1 cell cycle arrest, which can lead to cytotoxicity (Wu et al., 2002). Indeed, in the UM-PEL-1c, IFA for the early lytic protein vGPCR showed that the percent of apoptotic cells, as determined by YO-PRO-1/PI staining closely correlated with lytic reactivation, indicating that KSHV lytic replication could be causally associated with cytotoxicity. While single drugs alone tend not to induce robust KSHV lytic reactivation in PEL (Yu et al., 1999), the combination of Btz and SAHA synergized to induce KSHV lytic replication and enhanced apoptosis of PEL cells, an effect that correlated in vivo to prolonged survival.

In addition to viral lytic induction, Btz and SAHA might promote PEL cell apoptosis by targeting latent KSHV mechanisms known to prevent apoptosis in PEL such as NF-κB constitutive activation by viral Fas-associated with death domain (FADD)-like interleukin-1β-converting enzyme (FLICE)/caspase 8-inhibitroy protein (vFLIP) and p53
inactivation by KSHV latency-associated nuclear antigen (LANA). While Btz is a known inhibitor of NF-κB signaling, in PEL cells Btz led to IκB-α phosphorylation and increased NF-κB (p50/p65) nuclear activity in vivo. These results concur with a recent study demonstrating that Btz induces NF-κB (p50/p65) activity in a multiple myeloma through the phosphorylation of IκB kinase (IKK-β) (Hideshima et al., 2009). NF-κB can activate caspase-8 as a result of death receptor (such as Fas/CD95) signaling. While we observed activation of caspase-8 with Btz, whether death receptor-mediated signaling or NF-κB activation induced by Btz and SAHA in PEL contributed to cell death remains to be determined in future studies. Another possible explanation for the extensive apoptosis seen in our PEL xenografts in response to Btz treatment was stabilization of p53 protein by inhibiting its proteasomal degradation along with the accumulation of one of its targets, the cyclin dependent kinase inhibitor p21. This increase in p53 activity is consistent with cell cycle arrest and a pro-apoptotic phenotype mediated by pro-apoptotic regulators such as Bax that was also increased. Additional mechanisms by which the combination of Btz and SAHA might cause apoptosis is through ER stress. However, of the proteins known to be upregulated by the UPR, only CHOP expression increased significantly after Btz in UM-PEL-1 xenografts. Previously, we showed that c-myc, another cell cycle and apoptosis regulator, was downregulated with Btz (Sarosiek et al., 2010b). Indeed, this again was the case, irrespective of the addition of SAHA.

Histone hyperacetylation resulting from Btz and SAHA treatment could also contribute to PEL cell apoptosis. While SAHA increased histone acetylation, likely contributing to chromatin remodeling, Btz also led to progressive accumulation of acetylated histones. Indeed, when combined, SAHA and Btz led to a sustained
irreversible accumulation of hyperacetylation, which has been shown to have pro-apoptotic effects in neoplastic models (Kikuchi et al., 2010; Miller et al., 2009; Sato et al., 2011).

The powerful induction of lytic KSHV replication with concomitant inhibition of virus production showed a clinically desirable outcome of the Btz/SAHA combination. To understand the nature of this inhibition, we analyzed viral DNA loads in vitro and we found that the Btz-treated UM-PEL-1c harbored significantly greater copies of viral DNA than the control and SAHA-treated cells. We reasoned that since Btz was inhibiting lytic gene expression, the accumulation of intracellular viral DNA could be a reflection of DNA replication along with failure to complete the lytic replicative cycle. The results from viral infection assays support this hypothesis as PEL cells stimulated with Btz or Btz/SAHA produced fewer infectious virions into the supernatant for de novo infection of 293 cells. This was confirmed in vivo: mice treated with Btz had significantly less encapsidated viral DNA in the ascites than those treated with SAHA alone. The inhibition of infectious KSHV production by Btz is supported by a previous in vitro study describing Btz inhibition of virion production (Brown et al., 2005). This effect likely results from the dependency of KSHV on the proteasome throughout the viral replicative cycle, which has also been described in the context of other herpesvirus (Klass et al., 2005; Randow and Lehner, 2009). In studies presented herein, while Btz induced the expression of most KSHV lytic genes, the transcription of several KSHV lytic genes were affected differentially, indicating that inhibition of the proteasome has gene-specific effects on viral lytic replication. For instance, transcription of RTA and ORF45 genes was synergistically enhanced by combining Btz and SAHA, while SAHA-induced K8/b-
Zip expression was inhibited by Btz. This may be a significant event as the K8/b-Zip protein coordinately activates, along with RTA, the expression of some KSHV lytic genes (Lefort and Flamand, 2009). K8/b-Zip is also involved in the negative regulation of RTA, thus helping to re-establish latency (Liao et al., 2003). K8.1, a late lytic gene which transcribes a key glycoprotein and is transcribed from the same locus as K8/b-Zip, was found to be inhibited at the mRNA and protein levels. While we did not interrogate all ORFs encoded by KSHV, it is likely that there are other genes similarly inhibited by proteasome inhibition. Although it has been previously reported that Btz can reactivate EBV expression (Fu et al., 2007), we did not observe significant reactivation of EBV early lytic genes in PEL xenografts.

Figure 2.13. Proposed model for mechanism of action for SAHA, Btz and the combination. SAHA leads to lytic cycle induction leading to the full replicative cycle, leading to virus production and apoptosis. Btz also leads to lytic cycle induction but it also blocks late lytic gene expression leading to apoptosis in the absence of virus production. In the case of the combination SAHA and Btz synergize in inducing the lytic cycle, however the presence of Btz inhibits the completion of the lytic cycle resulting in massive apoptosis in the absence of virus production.
In summary, the results from this study point to a novel treatment strategy for KSHV-infected PEL. Using the Btz/SAHA combination allows for an extremely high viral inductive capacity while at the same time blocking infectious virus production, thus ensuring destruction of KSHV-infected PEL cells. Given the observed anti-KSHV effect of Btz in stalling full lytic replication and virion production, and the enhanced effect of Btz and SAHA on apoptosis, this study provides a strong rationale for combining Btz with SAHA as a potent anti-PEL therapy, especially in the setting of HIV and immunosuppression. Based on our findings, clinical trials combining proteasome and HDAC inhibitors should be strongly considered for the treatment of PEL and other γ-herpesvirus related malignancies.
CHAPTER THREE: DEVELOPMENT AND CHARACTERIZATION OF VIRALLY PRODUCTIVE MOUSE MODELS OF KS

Background

In the previous chapter we established in a direct xenotransplant model of PEL the efficacy of using the lytic-inductive paradigm for the treatment of the KSHV driven lymphoma. The antiviral activities of Btz allowed us to use SAHA, a potent lytic inducer of KSHV replication, to inhibit PEL growth while at the same time ensuring that there is not a concurrent increase in viremia in vivo. Another KSHV-driven cancer is Kaposi’s sarcoma, which unlike the lymphomatous effusions of PEL, is a solid tumor with a very different tumor microenvironment. However, like PEL, KSHV is largely latent in the tumor, and in theory, the lytic-inductive paradigm could also be used to inhibit tumor growth or induce remission. Although our lab previously established a murine animal model of KS (mECK36), the model was characterized as having an abortive lytic replicative cycle and therefore did not produce virions in the mouse. Therefore, it may not be suitable to test the antiviral activities of Btz or other such antivirals in the context of the lytic inductive paradigm. Our next goal was to optimize the mECK36 model by replacing the BAC36 construct with a fully infectious recombinant KSHV in an attempt to create a productively infected murine model of KS.

