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The Role of NF-kappaB in Kaposi's Sarcoma-Associated Herpesvirus Pathogenesis

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The Role of NFkappa-B in Kaposi's Sarcoma-Associated Herpesvirus Pathogenesis

by

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DISSERTATION

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To my parents

For their enduring love, support and encouragement

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ABSTRACT

Kaposi's Sarcoma-ssociated Herpesvirus (KSHV), is the most recently discovered human tumor virus, as well as the causative agent of Kaposi's Sarcoma (KS), the most common neoplasm in AIDS patients. As a human pathogen, KSHV has evolved to interact with many cellular signaling pathways, especially those involved in control of infection and inflammation. Primary among these pathways is that which leads to the activation of the transcription factor, Nuclear Factor Kappa B (NFκB). NFκB is a master regulator of the pro-inflammatory transcription program and an integral part of the human body's system of innate immunity. This dissertation explores the interactions of KSHV with the NFκB pathway in terms of both the pathology of the KS lesion, contributing to both the inflammatory microenvironment and the presence of spindle cells, as well as the role played by NFκB in the delicate balance between latency and lytic replication.

KS is an inflammatory lesion induced by infection of endothelial cells with the KSHV. Infected endothelial cells assume an elongated (spindle) shape that is one of the histologic signatures of KS. We found that this spindling phenotype can be attributed to expression of a single viral protein known as vFLIP, a known activator of NFκB. vFLIP expression in spindle cells also induces production of a variety of proinflammatory cytokines and cell surface adhesion proteins that likely contribute to the inflammatory component of KS lesions.

Like all herpesviruses, KSHV can produce either latent or lytic infection. We have examined in detail the effects of NFκB activation and inhibition on KSHV replication. In accord with earlier work, we find that inhibition of NFκB signaling in KSHV infected lymphoma cells is associated with enhanced lytic reactivation of KSHV. Similarly, in KSHV infection of primary endothelial cells, but no other tested cell line, inhibition of NF-

κB signaling leads to an increase in lytic replication. However, counter-intuitively, NFκB signaling is strongly and consistently activated in lytically infected cells of all lineages tested. This indicates that the relationship of NFκB activation to latency and lytic reactivation is not uniform, but is dependent on the cellular context.

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Introduction

Kaposi's Sarcoma Associated Herpesvirus

Kaposi's Sarcoma Associated Herpesvirus (KSHV), also called Human Herpesvirus 8 (HHV-8), is the most recently discovered human herpesvirus. Like Epstein-Barr Virus, KSHV is a member of the lymphotropic gammaherpesvirus family and is the first human rhadinovirus, a sub-family that includes Herpesvirus Saimiri (HSV) and Rhesus monkey rhadinovirus (RRV). KSHV was discovered in 1994 using representational difference analysis from a KS lesion (14) and has since been implicated as the causative agent of Kaposi's Sarcoma, the most common neoplasm in untreated AIDS patients, as well as the rare B cell lymphoma Primary Effusion Lymphoma (PEL), and a variant of the lymphoprofilferative disease Multicentric Castleman's Disease (MCD). Seroprevelance of KSHV infection varies widely and correlates with geographic location, ranging from 3% in Northern Europe and North America to 50-60% in parts of sub-Saharan Africa.

Infection of B cells by KSHV is associated with the development of rare proliferative diseases. The plasma cell variant of MCD has been linked to KSHV infection and is characterized as a lymphoproliferative condition associated with expanded germinal centers and increased B cell proliferation. More common than MCD, though still very rare, PELs present as malignant effusions in serosal cavities, not associated with a solid tumor mass. PELs are clonal and bear few markers of normal B cells, but have been shown to have rearranged immunoglobulin loci (2). PEL cells are uniformly infected with KSHV, grow readily in culture and have been shown to form tumors in NUDE mice (65).

KS is the most significant disease associated with KSHV infection, and was first described in the early 19th century by Moritz Kaposi (48). Originally described as red nodules and plaques appearing primarily on the skin of elderly Mediterranean men, the twentieth century brought to light several more manifestations of the disease. Since the original identification of KS, it's occurrence has been described in four distinct forms: Classic-KS, a rare indolent form found primarily in elderly men of Mediterranean descent; AIDS-associated-KS, a more aggressive form found in untreated AIDS patients; Endemic-KS, found primarily in sub-Saharan Africa; and Iatrogenic-KS, found associated with immunosuppressive therapies such as in transplant patients.

Since the discovery of KSHV, epidemiological studies have drawn clear links between KSHV infection and the development of KS. Significantly, KSHV infection was found to be less common in populations at low risk for development of KS, while populations at high risk for KS have shown increased rates of infection, and among those infected with KSHV, detection of viral DNA in the blood is predictive of development of KS (15, 33, 34, 50, 54, 63, 72, 74, 91, 92). Additionally, and perhaps most convincingly, in a long term study of gay men in San Francisco, KSHV infection was shown to precede KS development and to be predictive of increased risk of tumor formation (59). KSHV infection, however, appears to be necessary, but not sufficient, for KS development. The need for the involvement of an additional cofactor(s) is clear when considering that rates of KS incidence are low even among KSHV-infected patients (47). In the case of AIDS-associated KS, it is clear that HIV infection serves as a cofactor for KS development. Considered to be a rare disease in North America and Europe throughout most of history, the AIDS epidemic produced a spike in KS incidence in these parts of the world as well as in sub-Saharan Africa. Furthermore, the dramatic reduction in KS incidence that has followed the widespread treatment of AIDS patients

with highly active retroviral therapy (HAART) illustrates the role of HIV as a cofactor for KS development in this population (35, 38, 45).

Despite the “sarcoma” label, KS does not meet the criteria of a traditional cancer. Unlike most tumors, which are composed of a single cell type, the KS lesion is complicated, consisting of several different cell types. KS lesions can be multicentric and are found primarily on the skin of the lower extremities. However, in more advanced cases, and particularly in the AIDS-associated form, lesions can be found on internal organs (especially the lungs and gut), necessitating treatment with chemo- or radiation therapy. Histologically, the KS lesion is characterized by abnormal angiogenesis, leading to the extravasation of erythrocytes, giving the lesion its red color; inflammation, in the form of production of chemokines and cytokines as well as infiltration of inflammatory cells; and proliferating spindle cells (27).

The spindle cells, which are so named due to their distinctive elongated shape, are the principal proliferating cells within KS lesions. However, they lack the characteristics of a classical cancer cell, showing neither genetic instability nor an ability to grow in soft agar nor the capacity to form tumors in NUDE mice. Rather, they are considered the driving force of KS due to their recruitment of vascular and inflammatory elements to the lesion through the production of pro-inflammatory and angiogenic factors (27, 76). Infiltration of immune cells is evident in KS lesions and is thought to be an important part of KS pathogenesis and spindle cell survival, since cultured spindle cells require the secreted products of activated T cells for growth (27, 66). Spindle cells are believed to be of endothelial origin based on their expression of a number of surface markers including CD31, CD34, CD36, UEA1 lectin and EN4 (9). However, whether the originally infected endothelial cell is lymphatic or vascular in origin is still unclear, as

reprogramming of endothelial marker expression upon KSHV infection *in vitro* has been well documented (11, 46, 88). Within the KS lesion, spindle cells have been found to be almost exclusively latently infected with only a small minority (<3% of spindle cells) showing evidence of lytic viral replication (79).

As a canonical herpesvirus, KSHV displays two modes of infection, latent and lytic. The latent state of replication involves the persistence of the viral genome in the cell in an episomal form tethered to the host chromosome. During latency, very few viral genes are expressed. The expression of viral genes associated with KSHV latency are transcribed from two genetic loci. The first latency associated transcript includes the latency associated nuclear antigen (LANA), viral FLICE-inhibitory protein (vFLIP) and a viral homolog of cyclin D (vCyclin). LANA has been shown to be involved both in the maintenance of the viral episome in infected cells by tethering it to host chromosomes (3, 22, 70), as well as modulation of cell growth and survival by interacting with p53, Rb and GSK3β (30, 31, 71). The vCyclin protein functions similar to the human cyclin D, binding and activating cdk6 to induce cell proliferation (36, 55). Finally, vFLIP is a death effector domain-containing protein whose primary function is the potent activation of NFκB (16, 60, 80, 81) and whose expression in crucial for the survival of PEL cells (40). The K12 or Kaposin locus is the second latency associated transcript and gives rise a single messenger RNA encoding proteins termed kaposins A, B and C, none of which bear any similarity to human genes. Kaposin A is a transmembrane protein with weak transforming properties in Rat fibroblast cells (65), while kaposin B has been implicated in the stabilization of AU-rich element containing messages (61), most of which encode for cytokines and growth factors, a function which undoubtedly contributes to the inflammatory phenotype of the KS lesion. Infection of human endothelial cells with KSHV *in vitro* is a widely used model system for studying KS that faithfully reproduces

the predominantly latent KSHV infection (20, 25, 29, 39, 53, 64, 75). In some cases, upon infection with KSHV, these endothelial cells undergo morphological changes that approximate spindle cells, as well as producing inflammatory and angiogenic cytokines that are found in the KS lesion *in vivo* (20, 32, 39).