Replacing BAC36 for rKSHV.219 maintains a KSHV-dependent tumorigenic phenotype

Previously, we found that a bone marrow-derived murine endothelial cells (mEC) transfected with BAC36 (mECK36) resulted in a cell population capable of forming KS-
like lesions in immunocompromised mice, however the nature of infection was abortive lytic. It was unknown if the abortive lytic phenotype was due to the BAC36 construct, which has been shown to have duplications (Yakushko et al., 2011), or the murine cellular environment (Austgen et al., 2012b). We hypothesized that by replacing the BAC36 construct with rKSHV.219, a fully replication-competent infectious recombinant virus, we might be able to generate a model that produces virions and more closely recapitulates human KS. Upon infection, rKSHV.219 constitutively expresses GFP driven by an EF-1α promoter, along with the pac gene allowing for puromycin selection of essentially 100% infected cells. The recombinant construct also contains RFP driven by the KSHV replication and transcriptional activator (RTA)-dependent lytic polyadenylated nuclear (PAN) RNA promoter, allowing for the identification of cells undergoing early lytic replication (Vieira and O'Hearn, 2004). Replacing the BAC36 with rKSHV.219 was accomplished by culturing the mECK36 cells without hygromycin selection, allowing for the loss of the episomal BAC36, resulting in the non-tumorigenic KSHV-null mECK36 cell line, mECKSHVnull (mECKnull) (Mutlu et al., 2007). Three pieces of information indicated that the mECK null were not transformed: (1) the cells maintained contact inhibition in vitro, (2) they did not form colonies in a soft agar assay (Figure 3.1A), and (3) there was no tumor formation when injected into nude mice (0/10), confirming the concurrent loss of tumorigenicity with the loss of the KSHV genome.

The mECKnull cells were infected with rKSHV.219, and cultured under puromycin selection resulting in a population of >99% GFP positive cells (mECKnull.rK). Subcutaneous injection into nude mice resulted in unreliable tumorigenicity (4/7 mice). In an attempt to enrich for a more uniform population of tumorigenic cells, mECKnull.rK
were enriched for Prominin-1 (CD133), a marker of cancer stem cells and endothelial progenitor cells (Fonseca et al., 2008; Salven et al., 2003; Yu et al., 2011), a putative spindle-cell precursor (Ganem, 2010b; Mesri et al., 2010b) which was shown to be upregulated upon KSHV infection of lymphatic endothelial cells (Liu et al., 2010). CD133 was expressed on a minority of cells, 0.8% (data not shown), which were easily expanded under puromycin selection; we termed these CD133-enriched cells, mECKnull.rK133 cells. Both the unenriched and CD133 enriched population of mECnullrK formed colonies in soft agar (Figure 3.1A). It is widely agreed that KS spindle-cells are of the endothelial lineage (McAllister and Moses, 2007). As an initial characterization, we ran a transcriptional profile for the mECKnull.rK133 cells and performed unsupervised hierarchical clustering against multiple murine cell types, including endothelial cells. We found that mECKnull.rK133 cells clustered tightly with the mature aortic endothelial cells but not with stem cells, total bone marrow, or subcutaneous tissue (Figure 3.2).

A hallmark of KS-derived spindle cells is their maintenance of KSHV as an episome tethered by LANA to the host chromosomes (Ballestas et al., 1999). When spindle cells are cultured ex vivo, there is a rapid loss of the episome (Grundhoff and Ganem, 2004). To verify that the rKSHV infection was episomal in mECKnull.rK133, they were cultured in the absence of puromycin and GFP expression was followed by flow cytometry. By week 10 the population was KSHV negative as determined by flow cytometry for GFP (Figure 3.1B) and PCR for LANA (data not shown). A general characteristic of KSHV-infected endothelial lineage cells is that, in vitro, only a small proportion of spindle cells are harboring lytic KSHV at any one time (Moses et al., 1999). This is also the case with mECKnull.rK133. We observed that latency was tightly
maintained, as determined by an absence of RFP expression (Figure 3.1C, top panels). We compared the latent nature of the mECK\textsuperscript{null}.rK133 to the iSLK.219 cells, a tightly latent line derived from a primary KS tumor and reconstituted with KSHV by rKSHV.219 infection (Myoung and Ganem, 2011). Using qRT-PCR to detect viral lytic activity \textit{in vitro}, we found that the iSLK.219 expressed profoundly higher levels of RTA (fold change: 1389 ± 308), K8/K-bZip (fold change: 88.6 ± 12.4), viral interferon response factor-1 (vIRF-1) (fold change: 88.9 ± 6.8), vGPCR (fold change: 64.4 ± 4.4), and the glycoproteins, K8.1 (fold change: 106.5 ± 11.9) and gB (fold change: 177.3 ± 13.2), than the mECK\textsuperscript{null}.K133, indicating that the murine cells maintained KSHV in a tight state of latency \textit{in vitro}. To evaluate the ability of the mECK\textsuperscript{null}.rK133 to support lytic replication, they were induced with the histone deacetylase (HDAC) inhibitor, Trichostatin A (TSA), for 12, 24, 36h and 60h. When undergoing lytic replication the rKSHV.219 produces RFP; about 5% of cells underwent lytic replication as determined by RFP expression at 24h (Figure 3.1C, bottom panels). The induction of RFP expression correlated with a sustained increase in RTA and other lytic KSHV transcripts (Figure 3.1D). However, despite strong lytic induction, we were unable to detect the production of infectious virions \textit{in vitro}, regardless of whether induction was achieved chemically or by providing the RTA protein in \textit{trans} using BacK50, a baculovirus expressing RTA (Vieira and O'Hearn, 2004). This is consistent with the observations of others and suggests that, at least \textit{in vitro}, murine cells do not support all aspects of KSHV lytic replication (Austgen et al., 2012a; Mutlu et al., 2007). Regardless, qRT-PCR studies of cultured mECK\textsuperscript{null}.rK133 revealed robust transcriptional production of angiogenic growth factors and receptors as indicated by threshold cycle (Ct) values: vascular
endothelial growth factor (VEGF) A (Ct Range: 17.2-18), VEGFD (Ct Range: 16.6-17.1), VEGFR1 (Ct Range: 29.3-29.9), VEGFR2 (Ct Range: 34.1-35.5), angiopoietin1 (Ct Range: 25-25.4), angiopoietin-2 (Ct Range: 29.5-30.2), with $\beta_2$M (Ct Range: 15.7-16.5) strongly supporting their angiogenic potential, therefore we decided to evaluate the tumorigenic potential in vivo.
Figure 3.1. Characterization of mECKnull.133rK cells. (A) Soft agar assay shows that mECKnull cells do not form colonies in soft agar while mECKnull.rK and mECKnull.rK133 cells are colony inducing indicating that colony formation is dependent on KSHV infection. (B) Dendrogram shows that, similar to the mECK36 cells, mECKnull.rK133 cells are of the endothelial lineage and cluster with murine aortic endothelial cells. (C) Heatmap showing differential gene expression of the various mouse cells and subcutaneous tissue. Again, mECKnull.rK133 cluster with mature vascular endothelial cells, but not with stem cells, total bone marrow or subcutaneous tissue. (D) mECKnull.rK133 are almost uniformly GFP positive and maintain rKSHV in a tight state of latency as determined by the lack of RFP expression in uninduced cultures, but can be induced to express RFP with TSA. (E) qRT-PCR analysis shows that TSA induction of RFP coincides with an induction of sustained viral lytic gene expression as well. (F) rKSHV infection of mECKnull.rK133 is episomal as determined by flow cytometry for GFP in the absence of puromycin selection.
Figure 3.2 Dendrogram and heatmap demonstrate that, similar to the mECK36 cells, mECKnull.rK133 cells are of the endothelial lineage and cluster with murine aortic endothelial cells. ESC, embryonic stem cells; BM, total bone marrow; FLE, fetal liver erythroblasts; EC_NCadh, N-cadherin expressing endothelial cells; EC_VE+NCadh, VE+N-cadherin expressing endothelial cells; EC_VE_Cadh, VE-cadherin expressing endothelial cells; BM_MSC, bone marrow derived mesenchymal stem cells; AEC, aortic endothelial cells; mECK.219, primary bone marrow-derived mEC infected with rKSHV.219; mECKnull.rK133, mECKnull cells infected with rKSHV.219 and then enriched for Prominin-1; BM_Macro, bone marrow-derived macrophages; Macro, peripheral macrophages; HSC, hematopoietic stem cells; LSC, leukemia stem cells; GEC, glomerular endothelial cells; SubQ, subcutaneous tissue

mECKnull.rK133 form virally productive KS-like tumors in immunocompromised mice

To determine the KS-like tumorigenicity of the mECKnull.rK133 cells, we subcutaneously injected 3x10^6 into the hind flanks of immunocompromised mice. Solid tumors developed between four and six weeks post-injection (Figure 3.3A) with an incidence of 95% (38/40). In contrast, neither mECKnull nor mECK+KSHV.219 cells developed tumors by 8 weeks while the mECK+KSHV.219 cells (those not enriched for CD133) formed tumors only 60% of the time and with highly variable growth kinetics. Importantly, mECK+KSHVnull cells never formed tumors further confirming the KSHV-dependency of the mECKnull.rK133 tumorigenicity. Upon dissection, vibrantly GFP positive tumors were visualized under ultraviolet light (Figure 3.3B). Paraffin-embedded sections were H&E stained and evaluated by a pathologist who confirmed that the tumors were indistinguishable from the vascularized spindle cell sarcomas formed by mECK36 tumors previously generated by our lab and thoroughly characterized from the histopathological, host and viral gene expression perspective as KS-like tumors.
Examination of the H&E images reveals spindle cells arranged in bundles with extravasated red blood cells in slit-like spaces (Figure 3.3C). KS is distinguished in the laboratory from other angiosarcomas by antibody based screening for the major latency associated protein, LANA. Using immunofluorescence for the LANA protein of KSHV, it was determined that the majority of the tumor cells displayed the classical LANA punctuated nuclear staining while the overlying dermis was LANA negative (Figure 3.3D). To determine the breadth of KSHV gene expression in mECKnull.rK133 tumors, 19 viral transcripts spanning the entire viral replicative cycle and genome, were reverse transcribed by RT-PCR and visualized on an agarose gel (Figure 3.3E). Genes spanning the latent (LANA, Kaposin, vCyclin, vFLIP), immediate early (RTA, ORF45), early lytic (vGPCR, vIL6, SOX) and late lytic (K8.1, gB, ORF55) viral replicative cycle were all expressed in vivo, suggesting the potential for the full lytic replicative cycle to be transcribed. The broad and robust gene expression across the KSHV genome prompted us to determine if rKSHV replication was complete in the tumors resulting in mature virion production. Tumors were analyzed by transmission electron microscopy (TEM). Noteworthy, TEM imaging revealed herpesvirus-like particles between 100-200 nm (Figure 3.3F). We identified intracellular and extracellular virus-like particles at varying stages of maturation, including empty capsids (black arrow 1), virus-like particles inside intracellular vesicles (black arrow 2), some budding from cellular membranes (black arrow 3) and more mature particles with glycoprotein spikes (black arrow 4) (Figure 3.3F). To verify the lytic inducibility of the tumor cells, tumors were dissociated using collagenase and single cell suspensions were plated in culture. After 24 hours, the cells were stimulated with TSA. 24 hours later, fluorescence microscopy for RFP showed
RFP positive cells, indicating that the tumor cells are fully capable of being induced into lytic replication (Figure 3.3G).

Figure 3.3. mECK<sup>null</sup>.rK133 cells form virally productive rKSHV-induced tumors in immunocompromised nu/nu mice. (A) Subcutaneously injected mEC133rK cells form tumors in immunocompromised mice in 4-6 weeks. (B) The tumors are vibrantly GFP expressing, indicating that they harbor the rKSHV. (C) Pathologically, they are composed of spindle-cells with RBCs in slit-like vasculature (black arrows). (D) The spindle cells of the tumor are express the viral nuclear antigen, LANA. (E) Gene expression in vivo reveals the presence of transcripts that span the entire KSHV genome and replicative potential. (F) Electron microscopy revealed the presence of herpesvirus-like particles in vivo 12 weeks post-inoculation. (G) Ex vivo derived tumor cells can be induced into lytic replication.
To determine the extent of latently and lytically infected cells, tumors were collagenase dissociated and analyzed by flow cytometry for GFP and RFP. Tumors displayed a GFP positivity of up to 60%, while RFP expressing cells were always less than 1% of the population (data not shown). We also found that the pan-endothelial marker, CD31, was expressed by 30-60% of rKSHV-infected tumor cells, as determined flow cytometry for CD31 and GFP, further confirming the endothelial nature of the mECK\textsuperscript{null}.rK133 tumor cells (Figure 3.4A). KSHV infection of blood vascular endothelial cells induces lymphatic vascular endothelial differentiation and vice versa, leading many to suggest that this reprogramming contributes to the heterogeneity of markers in KS spindle cells (Hong et al., 2004; Wang et al., 2004). Examination of lymphatic and blood vascular angiogenic transcription indicated extensive expression of genes related to lymphatic and vascular angiogenesis (data not shown). KS-associated angiogenic endothelial heterogeneity was also seen at the protein level as mECK\textsuperscript{null}.rK133 tumors were found to express markers implicated in KS pathogenesis such as podoplanin, a lymphatic endothelial KS marker, as well as the vascular marker VEGFR2 and CD31 (Figure 3.4B).

To interrogate the state of the virus \textit{in vivo}, we began analyzing viral transcription with qRT-PCR and found that multiple KSHV lytic transcripts were increased relative to cells in culture, suggesting that the \textit{in vivo} environment is more conducive to lytic replication (Figure 3.4C). While flow cytometry suggested there was very little RFP expression, but qRT-PCR suggested strong upregulation of lytic genes, we wanted to further confirm the nature of the RFP, as it may be used as an indicator of viral lytic replication. We used anti-RFP antibody to facilitate and enhance RFP detection in frozen
sections with *in vitro* induced cells as positive control and mECK36 tumors, which only express the BAC36 encoded GFP, as negative staining controls. We found that, contrary to *in vitro* culture, which totally lacked RFP, but confirmatory by the flow cytometry, very few cells expressed RFP *in vivo* (Figure 3.4D, left panel) and it is these few cells that contribute to the increase in lytic gene transcription we found by qRT-PCR.