While the default pathway for KSHV infection appears to be latency, a small fraction of infected cells show evidence of lytic gene expression. The lytic state of infection involves the expression of the vast majority of the viral genes and culminates in the production of more KSHV virions, lysis of the cell and spread of infection. Expression of lytic genes takes place in a regulated cascade. The first set of genes expressed upon lytic reactivation, termed the immediate early genes, are usually regulatory proteins affecting viral and cellular gene expression. These are followed by the delayed early lytic genes which prepare the cell for viral DNA replication and virion production, and finally the late genes which are largely structural proteins and whose expression is dependent on viral DNA synthesis. Although lytic replication is seen in only a small fraction of cells in KS, the expression of these genes in a small subset of cells is thought to be important for the development of KS involving both autocrine and paracrine signaling (87) . Additionally, because the latent episome itself is unstable and readily lost from cells (39), these lytically infected cells are likely to contribute to the maintenance of a population of KSHV infected cells.

The switch from latent to lytic replication remains the subject of much interest in the field. Exogenous expression of the Replication and Transcription Activator (RTA) protein, an immediate early protein, is sufficient to initiate the lytic gene program (37, 58, 82). Additionally, over-expression of a dominant negative form of RTA (57), or null mutations in the RTA gene (94) are sufficient to block all forms of lytic reactivation.

These lines of evidence suggest that RTA plays a pivotal role in regulating the switch from latency to lytic replication. Experimentation *in vitro* has shown that RTA expression and subsequent lytic reactivation can be induced by exposure to certain stimuli such as phorbol esters or histone deacetylase inhibitors. Details surrounding the initiation of RTA expression *in vivo* remain largely unknown, although inflammatory cytokines, hypoxia and stress response mediators such as epinephrine, have all been suggested to cause lytic reactivation (12, 13, 42, 62, 89). Recent work suggests that the NFκB transcription factor may play a role in regulating this process by repressing lytic gene expression when NFκB is activated (7, 52, 78).

NFκB

Nuclear factor- kappa B (NFκB) is a family of highly conserved transcription factors that have been the focus of much investigative and therapeutic interest as they lie at an intersection of microbiology, immunology and cancer biology. NFκB family members play a key role in immunity, regulating expression of cytokines and growth factors; as well as in the delicate balance of cell death and proliferation, serving as a significant point of regulation in cancer progression and viral and microbial pathogenesis.

The term NFκB refers to five proteins: RelA (p65), RelB, c-Rel, p50/p105 and p52/p100, all of which share a common N-terminal REL-homology domain (RHD) and are found in the cell as hetero- and homodimer pairs. These common RHDs mediate DNA binding and dimerization of the proteins, while their divergent C-terminal ends are responsible for transcriptional activation. p50 and p52 can be sub-classified from the

other three, as they require additional processing for mature protein production. In the case of p50/p150, p50 is generated cotranslationally by proteaome-mediated processing concurrent with p150 translation, while p52 is generated via posttranslational cleavage of p100. Additionally, these two family members lack transactivation domains at their C termini and therefore serve as functional inhibitors when associated as homodimers. In resting cells, NF-κB transcription factors primarily reside in the cytoplasm associated with IκB proteins.

The classical NFκB pathway is mediated through the p65/p50 heterodimer (from here on referred to simply as NFκB) and will be the primary form discussed. Classical NFκB signaling is initiated upon serine phosphorylation of IκBα at residues 32 and 36 (in complex with NFκB) by the IκBα-kinase (IKK). IκBα is subsequently polyubiquitylated and degraded by the 26S proteasome (8, 26). The newly liberated NF-κB heterodimer rapidly translocates into the nucleus upon the exposure due to unmasking of its nuclear localization signal (86), otherwise occluded by IκB. An additional level of regulation exists at this point, as IκBα is an NFκB-responsive gene and therefore serves to create a negative feedback loop to dampen the response (84).

IKK is a large complex comprised of three core subunits: the catalytic units IKKα/1, IKKβ/2 and the regulatory subunit IKKγ/NEMO. Activation of the complex is thought to depend on transautophosphorylation of the IKKβ subunit leading to Lys-63 ubiquitination (Ub) of NEMO (85). In contrast to Lys-48 Ub, which leads to proteasomal degradation, Lys-63 coupled Ub serves to facilitate signaling by allowing for interactions with Ub-binding proteins (19).

In addition to translocation of the NFκB heterodimer into the nucleus, a further layer of regulation via post-translational modification of p65 exists. Maximal transcriptional activation of NFκB is associated with phosphorylation of p65, allowing for recruitment of transcription cofactors (17); additionally, acetylation of NFκB plays a role in regulating its interactions with IκBα as well as transcriptional co-activators (18).

Activation of NFκB upstream of IKK is extremely diverse and differs by cell type and origin of the stimuli. NFκB is known to be activated downstream of the TNFα receptor (TNFR) superfamily, the family of Toll-like receptors, T cell receptor, B cell receptor and several others, situating it as a central regulator of immune cell signaling. Adapter proteins between these cell surface receptors and IKK vary largely. Include among them are the TNF-receptor-associated factors (TRAFs) which are involved in TNFα-induced activation of NFκB, and serve to recruit IKK kinases such as receptor interacting protein 1 (RIP-1) in proximity with the TNFR complex (4, 43).

Genes regulated by NFκB transcriptional activation are as divergent and context-dependent as their initiating stimuli. However, NFκB activation is most often described as a pro-survival or anti-apoptotic signal. This is, first and foremost, do to its regulation of a number of inhibitors of apoptosis, both death receptor-associated and mitochondrial. Among these are the c-IAP proteins, one of the first recognized NFκB regulated genes, which serve as inhibitors of caspase activation (24).

The p65/p50 canonical pathway is not the sole pathway associated with NFκB signaling. A subset of NFκB stimuli, such as CD40 and LPS, activate signaling through the non-canonical NFκB pathway. Initiation of this pathway can occur through the

activation of IKKα via the NFκB-inducing kinase (NIK). Activation of IKKα leads to ubiquitination and processing of p100 by the proteasome resulting in formation of the p52 subunit (77). Subsequently, p52/RelB heterodimers are formed, which have greater affinity for distinct transcriptional response elements as the p65/p50 heterodimers, leading to transcriptional activation of a different subset of NFκB-responsive genes (68).

Recent studies have also shown that alternative modes of activation of the canonical NFκB pathway exist. For example, genotoxic stimuli can lead to activation of IKKβ first through IKKγ activation. This involves the translocation of IKKγ into the nucleus where it is sumoylated and phosphorylated by the ataxia telangiectasia mutated (ATM) kinase, leading to the export of IKKγ from the nucleus in complex with ATM, and activation of the IKK complex (93). Additionally, cases of IKK-independent activation of the canonical NFκB pathway, such as the IKK-independent phosphorylation of IκBα upon UV light exposure or hypoxia, have also been reported (69).

NFκB and viruses

The allocation of NFκB as a master regulator of immune function and cell growth and apoptosis positions it as a prime target for viral interference, especially in the context of malignancies of viral etiology. Additionally, the association of chronic inflammation, via NFκB activation by infection, with malignant cell growth (49); and the common observation of REL gene amplification in B cell malignancies (23) draws particular attention to the NFκB pathway in the study of gammaherpesviruses.

In the context of viral infection, NFκB activation is exploited for its effects on transcription of cellular genes as well as viral genes. In fact, several viruses are know to encode NFκB binding sites within their own viral promoters. This list includes Human Immunodeficiency Virus (HIV), Cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) among others. The best characterized of these interactions is that of HIV, where NFκB activation can promote LTR driven transcription (44).

The best characterized example of viral modulation of the NFκB pathway is undoubtedly that of the Human T Cell Leukemia Virus (HTLV-1), a retrovirus whose infection is associated with adult T cell leukemia (96). The HTLV-1-encoded protein Tax is an NFκB-activating protein that has been shown to be essential for viral replication and pathogenesis, as well as being necessary and sufficient for HTLV-1-mediated transformation of T cells (1, 90). Moreover, mutants of Tax that are unable to activate NFκB no longer immortalize T cells (73). Tax has been shown to shuttle between the nucleus and cytoplasm of HTLV-1 infected cells, and is associated with activation of the NEMO subunit of IKK, phosphorylation and degradation of IκBα, and sustained induction of NFκB signaling (83, 95).

Gammaherpesviruses have their share of NFκ-B interacting proteins. Epstein Barr Virus (EBV) is a γ_1-herpesvirus, which like KSHV, shows a natural tropism for B cells. EBV infection of primary B cells *in vitro* leads to efficient transformation of the cells, a process which is dependent on the expression of the viral protein LMP-1 (21). LMP-1 expression has been associated with NFκB activation and has been suggested to act with many different upstream members of the pathway to effect full activation (6). This activation of NFκB has been shown to be essential for the survival of the EBV-

transformed B cells (10).

Similar to EBV, KSHV-infected lymphoma (Primary Effusion Lymphoma, PEL) cells show persistent NFκB activation and are dependent on this signaling for survival (40). KSHV infection of PEL cells is almost exclusively latent, a state in which only a small subset of the viral genes are expressed. Of these few latent genes, vFLIP has been shown to be a potent activator of NFκB via TRAF 2 and 3 and the NEMO subunit of IKK (28, 41, 56). Additionally, KSHV encodes three other NFκB-activating proteins in the lytic cycle. These include a viral G-protein coupled receptor (vGPCR), the expression of which has been associated with increased expression of several NFκB target genes (67); K15, a protein similar to EBV LMP-1 which has been shown to interact with TRAF 2 (5); and K1, an ITAM-containing membrane-associated signaling molecule.

In recent years, several reports have described complicated interactions between the NFκB signaling pathway and KSHV infection in the context of PEL cell lines (7, 40, 51, 52, 78). These studies have shown increased lytic gene induction upon chemical inhibition of NFκB (7), enhanced apoptosis upon chemical inhibition of NFκB or knockdown of vFLIP (40, 51), and even production of defective KSHV particles upon genetic inhibition of NFκB in TPA-induced PEL cells (78). Additionally, inhibition of transactivation of lytic gene promoters by RTA, the KSHV lytic switch protein, was shown in the context of overexpression of the p65 subunit of NFκB (7). Taken together, these accounts have led some to suggest a model of suppression of lytic gene expression and function by NFκB activation in PEL cells.

The following chapters will address the interactions of KSHV with the NFκB pathway as it relates to the infection of primary human endothelial cells *in vitro*. The second chapter explores the contribution of vFLIP-mediated NFκB activation in the development of the unique histopathology of KS, as modeled *in vitro*. The third chapter will address the interaction between NFκB activation and the balance between KSHV latency and lytic growth in cells of different origins. Finally, the fourth chapter will describe our use of gene expression profiling and bioinformatics assays to better characterize the NFκB-dependent development of the spindle cell morphology in vFLIP expressing human endothelial cells.

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CHAPTER 2:

Activation of NFκB by the latent vFLIP gene of KSHV is required for the spindle shape of virus-infected endothelial cells and contributes to their pro-inflammatory phenotype

ABSTRACT

Kaposi's sarcoma (KS) is an inflammatory angioproliferative lesion induced by infection of endothelial cells with the KS-associated herpesvirus (KSHV). Infected endothelial cells assume an elongated (spindle) shape that is one of the histologic signatures of KS. In vitro, latent viral infection of primary endothelial cells (but no other cell type) strikingly recapitulates these morphologic findings. Here we report that the spindling phenotype involves major rearrangement of the actin cytoskeleton and can be attributed to expression of a single viral protein known as vFLIP, a known activator of NFκB. Consistent with this, inhibition of NFκB activation blocks vFLIP-induced spindling in cultured endothelial cells. vFLIP expression in spindle cells also induces production of a variety of proinflammatory cytokines and cell surface adhesion proteins that likely contribute to the inflammatory component of KS lesions.

INTRODUCTION

Kaposi's sarcoma (KS) is a complex angioproliferative lesion that is the most common neoplasm in patients with untreated AIDS, though it also exists in an HIV-independent form. All KS lesions have three histological components – proliferation, inflammation and neoangiogenesis. The principal proliferating element in KS is the so-called spindle cell, so named because of its distinctive, elongated, spindle-like shape. Spindle cells have long been thought to be the driving force of KS, responsible for the recruitment of the inflammatory and angiogenic components of the lesion; consistent with this, cultured spindle cells produce a number of proinflammatory and angiogenic factors(12, 34). The inflammatory infiltrate is also felt to be an important part of KS pathogenesis, since cultured spindle cells require the secreted products of activated T cells for growth(12, 28). The histogenesis of spindle cells has long been debated. Based on the expression of markers such as CD31, CD34, CD36, UEA1 lectin and EN4 by spindle cells, it is generally believed that the cells are most likely of endothelial origin (7). However, the exact endothelial lineage from which spindle cells are derived remains unclear. A number of lines of evidence favor the notion that KS is derived from cells of lymphatic rather than vascular endothelial origin. For instance, KS tumors are never observed in tissues (e.g. brain) that are devoid of lymphatics (47). In addition, KS cells consistently stain for markers of lymphatic endothelium, e.g. VEGF-R3, LYVE-1 and podoplanin (21, 36, 46). However, the pathogenetic significance of the latter observations has been rendered ambiguous by recent findings that KSHV infection of lymphatic or vascular endothelial cells can reprogram their expression of endothelial markers(17, 45).

Central to the pathogenesis of KS is infection by the KS-associated herpesvirus, KSHV (also called human herpesvirus 8). Like all herpesviruses, KSHV has two modes

of infection, latent and lytic. In KS tumors, KSHV selectively infects the spindle cells (5), most of which are latently infected (37); only a small subpopulation of spindle cells support lytic KSHV growth. Latent gene expression is therefore considered central to the development of KS. While many established cells in culture can be latently infected by KSHV, most such cells display no phenotype (2). However, recent studies from several groups show that primary vascular endothelial cells from dermal microvasculature (9) or umbilical vein (15, 30, 42) can undergo conversion from cuboidal to spindle-like morphology upon infection by KSHV *in vitro*, strongly reminiscent of KS tumor cells.

Here we show that such morphologic changes are due to the cell-autonomous action of latent viral gene products, and occur in both lymphatic and vascular endothelium. By expressing the known KSHV latency genes individually in primary endothelial cells, we show that this morphologic change can be effected by a single viral gene encoding the vFLIP protein. This protein has previously been shown to be an anti-apoptotic factor (10, 16, 38) that acts by upregulating NFκB activity (1, 13, 25). We show that NFκB induction by vFLIP is required for spindling, and in addition results in upregulation of numerous pro-inflammatory cytokines by endothelial cells. Thus, in addition to its widely recognized anti-apoptotic functions, vFLIP also induces the development of cell-autonomous morphologic changes and contributes to the inflammatory microenvironment – two features that have long been recognized as signatures of Kaposi's sarcoma.

MATERIALS AND METHODS

Cells and KSHV infection

HUVECs were purchased from Clonetics and cultured in EGM-2 media supplemented with the microvascular supplement pack (Clonetics). LECs and BECs were isolated and cultured as previously described (32). BCBL-1 cells were carried in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamine and β-mercaptoethanol.

KSHV was concentrated from supernatants of induced BCBL-1 cells as previously described (2, 23). KSHV infections were done in media containing 2 μg/ml polybrene and incubated with cells for two hours after which cells were rinsed and media was added back.

Retrovirus production and infection

Retroviruses were produced using the amphotropic Phoenix packaging cell line transfected with the Moloney Murine Leukemia Virus based vector pBMN(IRES Puromycin resistance). Phoenix cells were transfected using FuGENE 6 (Roche) according to the manufacturer's specifications. 36 hours after transfection supernatants were collected and concentrated at 5,000rpm for 16hrs. Concentrated retroviruses were resuspended in EGM-2 media with 6 μg/ml polybrene and filtered through a 0.2μm filter. Concentrated retrovirus was diluted in EGM-2 media with 6 μg/ml polybrene and applied to cells. These cultures were spun at 2,000rpm for 1.5 hours after which virus-containing media was removed and regular culture media was added back. In cases were selection

was employed, 24 hours after transduction media containing the selective agent was added to cells at the stated concentration.

Immunofluorescence, cytokine array, ELISA and flow cytometric analysis

Immunofluorescence assays were performed as previously described using either polyclonal antibody to LANA (A. Polson and D.G., unpublished data) or monoclonal antibody to K8.1 (gift L.Wu and B. Forghani) or anti-p65 (Santa Cruz Biotechnology)(2). Secondary antibodies anti-rabbit and anti-mouse FITC and anti-mouse rhodamine (Santa Cruz Biotechnology) were used at 1:300. FITC-coupled phalloidin (Sigma) was used at 1:200.

Human Cytokine Antibody Array V was purchased from RayBiotech and performed according to manufacturer's instructions. Arrays were performed with EGM-2 basal media + 2% FBS conditioned by HUVECs for 24hrs. ELISAs were performed according to the manufacturer's instructions (BD Bioscience) using media conditioned for 24hrs.

For flow cytometry, HUVECs were incubated with anti-VCAM-1 antibody at a 1:100 dilution (BD PharMingen) for 30 minutes on ice. APC conjugated goat anti-mouse secondary (Caltag) was used at 1:250 dilution also for 30 minutes on ice. Analysis was done using a Becton Dickinson FACScalibur.

NFκB EMSA

Nuclear enriched lysates were made from cells by incubation with hypotonic buffer (20mM HEPES pH7.8, 5mM KCl, 1.5mM $MgCl_2$, 1mM DTT and protease inhibitors), followed by pelleting and disruption of nuclei by incubation in high salt buffer (0.4M KCl, 50mM HEPES pH7.9, 0.1% NP40, 0.5μM EDTA, 10% glycerol and protease inhibitors). 5μg of lysate was incubated with ^{32}P-labled oligonucleotide encoding the

NFκB consensus sequence (Santa Cruz Biotechnology) and dI:dC DNA (Sigma) in binding buffer (20μM HEPES pH7.9, 50mM KCl, 10% glycerol, 1mM EDTA, 1mM $MgCl_2$, 1mM DTT). In cases of competition, unlabeled wildtype or mutant oligonucleotides (Santa Cruz Biotechnology) were included in 250-fold excess. For supershifting, 1.5μl of anti-p65 (Santa Cruz Biotechnology) were included in the binding reaction. Binding reactions were incubated at room temperature for 30 minutes without the labeled probe and an additional 30 minutes after addition of the probe. Complexes were resolved in a 1x TBE, 4% acrylamide gel.

Luciferase Assays

HUVECs were transduced with retroviruses encoding either vFLIP or empty vector and subjected to selection. Once the vFLIP expressing cells had fully developed into spindle cells, the cells were transfected in OptiMem media (Gibco) with 0.6μg NF-κB-luciferase vector and 0.4μg β-galactosidase encoding vector using 0.25μl Jurkat transfection reagent (Mirus). The DNA-transfection mixture was incubated on the cells for 4 hours after which the cells were rinsed and regular media was added back. After 48 hours, luciferase and β-galactosidase assays were carried out according to the manufacturer's specifications (Promega).