Given the similarities to KS and the ability of the tumors to produce rKSHV, we wanted to determine if the model may be useful for *in vivo* testing of novel antiviral and antitumor strategies. An excellent way to identify potent antivirals would be to induce greater viral replication in an *in vivo* tumor setting, and then challenge the antiviral candidate to inhibit virus replication and production. Furthermore, certain anti-herpesviral strategies rely on combining lytic inducers with antivirals for antitumor effects. To determine the ability of the tumors to be further induced into lytic replication, mice were treated with a short course of Vorinostat (SAHA) a FDA approved HDAC inhibitor for 4 days. Compared to uninduced mice, qRT-PCR studies confirmed increased viral lytic gene expression (Figure 3.4E) while immunofluorescence verified enhanced RFP expression *in vivo* (Figure 3.4D, right panel). The ability of rKSHV to infect bone marrow-derived mEC *in vitro* and the presence of rKSHV particles *in vivo* suggested that released rKSHV may be able to infect extratumoral sites. This would be an important aspect of the model as potent antivirals could be identified by the decreased spread of rKSHV *in vivo*. The levels of KSHV in extratumoral sites were low, so we used various methods to detect presence of virus: DNA quantification, RNA detection and cellular GFP expression. We were able to detect viral DNA in the bone marrow and whole blood of tumor bearing mice (Figure 3.4F), and confirm that the DNA detected in
the bone marrow correlated with transcription of LANA, Kaposin and GFP (Figure 3.4G). Further, GFP positive cells were isolated from the spleen (Figure 3.3H) lymph nodes of tumor bearing mice (Figure 3.4I). This is interesting as the mECK36 model developed previously in our lab by transfecting the BAC36 construct into bone marrow-derived endothelial enriched cells did not exhibit metastasis \textit{in vivo}. Despite extensive investigations KSHV could not be detected in non-tumor tissue, KSHV and KSHV-infected cells remained restricted to the tumor in the mECK36. This suggests that the ability of the rKSHV.219 to complete a full lytic cycle may contribute to either: the ability of the virus to spread and initiate \textit{de novo} infection within the murine host, or allow for the KSHV-infected tumor cells to metastasize and establish themselves in a new milieu. Regardless, whether these cells represent migration of KSHV-infected cells from the primary tumor, or \textit{de novo} infection \textit{in vivo}, remains to be determined. Ways of determining between migration and \textit{de novo} infection include: 1.) Inoculating a female mouse with male tumor cells and using FISH to determine if the splenic cells contain a Y-chromosome. If so, this would indicate that KSHV gave the cells a metastatic potential. If not, if the splenic cells contained two X-chromosomes, this would indicate that the virus was produced in the tumor and was able to establish \textit{de novo} infection \textit{in vivo}. A second way to determine metastasis vs. \textit{de novo} infection would be to evaluate the LTR of the viruses. If the viral LTRs are of the same size in the spleen and tumor, this would suggest metastasis. If not, it would suggest \textit{de novo} infection as LTRs differ upon viral DNA cleavage and packaging during lytic replication.
Figure 3.4. mECK\textsuperscript{null}.rK133 tumors express endothelial markers important in KS and rKSHV can be detected in distant areas of the mouse. (A) Cells from a dissociated tumor were immunostained for CD31, an angiogenic marker and analyzed by flow cytometry for GFP/CD31 co-expression. The vast majority of rKSHV infected cells also express CD31. (B) Tumors were found by immunofluorescence to express high levels of VEGFR2, podoplanin and CD31. (C) qRT-PCR reveals that KSHV lytic gene expression is increased \textit{in vivo} relative to \textit{in vitro}. (D) \textit{In vivo} RFP expression is low in uninduced mice but increases with SAHA treatment. An anti-RFP antibody was used to enhance RFP detection. (E) KSHV lytic gene transcription is also increased in tumors of mice treated with SAHA. (F) 10x10^6 cells purified from bone marrow and DNA isolated from 500\mu l whole blood indicate the presence of KSHV DNA in extratumoral sites. (G) Bone marrow extracted from a representative tumor-bearing mouse was compared to a non-tumor bearing mouse for the presence of KSHV transcripts. RT negative control was run to verify the absence of DNA in the RNA samples. (H) GFP expressing cells isolated and expanded from the spleen of mECK\textsuperscript{null}.rK133 tumor bearing mouse were LANA positive. (I) GFP positive cells were found in the lymph nodes of mECK\textsuperscript{null}.rK133 tumor bearing mice. IFA of native GFP (IgG control antibody) and GFP enhanced by fluorescent detection is shown.
CHAPTER FOUR: KSHV INFECTION OF MURINE PRIMARY BONE MARROW-DERIVED ENDOTHELIAL-LINEAGE CELLS LEADS TO TUMORIGENESIS WITH A KS-LIKE PHENOTYPE

Background

The origin of the KS spindle cell remains one of the great enigmas in KS pathobiology. Spindle cells display hallmarks of many cell types and a large body of work has shown that many cell types are infectable by KSHV in vitro (Bechtel et al., 2003). However, in vivo, the cellular distribution is much more limited (Ganem, 2010b). Further, KSHV infection of vascular endothelial cells induces lymphatic differentiation and while lymphatic cell infection leads to vascular endothelial phenotype (Hong et al., 2004; Wang et al., 2004). These and other findings in vivo, such as the fact that KS-like endothelial precursor cells have been found circulating in the peripheral blood of KS patients (Browning et al., 1994), have led some to speculate that the spindle cell precursor is an endothelial-like progenitor cell. Our previous results of obtaining a tumorigenic phenotype via BAC36 transfection of primary bone marrow-derived endothelial cells also supports the progenitor hypothesis of spindle cell origin. Further, it has been speculated that using genetically engineered mice may be an excellent way to interrogate host specific mechanisms of KSHV-pathogenesis and creating a protocol in which to generate the spindle-precursor cells from the marrow of knock-in/out mice may be beneficial (Mutlu et al., 2007). Therefore, using purified bone marrow from immunocompromised athymic nude mice, we wanted to determine if we could infect primary endothelial-like cells (mEC) and obtain a KS-like tumorigenic phenotype.
Results

To determine if the tumorigenicity of the mECK\textsuperscript{null}.rK133 cells was restricted to murine cellular environments conditioned by prior KSHV infection, we tested the tumorigenic potential of rKSHV.219 infection of primary bone marrow-derived mEC. Bone marrow cells were flushed from murine femurs and the cells were allowed to adhere in culture in supplemented endothelial cell growth medium in a similar manner as previously described (Mutlu et al., 2007). After removal of the non-adherent cells, the adherent population was infected with high titer rKSHV.219. After puromycin selection, we obtained a population of cells that were determined to be >98% GFP positive and expressed LANA in the classic nuclear punctate pattern (Figure 4.1A, top panels). Interestingly, unsupervised hierarchical clustering of these cells showed that they are almost indistinguishable from the mECK\textsuperscript{null}.rK133 cells (Figure 3.1B and C) a progeny of the original mECK36 cells (Mutlu et al., 2007). Also, like the mECK36, mECK\textsuperscript{null}.rK133 and rKSHV infected MEFs reported by Augusten and Ganem, we were unable to recover virus from the supernatant of \textit{in vitro} induced mECrK cells regardless of the method used to induce viral lytic replication. To determine if these cells were also tumorigenic, \(3 \times 10^6\) cells were subcutaneously injected into the flanks of nude mice. Approximately 14 weeks later, we observed GFP expressing, KSHV-infected, KS-like tumor growth, similar to the kinetics we observed with the original mECK\textsuperscript{null}rK cells (Figure 4.1B and data not shown). Further, like the mECK\textsuperscript{null}.rK133 tumors, the majority of the GFP expressing cells also expressed CD31 on the cell surface (Figure 4.1C). Excised tumors were analyzed by TEM and found to contain herpesvirus-like particles of the same size and morphology as was seen in the mECK\textsuperscript{null}rK.133 tumors (Figure 4.1E). Further, like the
mECKnull.rK133, ex vivo-derived tumor cells could be further induced into lytic replication (Figure 4.1D) and viral DNA is easily detected in extratumoral sites such as the spleen and kidney (Figure 4.1F).

Figure 4.1. Primary bone marrow-derived mouse endothelial-lineage cells are tumorigenic when infected with rKSHV.219 (mECrK). (A) Fluorescence microscopy shows that mECrK cells are uniformly GFP positive and express LANA in the classic nuclear punctate manner. (B) Growth kinetics of 4 tumors were followed using caliper measurements beginning on day 40 post-subcutaneous injection. (C) Cells from a dissociated tumor were immunostained for CD31, an angiogenic marker and analyzed by flow cytometry for GFP/CD31 co-expression. About half of the CD31+ cells are also rKSHV infected as determined by GFP expression. The vast majority of rKSHV infected cells also express CD31. (D) Ex vivo derived tumor cells can be stimulated into lytic replication in vitro with TSA. (E) Electron microscopy revealed the presence of herpesvirus-like particles in vivo. (F) KSHV DNA copy number in the tumor, spleen and kidney is depicted for 3 mECrK tumor bearing mice. Bars represent the variability between the 3 mice.
Discussion