Inhibition of NFκB

HUVECs were treated with 4μM Bay 11-7082 (Calbiochem) in DMSO for 2 hours before being transduced with vFLIP encoding retrovirus as described above. After transduction, media containing Bay 11-7082 was added back to the cells.

RESULTS

The spindle cell phenotype and its viral etiology.

To examine the effects of KSHV on different populations of endothelial cells, we employed primary cultures of human umbilical vein endothelial cells (HUVEC), lymphatic endothelial cells (LEC) and blood endothelial cells (BEC) established by previously published methods (32). Cells were infected at high multiplicity by KSHV virions concentrated from the medium of BCBL-1 cells, a B-lymphoma line previously shown to produce infectious KSHV virions after TPA stimulation (33). In all three lines, virus infection and spread results in a monolayer culture which, after a number of days, is virtually entirely latently infected, as judged by staining for the viral Latency-Associated Nuclear Antigen (LANA) (Fig 1a-c, green); at this point, few cells (<2-5%) stain for the lytic marker, K8.1 (Fig 1 a-c, red). Remarkably, all three lines display dramatic elongation to the spindle cell shape characteristic of KS tumor cells (Fig. 1 d-f). Phalloidin staining (Fig1, g,h,i) revealed that the elongated cells had undergone a dramatic rearrangement of the actin cytoskeleton, with the prominent formation of parallel arrays of actin cables. Interestingly, we did not observe immortalization of these cells, which went on to sensence with kinetics similar to uninfected cells (data not shown).

Since latently-infected cells are known to produce a number of cytokines and other paracrine factors (12), we considered the possibility that spindling might be due to extracelluar signaling molecules produced in this fashion. Accordingly, we tested the effect of conditioned medium from KSHV-positive LECs and BECs on uninfected primary LEC or BEC cultures; no spindling was observed in such conditions (data not shown). While we cannot rule out the possibility that paracrine signaling might be a cofactor for spindling, it is clearly not sufficient to induce this morphology. The fact that spindle cells

maintain their morphology even at low cell density (not shown) is more consistent with the process being largely or entirely cell-autonomous.

To determine what viral gene(s) are responsible for this phenotype, we tested individual KSHV latency genes for their ability to recapitulate this phenotype upon introduction into uninfected cells; HUVEC cells were chosen for this purpose because they are easier to prepare and maintain. Since these cells are poorly transfectable, we employed a retroviral vector to deliver each of the known KSHV latency genes: LANA, v-cyclin, vFLIP, kaposin A (ORF K12) and kaposins B and C (this vector may also encode small quantities of kaposin A). Early passage HUVECs were transduced at high MOI with the individual retroviruses and subjected to selection with puromycin (0.5μg/ml). After 3 days, cells were scored for changes in their morphology. As shown in Figure 2A, expression of LANA, v-cyclin, the K12 locus and kaposin A alone failed to induce morphologic changes in the selected cell lines. However, a different result was obtained with the vFLIP vector – cells expressing this protein displayed dramatic elongation and spindling virtually identical to that observed by authentic KSHV latency. This change was also observed in LECs and BECs infected with the same vector (Fig 2B), affirming that the results apply to those endothelial lineages as well.

Activation of NFκB correlates with spindle cell development

Several reports have established that vFLIP is a potent activator of the transcription factor NFκB (1, 26, 38-40) vFLIP has been shown to interact with several upstream components of the NFκB activation pathway, including TRAF 1and 2, RIP, NIK and IKK (22,23), resulting in activation of IKK. This leads to phosphorylation and subsequent proteasome-dependent degradation of IκB, an inhibitory subunit that binds cytosolic NFκB and inhibits its nuclear import. This degradation releases active NFκB,

allowing it to translocate into the nucleus and activate the expression of its target genes. Based on these reports, we investigated whether development of spindle cells, as driven by vFLIP, correlated with increased activation of NFκB.

First, we affirmed that in primary endothelium stably expressing v-FLIP that NFκB was indeed activated. Spindled v-FLIP-positive HUVEC transductants were transfected with an NFκB-dependent reporter construct; in parallel, HUVECs transduced with an empty retrovirus were similarly transfected, and luciferase activity measured in both cultures. Fig 3A (bars 1, 2) shows that, as expected, vFLIP-expression resulted in substantial induction of NFκB activity. This was associated with enhanced nuclear translocation of p65 and increased binding of NFκB to oligonucleotides bearing NFκB recognition sites in nuclear extracts (C.G., unpublished results)

KSHV vFLIP is a member of a small family of viral FLIP proteins, all of which share partially homologous DEDs (death effector domains) (43). Classical viral FLIPs, like the E8 protein of equine herpesvirus, antagonize apoptosis by impairing recruitment of caspase 8 to Fas and other death receptors, but do not activate NFκB (3, 8, 43). Transfection of 293 cells with an E8 expression vector did not activate LUC expression from an NFκB-dependent reporter gene (Fig3A, bars 3-5). Importantly, transduction of HUVEC cells with an E8-expressing retrovirus did not lead to cell shape changes, under conditions in which transduction of KSHV v-FLIP led to efficient spindling (Fig 3B). Thus, endothelial spindling is closely correlated with NFκB activation.

vFLIP-expressing spindle cells show markers of inflammatory activation

In addition to its angioproliferative features, KS is also an inflammatory lesion. In early KS, inflammatory cells are usually a prominent feature, and inflammatory cells are a potential source of both proliferative and angiogenic signals (12). NFκB is a central

player in orchestrating the inflammatory response and its dysregulated expression has been linked to several inflammatory disease states (41). NFκB-regulated genes include markers of endothelial cell activation as well as several inflammatory cytokines and chemokines, many of these may play roles in KS histogenesis. Based on the potent activation of NFκB in vFLIP-expressing spindle cells, we asked if these cells showed evidence of upregulation of endogenous, proinflammatory NFκB-regulated genes. Using a cytokine array bearing numerous anti-cytokine antibodies, supernatant from fully spindled HUVECs expressing vFLIP were assayed for the presence of 79 cytokines (Fig 3C). Supernatant from HUVECs expressing only vFLIP was found to have increased amounts of IL-6 (in agreement with earlier data (1)), IL-8, GRO, RANTES, GCP2 and MIP3α as compared to supernatant from HUVECs expressing empty vector. All of these cytokines are known to be regulated by NFκB (24, 27, 35). ELISAs performed on supernatant from spindled vFLIP-expressing HUVECs and confirmed elevated amounts of the pro-inflammatory cytokines IL-6 and GMCSF (Fig 3D). Additionally, using flow cytometry we detected upregulation of VCAM-1 on vFLIP-expressing spindle cells (Fig 3E). VCAM-1 expression is a known marker of endothelial cell activation that is upregulated by NFκB (18, 29) and is found commonly on KS spindle cells (12). These data suggest that the expression of vFLIP and its subsequent activation of NFκB in endothelial cells contribute to the inflammatory microenvironment of the KS lesion.

Inhibition of NFκB activation prevents the formation of spindle cells

If NFκB induction is required for induction of the spindle cell phenotype, then inhibition of NFκB activation should block vFLIP-mediated spindling. Accordingly, we took advantage of the availability of an NFκB-inhibitor, Bay 11-7082, to explore the importance of this transcription factor in spindling of HUVECs. Bay 11-07082 blocks

NFκB activation by inhibiting phosphorylation of IκB-α (31). HUVEC cells were pretreated with Bay 11-7082 for 2 hours, and then transduced with the retrovirus encoding vFLIP at high MOI. After transduction, media containing Bay 11-7082 was added back and the cells were followed for several days. As seen in Figure 4A, treatment of HUVECs with Bay 11-7082 at 4μM (in DMSO) prevented the activation of NFκB in these cells, as judged by the lack of p65 staining in their nuclei. This inhibition of NFκB coordinately inhibited the development of the spindle cell phenotype, as these cells maintained their typical cobblestone morphology. In contrast, vFLIP transduced cells that received only the DMSO diluent readily developed the spindle cell phenotype and showed robust NFκB activation, as seen by the presence of p65 in their nuclei (Fig 4A).

Bay 11-7082 is a toxic drug with a narrow therapeutic window. To further demonstrate that NFκB activation is the central event in spindling, we took advantage of a mutant of IκB (IκB-SR) whose phosphorylation sites for IKK have been ablated. As a result, expression of this mutant results in irreversible sequestration of NFκB in the cytosol, since this form of IκB cannot be directed to the proteasome. Accordingly, we constructed a retroviral vector for IκB-SR, and used it (or an isogenic empty-vector control virus) to transduce HUVEC cells; following selection for several days, cells were superinfected with the vFLIP retrovirus at high MOI, and observed for another 5 days. As shown in Fig 4B, under conditions in which exuberant spindling occurred in the control cells, virtually no morphologic change was evident in IκB-SR expressing cells. (While it is likely that this effect results from impaired action of v-FLIP, we cannot entirely exclude the formal possibility that inhibition of NFκB reduces the accumulation of vFLIP

protein, since levels of vFLIP are too low for routine detection by immunoblotting even in the presence of active NFκB).