Few groups have achieved a KSHV-induced tumorigenic phenotype from the *de novo* infection of primary mammalian cells (Jones et al., 2012a). Although primary human endothelial cells (Flore et al., 1998), fetal mesenchymal stem cells (Parsons et al., 2004), and CD34+ hematopoetic precursors (Wu et al., 2006) are infectable and displayed phenotypes important in KSHV-pathogenesis, none formed tumors in immunocompromised mice. Our ability to reproduce a tumorigenic phenotype in primary murine cells paves the way for future studies on both the phenotype of the original spindle cell precursor in the mouse which may correlate with the human precursor. To determine if the tumorigenicity of the mECK<sup>null</sup>.rK133 cells was restricted to murine cellular environments conditioned by prior KSHV infection, we tested the tumorigenic potential of rKSHV.219 infection of primary bone marrow-derived mEC. Bone marrow cells were flushed from murine femurs and the cells were allowed to adhere in culture in supplemented endothelial cell growth medium in a similar manner as previously described (Mutlu et al., 2007). After removal of the non-adherent cells, the adherent population was infected with high titer rKSHV.219. After puromycin selection, we obtained a population of cells that were determined to be >98% GFP positive and expressed LANA showed that they are almost indistinguishable from the mEC<sup>null</sup>.rK133 cells a progeny of the original mECK36 cells (Mutlu et al., 2007). This is a key fact suggesting that from a bone marrow cell population enriched in heterogeneous endothelial lineage cells, both rKSHV infection and BAC36 transfection were able to establish an infection in, and promote survival of, a KS-like progenitor cell, i.e cell type that when infected with KSHV is capable of forming KS-like tumors in nude mice.
Alternatively, it is tempting to speculate that KSHV, in the right progenitor environment, induces transdifferentiation of the cell(s) to the same endothelial lineage. KSHV-infected, KS-like tumor growth, similar to the kinetics we observed with the original mECnull.rK cells. Interestingly, like the mECKnull.rK133 but unlike the mECK36 tumors, we were again able to see viral particles by electron microscopy suggesting that murine cells are capable of producing KS-like herpesviruses and that the BAC36 in murine cells leads to an abortive lytic infection (Mutlu et al., 2007).

In summary, we have constructed two productively infected in vivo models of KS. By reproducing a tumorigenic phenotype in de novo infected mEC, we have also shown that the tumorigenicity induced by KSHV is not reliant upon a cellular environment preconditioned by previous KSHV infection. The fact that primary mouse bone-marrow-derived mEC can become tumorigenic when infected with rKSHV, opens the possibility of delineating KS and KSHV biology in genetically engineered mice. Furthermore, these types of productively infected, inducible models should be ideal for the testing of novel targeted antiviral strategies and interrogating viral/host mechanisms of KSHV-induced pathogenesis.
CONCLUDING REMARKS

While the prevalence of PEL and KS have declined dramatically in the developed world, they remain responsible for significant morbidity and mortality in the developing world. Further, current treatments are often associated with considerable toxicity. Developing effective therapies for KSHV-induced malignancies has been hindered due to a lack of animal models that loyally recapitulate the cancers. The fact that these cancers are induced by a herpesvirus means they should be perfect substrates for highly specific tumor cell-targeted therapies as the virus provides many therapeutic targets that do not exist in the normal host tissue. One therapeutic strategy that has been proposed is termed lytic-inductive therapy, or virus-activated cytotoxic therapy, and proposes to kill the virally infected cells by inducing lytic replication of the virus. However, unwanted sequelae could include worsening disease due to the induction of lytic genes that may aid in oncogenesis or increased viremia in the immunocompromised host. Therefore, a potent antiviral needs to be administered along with the lytic inducing agent to inhibit these effects. We used a direct xenograft transplant model of PEL, thereby abrogating any cell culture induced changes and maintaining the in vivo phenotype. We used this model to test the lytic inductive paradigm for treating this largely latent tumor by inducing viral lytic replication with SAHA, an HDAC inhibitor and bortezomib (Btz), a proteasome inhibitor. While we previously reported that Btz was an inducer of the lytic cycle in this model, we advanced these findings in these studies to show that Btz is more importantly, a potent antiviral agent. The combination of Btz and SAHA led to synergistic increase in viral lytic replication, enhanced survival of PEL inoculated mice and massive apoptosis of the PEL cells, all in the absence of infectious virus production.
This is an ideal outcome as PEL is most often found in individuals with AIDS and are profoundly immunosuppressed. These findings strongly support the concept of the lytic-inductive paradigm for treating these highly aggressive tumors and provide a framework for further pre-clinical studies and Phase I trials in individuals with PEL refractory to conventional therapies.

Kaposi’s sarcoma is generally studied using in vitro systems or animal models that are limited in their abilities to recapitulate key aspects of KSHV-induced Kaposi’s sarcoma. Herein, we have described two new models based on infection with a replication competent fully infectious KSHV. The KS-like tumors are composed of spindle-cells harboring the recombinant KSHV and further, electron microscopy showed that the tumors are capable of producing virions. These findings are significant as this is the first time a virally productive mouse model of KS has been reported. These systems may be useful for multiple reasons. First, as the cells are infected with rKSHV, a virus that expresses GFP constitutively and RFP upon lytic reactivation, the latent vs. lytic nature of the virus can be studied relatively easily as the lytic cells are red and able to be discerned from the latent cells. Second, having established a system in which primary cells become oncogenic when infected with KSHV paves the way for interrogating viral/host mechanisms of pathogenesis using genetically engineered mice, where host genes can be manipulated and studied for contributions to cellular immortalization by KSHV. Finally, the fact that the tumors reproduce both host and viral KS biology make these systems an excellent choice to be used for elucidating novel antitumoral and antiviral therapies in an in vivo setting.
APPENDIX A: Material and Methods

Generation of mECK\textsuperscript{null}.rK133 and mECrKSHV.219 (mECrK). mECK\textsuperscript{null}.rK133 cells were generated as described (Mutlu et al., 2007) by allowing mECK36 cells to lose the BAC36 construct through serial passage in culture without hygromycin selection for BAC36. This resulted in mECKSHV\textsuperscript{null} (mECK\textsuperscript{null}) cells. mECK\textsuperscript{null} were then infected with rKSHV.219 and expanded under puromycin selection at 1-2µg/mL, these are mECK\textsuperscript{null}.rK cells. Finally, Prominin-1 (CD133) expressing cells were enriched from the mECK\textsuperscript{null}.rK cells using magnetic beads (Multenyi Biotec) as per manufacturer’s protocol and expanded under puromycin selection at 1-2µg/mL. These are termed mECK\textsuperscript{null}.rK133 cells. Primary mECs were generated from the bone marrow of 10 week old Athymic NCr- nu/nu mice from the National Cancer Institute (National Institutes of Health). Mouse femurs were flushed with PBS, bone marrow cells were put in culture in for 3 days. Non-adherent cells were washed away and medium was replaced every 3 days for two weeks to allow for expansion of the adherent cells. All murine cells were cultured in endothelial cell (EC) growth medium (mEC medium): DMEM supplemented with 30% FBS (Gemini Bioproducts), 0.2 mg/ml Endothelial Cell Growth Factor (ECGF) (Sigma-Aldrich), 0.2 mg/ml Endothelial Cell Growth Supplement (ECGS) (Sigma-Aldrich), 1.2 mg/ml heparin (Sigma-Aldrich), insulin/transferrin/selenium (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and BME vitamin (VWR Scientific). NIH3T3 cells were purchased from American Type Culture Collection. HEK293 cells were cultured in DMEM with 10% FBS (Gemini Bioproducts) and 1% penicillin-streptomycin (Invitrogen)
**Soft agar assay.** Base agar was made by combining melted 1% agar with 2X mEC medium to give a 0.5% Agar/1X mEC medium solution. 1.5mL was added to each well of a 6 well plate and allowed to set. Five thousand cells were plated on top of base agar in 0.7% agar/2X mEC medium in triplicate in 6-well plates. The cells were fed every 3 days with 1mL of mEC medium (described above). Colonies were photographed at 4 weeks. Only colonies larger than the mean size of the background colonies in the NIH3T3 negative control wells were considered.