DISCUSSION

These findings establish that expression of the latent vFLIP gene of KSHV is sufficient to cause the morphologic changes that underlie the spindle cell phenotype, and demonstrate that the activation of NFκB is required for development of this phenotype. We made concerted attempts to demonstrate that NFκB activation is also necessary for spindling in the context of authentic viral latency, by asking if IκB-SR expression could block spindling following infection with KSHV virions. However, we found that NFκB inhibition resulted in dramatic increases in cell death of the monolayer following KSHV infection, making scoring of spindling impossible. We believe this phenomenon to be due to increased lytic reactivation of the virus in cells unable to activate NFκB, as has previously been suggested by Brown et al. (6) on the basis of reporter gene studies.

V-FLIP expression also upregulates genes that contribute to the inflammatory microenvironment of KS. Other described activities of vFLIP – e.g. binding of procaspase 8 or inhibition of Fas-mediated caspase 8 activation(3, 10) – are unlikely to be required for spindling, since selective inhibition of NFκB activation blocks the phenotype, and since other FLIPS with these activities (e.g. E8) lack the capacity to induce spindling. Interestingly, although NFκB activation is usually an anti-apoptotic signal (22), neither we nor others (9, 15, 30, 42) have observed lifespan prolongation of KSHV-infected HUVEC cells. [This is in contrast to an earlier report in which viral infection was associated with long-term outgrowth of endothelial cells; however, in that study, the clones that did emerge largely did not retain the viral genome (14), in keeping with the known instability of KSHV latency *in vitro* (15, 23). We do not know the reason for this discrepancy]. Nonetheless, these observations do not exclude that vFLIP confers a survival advantage on spindle cells in the complex inflammatory milieu of an

infection *in vivo* – for example, by reducing the sensitivity of cells to TNF-induced cell death. Since KSHV-infected spindle cells accumulate progressively during the evolution of KS (11) they certainly must have a survival/growth advantage *in vivo*, and it seems very likely that vFLIP's antiapoptotic action would play a role in this.

This is the first report suggesting a role for vFLIP in cytoskeletal rearrangement. How this cytoskeletal alteration is engendered remains unknown, but it is clear that NFκB activation is responsible, as it is for vFLIP's known anti-apoptotic effect(16). Surely, the morphologic change reflects activation of a different set of NFκB target genes from those involved in blocking programmed cell death. Since these cytoskeletal changes are specific to endothelial cells, either there are endothelial –specific NFκB targets, or the action of one or more NFκB targets must require endothelial-specific cofactors. The linkage of NFκB activation to cytoskeletal rearrangement was unanticipated. Our review of the literature on NFκB and the cytoskeleton revealed no prior instances in which activation of this factor has been linked to cytoskeletal rearrangement, although the converse has been observed – for example, NFκB activation has been observed in response to Rac1 activation (and actin rearrangement) by endothelial shear stress, and to agents that disrupt microtubules (4, 19, 20, 44).

By contrast, the anti-apoptotic and pro-inflammatory effects of vFLIP are observed in many cell types. For example, in B cells, elegant studies of Cesarman and coworkers reveal that continuous vFLIP expression is essential for B cell survival in primary effusion lymphoma (PEL) caused by KSHV (16). vFLIP also blocks apoptosis in myeloid cells deprived of growth factors (38).

NFκB induction therefore plays pivotal and overlapping but non-identical roles in the lymphoid and endothelial disorders linked to the virus.

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Fig 1. Infection of endothelial cells with KSHV causes the formation of spindle cells.
Primary HUVEC, lymphatic endothelial cells (LEC) and blood endothelial cells (BEC) were obtained as described in Materials and Methods. (a-f) HUVECs, LECs and BECs were subjected to infection with KSHV at high MOI for 2 hours, rinsed and media was replaced. (a-c) green: LANA, red: K8.1, blue: DAPI. Images were taken at 3 days post infection. (g-i) Mock and KSHV-infected LECs and KSHV-infected BECs were stained with FITC-coupled phalloidin.

Fig 2. vFLIP expression alone causes spindle cell formation in HUVEC, LEC and BEC.
a. HUVECs were transduced with retroviruses encoding the individual latency-associated genes and subjected to selection with 0.5μg/ml puromycin. Images were taken 3 days post transduction.

b. LECs and BECs were transduced with retroviruses encoding vFLIP or empty vector. The cells were then selected with 0.25μg/ml puromycin. Images were taken at 2 days post transduction.

Fig 3. NFκB activation correlates with spindle cell formation and upregulates inflammatory cytokines and markers of endothelia activation.
a. *Bars 1, 2:* Spindled vFLIP-expressing HUVECs (1) and HUVECs expressing vector alone (2) were transfected with 0.5ug NFκB-luciferase reporter construct and 0.5ug β-galactosidase encoding plasmid to normalize for transfection efficiency. 48hrs post-transfection, luciferase and β-galactosidase assays were performed; bars show normalized levels of luciferase activity.
Bars 3,4,5: 293 cells were transfected with equal amounts of vector (3), vFLIP (4) or E8 (5) plasmids along with an NFκB-luciferase reporter and β-gal construct. Assays were performed at 48hrs post-transfection.

b. HUVECs were transduced with retrovirus encoding either vFLIP, E8 or empty vector and selected with puromycin as described before. Images were taken 3 days post transduction.

c. Supernatant conditioned for 24hrs by fully-spindled HUVECs expressing vFLIP was applied to the RayBio Human Cytokine Antibody Array V as per manufacturers

indications. Cytokines indicated by arrows are those found to be increased over conditioned media from vector-expressing HUVECs assayed I parallel.

d. Media from vFLIP or empty vector-expressing HUVECs were assayed for content of IL-6 or GMCSF by ELISA.

e. Spindle cells expressing vFLIP (thick line) or vector-transduced HUVECs (thin line) were stained for surface expression of VCAM-1 and examined by flow cytometry. Numbers next to histograms indicate geographic means of expression levels.

Fig. 4 Inhibition of NFκB prevents spindle cell formation.

a. HUVECs were pretreated with either 4μM Bay 11-7082 (lower panels) or DMSO (top panels) for 2 hours before being transduced at high MOI with retroviruses encoding vFLIP. After transduction, media containing either Bay 11-7082 or DMSO was added back. At 3 days post-transduction, the cells were photographed (right panels) or fixed and stained with a p65-specific antibody (left panels).

b. HUVECs were transduced at high MOI with retrovirus encoding for either the IκB super-repressor (lower panel) or empty vector (upper panel) and then selected in 0.5μg/ml puromycin for several days. The cells were then superinfected at high MOI with retrovirus encoding vFLIP, and their morphology was assayed at 5 days post-transduction.

Figure 1.

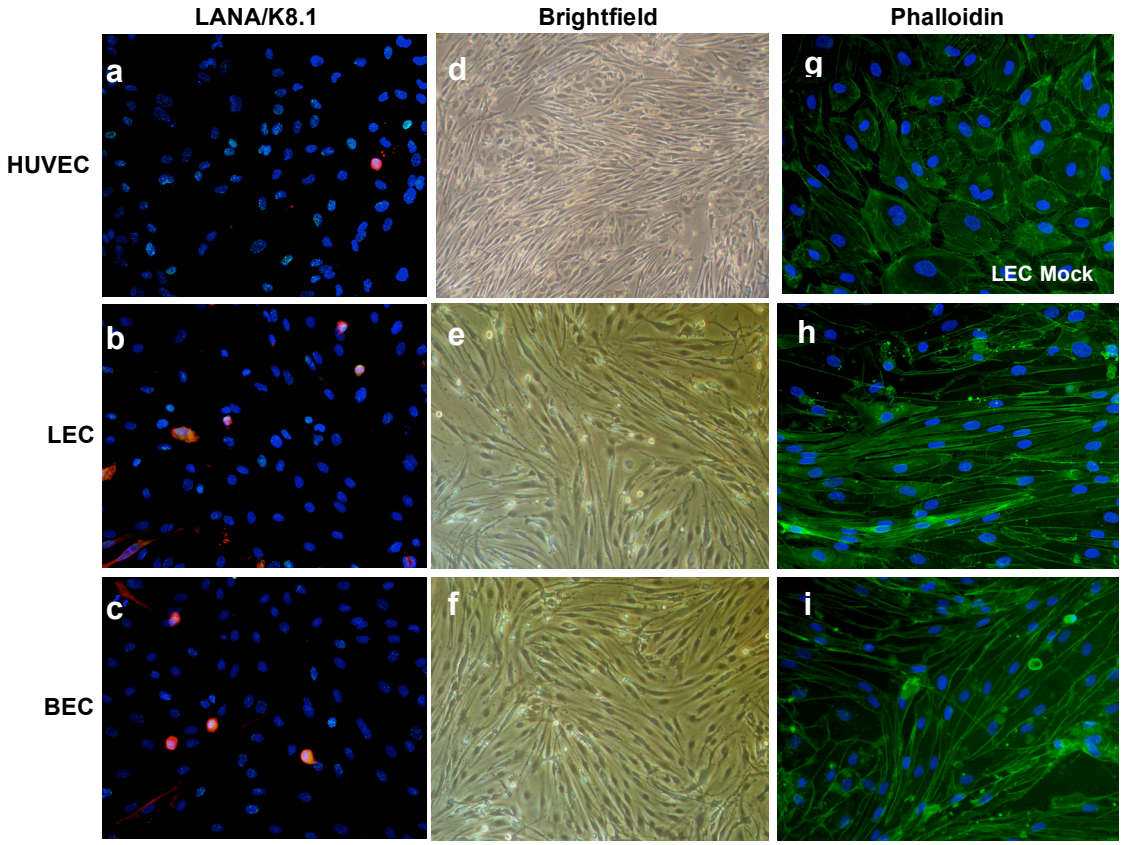
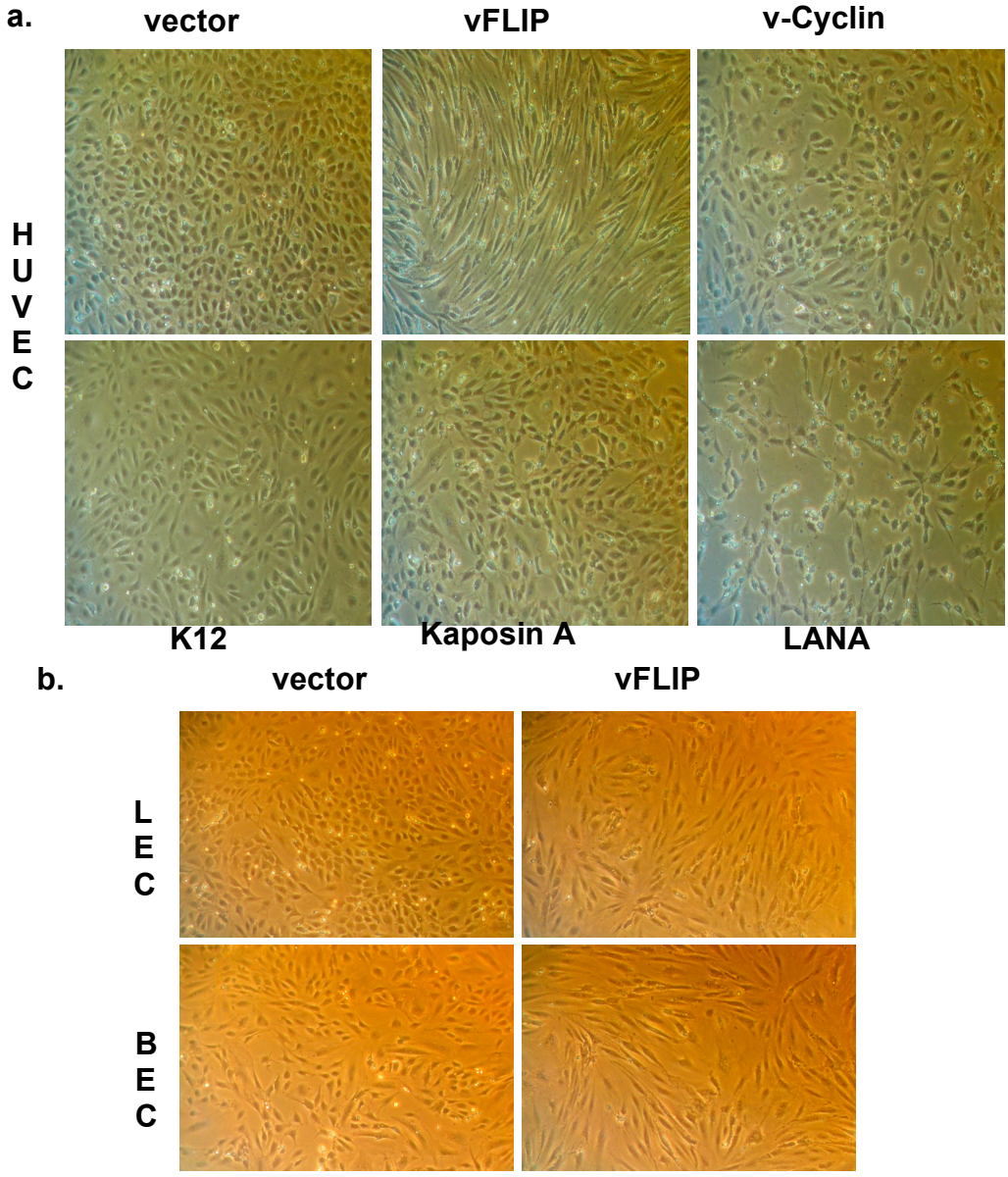

LANA/K8.1
Brightfield
Phalloidin
HUVEC
LEC
BEC
a
b
c
d
e
f
g
h
i
LEC Mock

Figure 2.


a.
vector
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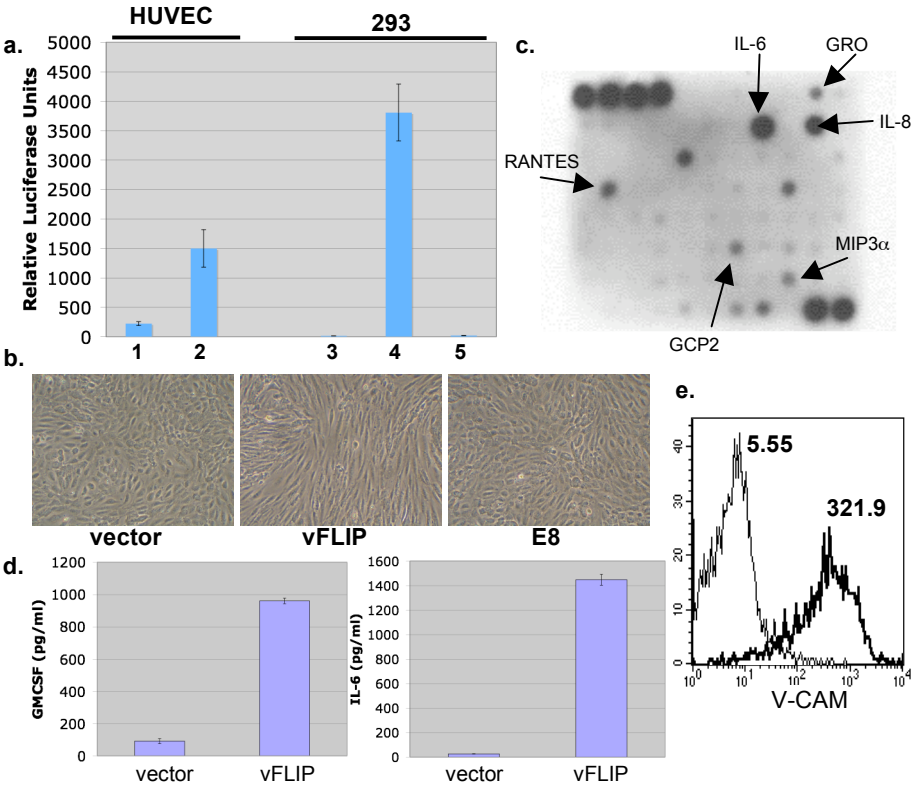
HUVEC
K12
Kaposin A
LANA
b.
vector
vFLIP
LEC
BEC

Figure 3.


a.
HUVEC
293
Relative Luciferase Units
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4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
2
3
4
5
b.
vector
vFLIP
E8
c.
IL-6
GRO
IL-8
RANTES
MIP3α
GCP2
d.
GMCSF (pg/ml)
1200
1000
800
600
400
200
0
vector
vFLIP
IL-6 (pg/ml)
1600
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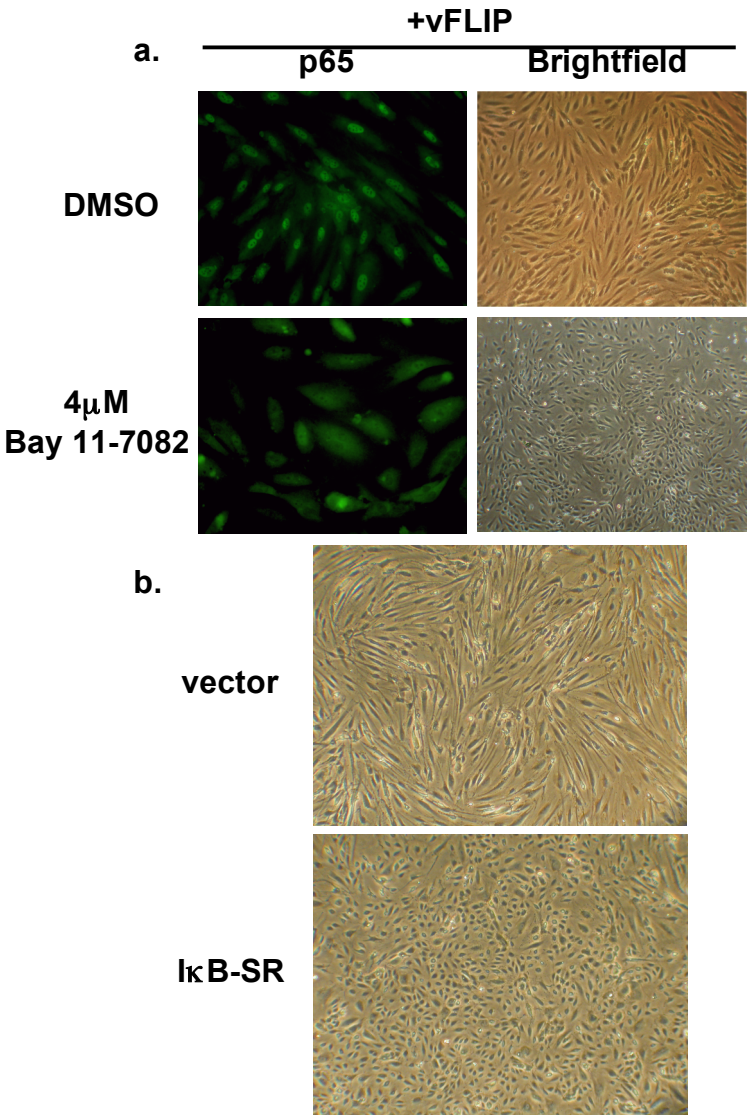
1200
1000
800
600
400
200
0
vector
vFLIP
e.
5.55
321.9
V-CAM

Figure 4.