**Light microscopy.** Tissue slices of rKSHV infected mouse tumors were fixed overnight in 10% buffered formalin, embedded in paraffin, and cut into 5µm sections and H&E stained.

**Transmission electron microscopy.** Tumors were excised and fixed overnight in 2.5% glutaraldehyde, 100mM sucrose, 0.05M phosphate (PO₄) buffer. After fixation, the segments were rinsed in 3 changes of 0.15M PO₄ buffer, pH 7.2 for 10min each. Samples were then fixed with 1% osmium tetroxide in 0.1M PO₄ overnight at 4°C. Following fixation, the segments were rinsed 3 times in 0.15M PO₄ buffer for 10min 3 times each. The tumors were then dehydrated through a graded ethanol series and rinsed twice in propylene oxide for 5min each. A 1:1 mixture of propylene oxide: Epon Araldite with DMP-30 (E/A) was added overnight at room temperature. Fresh E/A was added and the tissue was desiccated for 5 hours. Tissue was moved to embedding molds in fresh E/A, uncovered in 64°C oven overnight before embedding, sectioning and mounting on copper grids. The sections were viewed with a Philips CM-10 Transmission Electron microscope.
**Reagents.** Vorinostat was obtained from LC Laboratories. Trichostatin A, puromycin, and DMSO were obtained from Sigma-Aldrich. Bortezomib was obtained from Millennium Pharmaceuticals. All reagents were sterile filtered prior to use. SAHA (Vorinostat) was purchased from LC Laboratories. Primary antibodies to Bax, Bcl-X<sub>L</sub>, c-Myc, p21, p-IκB-α, caspase-8, acetyl-H3, total H3, GRP-78, GRP-94, P-eIF2α, total eIF2α and CHOP were from Cell Signaling; GAPDH, β-actin, IRF-4, c-FLIP and p53 were from Santa Cruz; total I B-α, Bcl-2 and CD30 were from BD Pharmingen; ATF-6 was from Imgenex; cIAP2 was from Abcam. YO-PRO-1 and propidium iodide (PI) were purchased from Invitrogen. LANA antibody was purchased from Abcam.

**Microarray analysis.** Using the Affymetrix MoGeneST_1.0 array, we compared gene expression levels of the mECK<sup>null</sup>rK133 cells and mECrK cells to data from 40 microarray samples representing 16 different mouse cell and/or tissue types also run on MoGeneST_1.0 array collected from GEO datasets. GEO accession numbers are as follows: Aortic Endothelial cells (AEC: GSM686077, GSM686078), Bone marrow derived mesenchymal stem cells (BM_MSC: GSM757750), N-Cadherin expressing endothelial cells (EC_NCadh: GSM859433, GSM859434), VE-Cadherin expressing endothelial cells (EC_VECadh: GSM859435, GSM859436), VE+N-Cadherin expressing endothelial cells (EC_VE+NCadh: GSM859437, GSM859438), total bone marrow (BM: GSM821069, GSM821070, GSM821071), undifferentiated embryonic stem cells (undiffESC: GSM839393, GSM839394, GSM839395), fetal liver erythroblasts (FLE: GSM842148, GSM842149, GSM842150), bone marrow-derived macrophages (BM_Macro: GSM787745, GSM787746, GSM787747), peripheral macrophages (Macro: GSM466436, GSM466437, GSM466438), hematopoietic stem cells (HSC: GSM862182,
GSM862184), leukemia stem cells (LSC: GSM862186, GSM862188), glomerular endothelial cells (GEC: GSM532931, GSM532932, GSM532933) and subcutaneous tissue (SubQ: GSM905121, GSM905122, GSM905123). Each sample was processed through QA/QC on GeneSpring 12 software (Agilent technologies). A batch bias was noted since the data was derived by many groups. Each batch was individually Quantile normalized and log2 transformed to median of all samples within the batch. The combined data was corrected for batch bias using the using the ComBat (Johnson et al., 2007) module on R/Bioconductor package. In order to clearly demarcate the clusters of samples, 7549 probes with most variation (SD ≥ 1.5) among all the 40 samples were subset. A hierarchical clustering was performed on the subset using GeneSpring. For clustering the Pearson centered similarity measure method with a centroid linkage rule was used.

**RNA Isolation and Quantitative real-time reverse transcriptase PCR (qRT-PCR).**

Total RNA was isolated using the RNeasy Plus kit or the AllPrep RNA, DNA, protein kit (Qiagen) for concomitant RNA/DNA isolation studies. DNase treatment of eluted RNA was done with Turbo DNase (Ambion) as per manufacturer’s instructions. 500ng of RNA was transcribed into cDNA using random primers and the Reverse Transcription System (Promega) according to the manufacturer’s instructions. Quantitative real-time reverse transcriptase PCR (qRT-PCR) reactions were run using SYBR Green PCR Master Mix (VWR) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Dissociation curve analysis verified specificity of products. To display the intratumoral transcription of the selected KSHV genes, a 3% agarose gel of the cDNA products was run which also confirmed the dissociation curve indicating that primers
were specific for single amplicons. Reverse transcriptase negative and non-template controls were run to verify purity of sample. Data were analyzed using the \( \Delta \Delta Ct \) method where target gene expression is normalized to the housekeeping gene by taking the difference between Ct values for target gene and housekeeping gene (\( \Delta Ct \)). This value was then compared to that of the normalized control sample (\( \Delta \Delta Ct \)). The fold change was then determined by the formula: \( 2^{-\Delta \Delta Ct} \). The following primer sets were used:

RFP (5'-AGGACGGCTGCTTCTCATCTAC-3', 5'-TGGTCTTCTTCTGCATCAG-3');

ORF73/LANA (5'-CCTGGAAGTCCCCACAGTGT-3', 5' AGACACAGGATGGGATGGAG-3');

vCyclin (5'-GCCGCGCTTTTTAACTTCTGAC-3', 5' AAATAGGGCGTGGCTTCTGAG-3');

vFLIP (5'- GGATGCCCTTTCTATGCAATGC -3', 5'- GGCGATAGTGTTGGGAGTGT -3');

Kaposin (5'-AGGCTTAACGGGTGTTGTTG -3', 5'- GTTGCAAACTCGTGTCCTGAA -3');

ORF50/RTA (5'-CAAGGTGTGCGGCTGTAGAGA-3', 5'-TCCCAAGAGGGTACCAGGTA-3');

ORF45 (5'-CATGGGATGGGTAGTGCAATGC -3', 5'-GGGTCTGCTGATGGGTAAGC -3');

ORF21/vTK (5'-ACGCCGTGTGCGGGATCTTG -3', 5'-GACGCCAAGTGAGTGCCC -3');