CHAPTER 3:

Effects of NFκB activation on KSHV latency and lytic reactivation are complex and context-dependent

ABSTRACT

Like all herpesviruses, Kaposi's sarcoma associated herpesvirus (KSHV) can produce either latent or lytic infection. The latent v-FLIP gene is a strong activator of NF-κB, and recent studies indicate that, in primary effusion lymphoma (PEL) cells, blockade of NF-κB activation is associated with enhanced lytic gene expression, while over-expression of p65 impairs expression of reporter genes driven by lytic promoters. This has led to the suggestion that NF-κB activation may promote latency by suppressing lytic reactivation. Therefore, we have examined in detail the effects of NFκB activation and inhibition on KSHV replication. In accord with earlier work, we find that chemical inhibition of NFκB signaling in PEL cells is associated with enhanced lytic reactivation of KSHV. Similarly, in *de novo* KSHV infection of primary endothelial cells, inhibition of NF-κB signaling (by stable expression of a non-degradable mutant of I-κBα) leads to an increase in lytic gene expression, enhanced cytotoxicity and enhanced virion production. By contrast, KSHV-infected human foreskin fibroblasts (HFF), which are also fully permissive for KSHV latent and lytic growth, show no increase in spontaneous lytic reactivation or cytotoxicity when NFκB is inhibited.

If NFκB activation is inhibitory to lytic gene expression, one might expect its activation to be suppressed during the lytic cycle. However, we find that NFκB signaling is strongly and consistently activated in lytically infected cells of all lineages tested. Together these data indicate that (i) the relationship of NFκB activation to latency and lytic reactivation is not uniform, but is dependent on the cellular context; and (ii) even though NF-κB activation is inhibitory to lytic gene expression in some contexts, such inhibition is routinely bypassed or overridden during lytic growth.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV, also called human herpesvirus 8) is the etiologic agent of Kaposi's sarcoma (KS), an inflammatory and proliferative lesion affecting microvascular endothelium. KSHV also targets B lymphocytes, and is linked to two rare lymphoproliferative syndromes, multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL) (1, 16, 20). Like all herpesviruses, KSHV can execute two different genetic programs, known as *latency* and *lytic growth*. In latency, viral gene expression is strongly restricted, with only a handful of the virus' nearly 100 genes being expressed. The latent viral genome is maintained as a circular nuclear plasmid, and no viral progeny are produced. Latency is usually the default pathway following experimental infection in cultured cells (3). However, if latently infected cells are exposed to certain stimuli (such as: phorbol esters or HDAC inhibitors) latency can be disrupted and lytic replication triggered. The lytic program proceeds via a temporally regulated cascade of gene expression, in which lytic cycle-specific genes are activated in at least 3 classes: immediate-early (IE), delayed-early (DE) and late (L). Viral DNA replication follows DE gene expression, and progeny genomes are encapsidated into virions and released.

KS tumors are primarily latently infected, but a small number of cells in the lesion are lytically infected (21), and both latency and lytic replication have been postulated to play important roles in KS pathogenesis . A single viral gene, RTA (Replication and Transcription Activator) controls this genetic switch; forced expression of RTA in latency triggers lytic reactivation, and mutational inactivation of RTA blocks lytic reactivation. However, little is known of the physiologic stimuli that activate RTA expression to trigger lytic growth, and still less is known about how latency, once established, is stably

maintained (that is to say, how the expression of RTA and other lytic genes is prevented).

Recently, Brown et al (2003) demonstrated that PEL cells treated with t Bay 11-7082, a known pharmacological inhibitor of IKK and thus of NFκB activation, triggered enhanced spontaneous lytic reactivation. Because KSHV latency is associated with expression of the v-FLIP gene, which tonically activates NFκB by binding to NEMO and activating IKK (6, 15), latently infected cells have elevated NFκB activity. A model has accordingly been proposed that NFκB activation opposes lytic reactivation; in support of this notion, Brown et al (2003) reported that overexpression of p65 inhibits expression of luciferase reporter genes driven by several KSHV lytic promoters. Here we have examined the role of NFκB in lytic reactivation in several cell types other than PEL cells (which have been selected for stable latency *in vivo* and display a strong dependence on v-FLIP-mediated NFκB activation (10)). We find that the dependence of latency on NFκB activation is variable and dependent upon the cellular context – primary endothelial cells behave similarly to PEL cells, but equally permissive human fibroblasts show no enhancement of lytic reactivation when the NFκB activation pathway is inhibited. Moreover, NFκB is strongly activated during lytic replication in all cell types, indicating that if there is a block to lytic gene expression mediated by p65 it can be negated or bypassed in the context of the lytic program.

MATERIALS AND METHODS

Cells and KSHV infection

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and cultured in EGM-2 media supplemented with the endothelial supplement pack (Clonetics). Human foreskin fibroblasts (HFFs) were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin and l-glutamine. BCBL-1 cells were carried in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, glutamine and β-mercaptoethanol. KSHV was concentrated from supernatants of induced BCBL-1 cells as previously described (3, 14). KSHV infections were done in media containing 2μg/ml polybrene and incubated with cells for six hours, after which cells were rinsed and media was added back. In the case of lytic infection, Adeno-RTA ($1x10^9$ particles/ml) was incubated in media plus 0.5ug/ml polylysine (Sigma) for 2 hours before being applied to cells after the removal of the KSHV containing media.

Retrovirus production and infection

Retroviruses were produced using the amphotropic Phoenix packaging cell line transfected with the Moloney Murine Leukemia Virus based vector pMXpie (a kind gift from Lewis Lanier). Phoenix cells were transfected using FuGENE 6 (Roche) according to the manufacturer's specifications. 36 hours after transfection supernatants were collected and concentrated at 5,000rpm for 16hrs. Concentrated retroviruses were resuspended in EGM-2 media with 6μg/ml polybrene and filtered through a 0.2μm filter. Concentrated retrovirus was diluted in EGM-2 media with 6μg/ml polybrene and applied to cells. These cultures were spun at 2,000rpm for 1.5 hours after which virus-

containing media was removed and regular culture media was added back. In cases were selection was employed, media containing the selective agent was added to cells at the stated concentration 24 hours after transduction.

Immunofluorescence and Western Blotting

Immunofluorescence assays were performed as previously described using anti-ORF59 (ABI) and an anti-mouse rhodamine (Santa Cruz Biotechnology) secondary antibody was used at 1:300.

Lysates for western blots were obtained by rinsing cells twice with ice cold PBS, scraping adherent cells from the plate, spinning cells at 1000rpm for 5min and resuspending pellets in 2-3x the pellet volume of RIPA lysis buffer. Lysates were quantified using Biorad Protein Assay. 20ug of protein were run on 4-15% Tris-HCl gels and protein was transferred to PVDF at 100mV for 1hr at 4°C. Blots were blocked for 1hr at room temperature in 5% milk in TBS plus 0.1% Tween. Primary antibodies were incubated overnight at 4°C at 1:1000 anti-PARP (Cell Signaling Technology), 1:5000 anti-β-actin (A1978, Sigma), 1:2000 anti-phospho-IκBα (Cell Signaling Technology), 1:500 anti-IκBα (BD Biosciences); secondary antibodies, anti-mouse-HRP and anti-rabbit-HRP (Santa Cruz) were incubated at 1:5000 for 1hr at room temperature. Signal was detected using ECL Detection Reagent (Amersham).

Bay 11-7082 treatment of BCBL-1 cells

Bay 11-7082 was purchased from Calbiochem, diluted in DMSO and used at the concentrations stated. For reactivation assays, BCBL-1 cells were resuspended in media containing Bay 11-7082, incubated for 30 minutes, then spun down and resuspended in fresh media. RNA and protein lysates were harvested at 48hrs post Bay 11-7082 treatment.

KSHV virion DNA isolation

Media was removed from virus-producing cells, filtered through a 0.45uM filter and stored at 4°C . 20u/ml DNase was added to filtered supernatants and incubated at 37°C for 1hr. Supernatants were then chilled on ice and spun at 15,000 rpm for 2 hours at 4°C. Media was then removed and pelleted virus was resuspended in 600ul lysis buffer (20 mM Tris-HCl pH 8, 10 mM EDTA, 100 mM NaCl, 0.5% SDS) and incubated at room temperature for 10 minutes. 0.7mg/ml Proteinase K and linearized plasmid encoding an unrelated malaria gene were diluted in 100ul lysis buffer and added to the 600ul resuspended virions. The 700ul total volume was incubated at 55°C for 2 hours; this was then extracted once with 700ul phenol/chloroform/isoamyl. 600ul of the aqueous phase was removed and to it was added 3ul glycogen, 100ul 3M Sodium Acetate and 700ul isopropanol; this was then spun at 15,000 rpm for 15 minutes at 4°C. The resulting pellet was rinsed once with 70% ethanol, spun and air dried before being resuspended in 85ul of water. 2ul of this was used in the subsequent Taqman and Sybergreen assays for PAN promoter and spiked-in malaria gene as a normalizing factor.