ORF36/vPK (5'-CCCCCGGTGTCCTGAAAC -3', 5'-ATCCTGGTGCCTGCAGCTGCC -3');
vCCL2/K4 (5'-CGTTTTATGCTGCGTGTAG-3',
5'-AGTTTTGGGAAGGCTCTGC-3');
vIRF1 (5'-GGAAGAAACAATGCGGAAATG-3',
5'-CGACTGGCTTTGTCGTGCAGTA-3');
vIL6 (5'-TGCTGGTCAAGTTGTGGTC-3',
5'-ATGCCGGTGACGGTACAGA-3');
ORF55 (5'-GCATTCCCCGCGCCTTTTGTATA-3',
5'-CTCGCGGCGGTATGTCGTCTCC -3');
ORF49 (5'-CGAGAAAGCCTTTAAGAT-3',
5'-GGTACGTGGCAGTCTGGATT-3');
ORF57 (5'-GGGTGGTTTGATGAGAAGGA-3',
5'-CGCTACCAAAATATGCCACCT-3');
ORF74/vGPCR (5'-TGTTGTGGTGAGGAGACAAA-3',
5'-GTTACTGCGCGCAGGCCACGT-3');
K7/vIAP (5'-CTGCCGCTTCACCTATGGAT-3',
5'-AACTGGCCTGGAGATTGAA-3');
K8.1 (5'-CACCACAGAACTGACCAGATG-3',
5'-TGGCACAAGGGTTACTAGAC-3');
ORF8/gB (5'-CTGGGGACTGTCATCCTGTT-3',
5'-ATGCTTCCTTACCCAGGTCTG-3');
Mouse GAPDH (5'-ACCCAGAAGACTGTGGATGG-3',
5'-CACATTGGGGGTAGGAACAC-3');
Mouse TBP (5'-CAGCCTTCCACCTATGCTC-3',
5'-TTGCTGCTGCTTCTTGTTT-3');
GFP (5'-ACGTAAACGGCCACAAGTTC-3',
5’-AAGTCGTGCTGCTTCATGTG-3’
Human GAPDH (5’-GAGTCAGGTTTGGTCTC-3’,
5’-GABAAGCTTCCCGTTCTCAG-3’)
K8/K-bZip (5’-GGCCCTAGGCGGCTCTCCC-3’,
5’-GGGAGGTCACGGGACGCTCT-3’);
ORF39 (5’-GCCGATATAGCAGGTCACG-3’,
5’-CAGCGAAGCACCCTCACCCG-3’);
ORF39/gm (5’-GCCGATATACGGCCACGC-3’,
5’-CAGCGAAGCACCCTCACCCG-3’);
EBNA1 (5’-GGTCCAGCCAGAAATTTGA-3’,
5’-TGGAATAGCAAGGCCAATTC-3’);
LMP1 (5’-GAGACCAACCACAGATGACT-3’,
5’-GTGCGCCTAGGTTTGTGAG-3’);
BRLF1 (5’-CTCGGGGTTACTGCGGGG-3’,
5’ ACCGGTCCGATCCCTAACGCC-3’);
BXLF1 (5’-GTTGGGCACCTGGTAGAGGCC-3’,
5’-CCAGGAAAGGAGGCCGGG-3’);
BGLF4 (5’-CTGGAAGCAGCCCGAGG-3’,
5’-CCCCTCGAGAGACCCAGGC-3’);
BZLF2 (5’-GGAGGATCCACCAGG-3’,
5’-AGGAGGAGGGCGGTTGGA-3’);
p21 (5’-GCCGAGGCAGGCCAGTTG-3’,
5’-CTGCCGCGGTTTTCGACT-3’);
p53 (5’-CTGCCCTCAACAGATTTGTTT-3’,
5'- CTCCGTCATGTGCTGTGACT -3');
c-myc (5'-CAGATCAGCAACAACCGAAA -3',
5'-GGCCTTTTCATTGTTTCCA-3')

**Flow Cytometry.** Tumors were excised, minced and further dissociated at 37°C with collagenase IV (Worthington Biochemicals) in DMEM, 0.5% bovine serum albumin, 1X pen/strep/fungi, and 2µL/mL DNase (Ambion) by shaking at 180rpm for 30-60 min. Dissociation was complete after final trituration using a 5mL pipette and then filtering the material through 70µM and then 40µM filters. Single cells were stained with anti-CD31 (BD Biosciences) or an isotype control antibody for 30 min at 4°C. After washing a secondary APC-conjugated antibody was added for 30 min incubation at 4°C. Cells were then washed with PBS and fixed with Cytofix fixation buffer (BD Biosciences). Flow cytometric analysis was performed on a Becton-Dickinson LSR analyzer (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.) software.

**Immunofluorescence.** Frozen sections and cytospin preparations were fixed in 10% buffered formalin for 10 min room temperature. They were then washed with PBS and permeabilized with 0.2% Triton-X in PBS for 30 min at 4°C, washed in PBS, and blocked with 10% goat serum (Dako) for 30 min. Primary antibodies were diluted to 1:100 in 2% goat serum and incubated at room temperature for one hour. Sections were then washed with PBS and secondary goat anti-rabbit or goat anti-mouse, as appropriate, antibodies conjugated to Cy3 or Cy5 (Molecular Probes, Invitrogen) were added at a 1:500 dilution for one hour at room temperature. Sections were washed, allowed to dry and mounted with ProLong Gold with DAPI (Invitrogen). All appropriate isotype
controls were included for negative controls. TSA induced cells were used as positive control for RFP antibody specificity. The anti-RFP antibody was counterstained with a Cy5 labeled secondary antibody. RFP visualized with the Cy3 channel co-localized with Cy5, indicating that the RFP antibody specifically stained RFP expressing cells. Uninduced cells, those expressing only GFP, were not stained by the anti-RFP antibody. MetaMorph 7.7 was used for relative quantification of K8.1/Cy3+ cells and normalized to DAPI+ nuclei using a pixel based approach. Images were acquired using Zeiss Axiovision 4.8.2 with a Hamamatsu ORCA-R2 CCD camera and Zeiss Axiovert 200M inverted fluorescence microscope, saved in Zeiss .ZVI file format, and batch exported to single channels monochrome 16-bit TIFF format using Axiovision File - Export - Batch command. Cy3 series and DAPI TIFF series were opened as respective stacks in MetaMorph. DAPI stack was thresholded, and the positive values were used to generate a binary mask stack. This mask was applied to the Cy3 stack to zero out non-cellular areas. MetaMorph Region Measurements command was used to quantify the number of Cy3+ thresholded pixels in the masked Cy3 stack vs. the number of pixels in the entire mask. Treated cells were normalized to untreated control. Error bars represent the variation in K8.1 expression between 12 fields at 10x magnification. Images were taken using Zeiss Axiovision 4.8.2 with a Hamamatsu ORCA-R2 CCD camera and Zeiss Axiovert 200M inverted fluorescence microscope.

**Isolation and rKSHV infection of bone marrow-derived endothelial-lineage cells.**

Under asceptic conditions, primary murine bone-marrow cells were flushed from the femurs of NCI athymic nu/nu mice and allowed to adhere to the cell culture dish for 48 hours with endothelial growth factor supplemented medium: DMEM (4.5 g/L glucose),
30% FBS, 1X MEM Vitamins, 1X Pen/Strep, 1X ITS, 20µg/mL endothelial cell growth supplement, 0.21 U/mL heparin, 0.4X endothelial cell growth factor. Non-adherent cells were removed, adherent cells were washed and supplemented medium was replaced for 2 weeks with medium changes every 3 days. The cells were then infected with rKSHV.219 in the presence of polybrene (8µg/ml) for 2 hours. 2 days later, puromycin was added to the culture to select and expand the infected cells.

**In vivo tumor studies.** 3x10^6 cells were injected into the hind flank of immunocompromised mice. Mice were monitored daily. For growth kinetics, tumors were measured by caliper and volume was calculated using the formula: l x w^2 x 0.52. For **in vivo** lytic induction, groups of 3 mice with tumor volumes of 50-150mm^3^ were treated with intraperitoneal injections for 4 days with 50mg/kg SAHA. On day 5, mice were sacrificed and tumors were snap frozen in LN2 for RNA extraction or in OCT for frozen section preparation. All animal studies were conducted under an IACUC approved protocol.

**Cell lines.** UM-PEL-1 cells (UM-PEL-1c) collected from mice were cultured in RPMI 1640 medium (Mediatech) supplemented with 10% fetal bovine serum (Mediatech) and penicillin/streptomycin (Gibco BRL). The BC1 cell line (KSHV+/EBV+), derived from HIV+ patient (Cesarman et al., 1995b) and BC3 cell line (KSHV+/EBV-), derived from an HIV-negative patient (Arvanitakis et al., 1996) were also cultured in the supplemented RPMI 1640 medium. HEK293 cells were cultured in DMEM medium (Mediatech) supplemented with 10% fetal bovine serum and penicillin/streptomycin.
**Western blot analysis and CD30 cell surface staining.** Whole-cell extracts, prepared by lysing 2x10^6 cells were immunoblotted with specified antibodies as described previously (Jiang et al., 2010). For cell-surface CD30-staining, 0.1x10^6 UM-PEL-1 cells obtained from peritoneal effusions of mice were washed with phosphate-buffered saline (PBS), resuspended in cold staining buffer [Hanks balanced salt solution with 2% FBS, and 1µg/ml blocker (BD Bioscience) and incubated for 15 min. FITC conjugated anti-CD30 antibody or isotype control were added for 30min followed by 3 washes and resuspended in cold staining buffer. Cells were analyzed on a BD LSR Analyzer (BD Biosciences).

**Flow Cytometry of dissociated tumors.** Tumors were excised, minced and further dissociated at 37°C with collagenase IV (Worthington Biochemicals) in DMEM, 0.5% bovine serum albumin, 1X pen/strep/fungi, and 2µL/mL DNAse (Ambion) by shaking at 180rpm for 30-60 min. Dissociation was complete after final trituration using a 5mL pipette and then filtering the material through 70µM and then 40µM filters. Single cells were stained with anti-CD31 (BD Biosciences) or an isotype control antibody for 30 min at 4°C. After washing a secondary APC-conjugated antibody was added for 30 min incubation at 4°C. Cells were then washed with PBS and fixed with Cytofix fixation buffer (BD Biosciences). Flow cytometric analysis was performed on a Becton-Dickinson LSR analyzer (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.) software.
Proliferation, cell cycle and apoptosis. Proliferation was assessed with CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega) following manufacturer’s instructions. For cell cycle studies, 1x10^6 UM-PEL-1c cells were incubated for 24h with PBS, Btz, SAHA or Btz/SAHA at indicated concentrations, collected, washed with PBS and fixed with 70% ethanol at 4°C. Cells were then washed with PBS and incubated with PI (50µg/ml) and 0.1mg/ml RNAse (Invitrogen) for 30-45 min. Cell cycle analysis was performed on a BD LSR analyzer (BD Biosciences). Apoptosis studies were performed using YO-PRO-1 and PI as reported previously (Sarosiek et al., 2010b).

In vivo PEL tumor studies. All animal studies were conducted according to an approved IACUC protocol. UM-PEL-1 cells (25×10^6) isolated from ascites of UM-PEL-1 tumor bearing mice were resuspended in 200µL ascites fluid and injected intraperitoneally (i.p.) into NOD/SCID mice. On day 3, mice were randomly assigned to DMSO (50μl), Btz (0.3 mg/kg; twice a week), SAHA (60 mg/kg; daily), or Btz/SAHA treatment groups treated i.p. for 3 weeks. Mice were monitored daily and sacrificed when moribund or exhibiting signs of discomfort.

NF-κB electrophoretic mobility shift assay (EMSA). UM-PEL-1 cells (25 × 10^6) isolated from ascites of tumor bearing mice were resuspended in 200 µL ascites fluid and injected i.p. into NOD/SCID mice. At day 7 post tumor cells injection, mice were treated i.p. with DMSO (50μl), Btz (0.3mg/kg), SAHA (60mg/kg), Btz/SAHA (0.3mg/kg/60mg/kg) and sacrificed 24h after treatment. Cells harvested from the
peritoneal effusions were used to prepare nuclear extracts for EMSA. NF-κB EMSA was performed as previously reported (Sarosiek et al., 2010b).

**TUNEL assay.** TUNEL assay was performed as per manufacturer’s instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche). Images were acquired using Zeiss Axiovision 4.8.2 with a Hamamatsu ORCA-R2 CCD camera and Zeiss Axiovert 200M inverted fluorescence microscope.

**Virion production assays and viral DNA quantification.** For testing effects on virion production *in vitro*, we used an infectivity read out. Briefly, 1x10^6 UM-PEL-1c cells were stimulated for 72h with 10nM Btz, 0.75µM SAHA or 10nM Btz/0.75µM SAHA. Supernatant was harvested, spun at 3000rpm for 10min and filtered through a 0.45uM filter (ThermoScientific). Polybrene (8µg/mL) was added to 1x10^6 uninfected HEK293 cells for 30min prior to the addition of the cleared supernatant. At 48h after infection, cells were fixed and immunostained for LANA. LANA positive cells in 10 random fields at 20x magnification were counted and averaged. For data presentation, one LANA+ cell was considered as one infectious unit.

For quantifying *in vivo* virion production, ascites fluid was removed and then spun at 3,200 x g for 15min at 4°C to remove cells and cellular debris. Polyethylene glycol (Abcam) was added to the cleared supernatant and incubated overnight at 4°C to precipitate the virus as per manufacturer’s instructions. Precipitated virus was pelleted and DNase treated to eliminate non encapsidated viral DNA. Encapsidated viral DNA was then isolated using the DNeasy Blood and Tissue kit (Qiagen), with the modification
of adding 3µg of carrier DNA as per manufacturer recommendations for samples with less than 5ng DNA (<10,000 copies), and quantified with qRT-PCR using primers to viral ORF73/LANA. To quantify, a KSHV DNA standard curve was generated by diluting a bacterial artificial chromosome containing the full KSHV genome (Zhou et al., 2002b) and amplifying the same region of LANA as was used for the experimental samples. Ct values for the BAC36 were converted into DNA copy number using the derivation of DNA mass formula: where \( n = \) DNA size in base pairs, \( m = \) mass, Avogadro’s number \( = 6.023 \times 10^{23} \) molecules/mole, average MW of a double-stranded DNA molecule \( = 660\) g/mole, as per ABI protocol. An ABI PRIZM 7300 sequence detector was used to run the reactions and amplification plots were analyzed using the SDS software. Non-template controls were run to verify absence of reagent contamination. All error bars represent SEM. To quantify intracellular KSHV DNA, total cellular DNA was harvested with the AllPrep RNA, DNA, and protein kit (Qiagen) and quantified using a Nanodrop spectrophotometer. 10ng/well was loaded in quintuplicate and viral DNA copy number was quantified as described above.

**Microarray analysis.** Using the Affymetrix MoGeneST_1.0 array, we compared gene expression levels of the mECK\textsuperscript{null}.rK133 cells and mECrK cells to data from 40 microarray samples representing 16 different mouse cell and/or tissue types also run on MoGeneST_1.0 array collected from GEO datasets. GEO accession numbers are as follows: Aortic Endothelial cells (AEC: GSM686077, GSM686078), Bone marrow derived mesenchymal stem cells (BM_MSC: GSM757750), N-Cadherin expressing endothelial cells (EC_NCadh: GSM859433, GSM859434), VE-Cadherin expressing
endothelial cells (EC_VECadh: GSM859435, GSM859436), VE+N-Cadherin expressing endothelial cells (EC_VE+NCadh: GSM859437, GSM859438), total bone marrow (BM: GSM821069, GSM821070, GSM821071), undifferentiated embryonic stem cells (undiffESC: GSM839393, GSM839394, GSM839395), fetal liver erythroblasts (FLE: GSM842148, GSM842149, GSM842150), bone marrow-derived macrophages (BM_Macro: GSM787745, GSM787746, GSM787747), peripheral macrophages (Macro: GSM466436, GSM466437, GSM466438), hematopoietic stem cells (HSC: GSM862182, GSM862184), leukemia stem cells (LSC: GSM862186, GSM862188), glomerular endothelial cells (GEC: GSM532931, GSM532932, GSM532933) and subcutaneous tissue (SubQ: GSM905121, GSM905122, GSM905123). Each sample was processed through QA/QC on GeneSpring 12 software (Agilent technologies). A batch bias was noted since the data was derived by many groups. Each batch was individually Quantile normalized and log2 transformed to median of all samples within the batch. The combined data was corrected for batch bias using the using the ComBat (Johnson et al., 2007) module on R/Bioconductor package. In order to clearly demarcate the clusters of samples, 7549 probes with most variation (SD ≥ 1.5) among all the 40 samples were subset. A hierarchical clustering was performed on the subset using GeneSpring. For clustering the Pearson centered similarity measure method with a centroid linkage rule was used.
References