Taqman and Sybergreen Real-time PCR assays

For all Taqman assays, 200ng of RNA was reverse transcribed using SuperScript III and oligo d(T) primer (Invitrogen) according to manufacturer's instructions. 2ul of this reaction was used in all subsequent real-time PCR Taqman assays. Primer and probe sequences used for lytic gene expression ORF50 is previously described (2). Other primers used were ORF 57 Forward: TGGACATTATGAAGGGCATCC, Reverse: CGGGTTCGGACAATTGCT; gB Forward: TCGCCGCACCAATACCATA, Reverse: CCTGCGATCTACGTCGGG; PAN promoter

Forward: GCCAGCTTGAGTCAGTTTAGCA, Reverse: CGAGCACAAAATCCATAGGTG; Malaria DNA Forward: AGGACCCGATCAACAACAT, Reverse: AAGCTGAACAAGAACGCGAT. Taqman reactions (all but malaria DNA) were performed using Taqman Universal Master Mix (Applied Biosystems) and SyberGreen reactions (malaria DNA) were performed using SybergreenER mastermix (Invitrogen) as per the manufacturer's specifications.

NF-κB EMSA

Nuclear enriched lysates were made from cells by incubation in hypotonic buffer (20mM HEPES pH7.8, 5mM KCl, 1.5mM $MgCl_2$, 1mM DTT and protease inhibitors), followed by pelleting and disruption of nuclei by incubation in high salt buffer (0.4M KCl, 50mM HEPES pH7.9, 0.1% NP40, 0.5μM EDTA, 10% glycerol and protease inhibitors). 5μg of lysate was incubated with ^{32}P-labled oligonucleotide encoding the NF-κB consensus sequence (Santa Cruz Biotechnology) and dI:dC DNA (Sigma) in binding buffer (20μM HEPES pH7.9, 50mM KCl, 10% glycerol, 1mM EDTA, 1mM $MgCl_2$, 1mM DTT). Reactions were incubated at room temperature for 30 minutes without the labled probe and an additional 30 minutes after addition of the probe. Complexes were resolved in a 1x TBE, 4% acrylamide gel.

NFκB luciferase assay

293T cells were transfected using Fugene 6 (Roche) as per the manufacturer's specifications with increasing amounts for the following constructs: pNFκB-luc (BD Biosciences), pCDNA3.1-RTA, pCD8 (Mylteni Biotech). 36 hours post transfection cells were passed over anti-CD8 MACS columns (Mylteni Biotech) to enrich for cells expressing CD8 and RTA. The separated cells were plated and allowed to recover

overnight. The next morning cells were either mock infected or infected with KSHV in triplicate. 48 hours post infection luciferase levels were assayed as per the manufacturer's specifications (Promega).

RESULTS

Inhibition of NFκB in PEL cells leads to increased lytic gene expression and apoptosis.

Although Brown et al. (2003) reported that NFκB inhibition upregulated lytic gene expression, other investigators have not observed lytic derepression in this setting (12). We therefore re-examines this issue. In accord with previous work (4), we found that inhibition of NFκB signaling in KSHV positive PEL cells leads to increased expression of an array of lytic genes. BCBL-1 cells, a line persistently infected with KSHV, were treated with a range of doses (1, 2, 4, 6μM) of the compound Bay 11-7082 (in DMSO), an irreversible inhibitor of IκBα phosphorylation, or a corresponding amount of DMSO. Total RNA was isolated from the cells and expression of several lytic genes was assayed by Taqman real time RT-PCR. As shown in Fig 1A, cells treated with Bay showed a marked increase in expression of an immediate early lytic gene (ORF 50), a delayed early gene (ORF 57) and a late lytic gene (gB). This increase was seen at all assayed doses and followed a dose-dependent trend.

NFκB inhibition in PEL cells has been reported to be associated with increased apoptosis of the PEL cells (11, 12). Accordingly, we examined apoptosis levels by immunoblotting for PARP, a downstream cleavage target of caspases 3 and 7 in the apoptotic cascade (Fig 1B). As expected, increased cleavage of PARP was seen in BCBL-1 cells treated with Bay 11-7082 (lanes 1,3,5,7) but not the corresponding vehicle, DMSO (lanes 2,4,6,8). Because apoptosis was assayed in the mass culture rather than at the single cell level, we do not know if the cell population experiencing lytic reactivation was the same or different from that undergoing apoptosis.

***De Novo* KSHV infection of endothelial cells in the context of NFκB inhibition leads to increased cytotoxicity, lytic reactivation and apoptosis**

Previous experiments investigating the link between the NFκB signaling pathway and the KSHV latent-lytic switch have been done almost exclusively in PEL lines (4, 10-12, 19). But PEL cells are very far removed from the initial latent infection, having been selected *in vivo* for stable episome maintenance despite rapid growth – a selection that we know requires epigenetic changes that are likely not present in KS-derived endothelial (spindle) cells or most latently infected cells established in culture (9). Therefore, PEL cells may not be fully representative of all cells in which latent KSHV infection can be observed. Accordingly, we have examined several other cell types in which *de novo* KSHV infection can produce latency, and which are permissive for lytic reactivation. These include primary human umbilical vein endothelial cells (HUVEC) and secondary human foreskin fibroblasts (HFF).

To examine the role of NFκB in HUVEC cells, we generated a HUVEC line stably expressing a degradation resistant mutant of IκBα. This version of IκBα (IκB super-repressor, IκBSR) contains two Ser/Ala mutations at positions 32 and 36, rendering it resistant to phosphorylation by the IκBα kinase (IKK) and therefore refractory to subsequent degradation by the proteasome. This results in a stabilization of IκBα-NFκB complexes in the cytoplasm of the cell, inhibiting the ability of the NFκB transcription factor to translocate into the nucleus. As previously described (8), HUVECs were transduced with retrovirus encoding IκBSR and selected for a short time with puromycin. These cells were assayed for inhibition of NFκB signaling by treatment with TNFα (10ng/ml) for 2hrs, isolation of nuclear extracts, followed by electromobility shift assay (EMSA) to determine NFκB DNA binding activity. Figure 2A shows complete inhibition

of inducible NFκB DNA binding in IκBSR HUVECs upon treatment with TNFα, in contrast to those expressing the empty vector, which display strong NF-κB induction.

Next, IκBSR and control HUVECs were infected with KSHV at levels that were previously determined to give 100% LANA positive cells and low (< 1%) spontaneous lytic reactivation; infected and mock-infected cells were then followed for 3 days. Figure 2B shows representative pictures of the mock- or KSHV- infected HUVEC monolayers at 66 hours post infection. Upon KSHV infection, IκBSR expressing cells showed reproducible increases in cytotoxicity as compared to control cells. In some cases, this toxicity was visible at time points as early as 18 hours post infection (data not shown), but was most pronounced at the 66 hour time point.

To determine whether the observed increases in cell toxicity correlated with increased spontaneous lytic reactivation of the virus, IκBSR and control HUVECs previously infected with KSHV were fixed and stained for the delayed early protein ORF 59 (Fig 2C). The lower panel of Fig 2C shows representative fluorescence images of cells stained for ORF 59 expression (Red) and DAPI (Blue) at 66 hours post infection (hpi). As is evident in the picture, and is quantified in the histogram in the upper panel, IκBSR HUVECs showed a significant increase in ORF 59 positive cells. In order to better quantify the increase in lytic gene expression in IκBSR HUVECs, total RNA was harvested from cells at 66hpi. Real time quantitative RT-PCR was performed to determine the relative abundance of genes that spanned the multiple phases of temporal regulation of the lytic cycle. As is shown in the upper panel of Fig 2D, all three lytic genes assayed, (ORF50, ORF57 and gB) showed increased expression in IκBSR HUVECs over control cells. Values are normalized to levels of the endogenous GAPDH gene for each sample, and are displayed as ddCT values (log base 2) showing the relative increase in expression of the genes in IκBSR HUVECs over control cells.

To determine whether the observed increase in lytic gene expression in the IκBSR HUVECs corresponded to an increased production of KSHV virions, supernatants from infected cells were collected at 42 and 66hpi. Supernatants were cleared of dead cells and cellular debris and the remaining virions were concentrated by centrifugation. Exogenous DNA was removed by DNase digestion; following inactivation of the nuclease, virions were lysed and virion DNA was extracted as described in the Materials and Methods section. Real time quantitative Taqman PCR was performed on the isolated virion DNA, and the abundance of viral genomes was quantified by measuring the relative levels of the PAN promoter sequence (Fig 2D, lower panel), normalized to a spiked-in control. IκBSR HUVECs showed increased production and release of virions into the supernatant at both the 42 and 66 hpi time points. In conjunction with the observed increases in staining for lytic protein and lytic gene expression, the increased production of KSHV virions supports the view that inhibition of the NFκB pathway in primary human endothelial cells leads to increased spontaneous lytic reactivation of KSHV upon *de novo* infection.

Although much of the cytotoxicity observed in the infected IkB-SR HUVECS could be attributed to cell necrosis from lytic infection, we also asked whether there was an increase in apoptosis in this setting. Lysates taken at 66 hpi were blotted with an anti-PARP antibody to assess the extent of PARP cleavage in the individual cultures (Fig 2E). Increased PARP cleavage was observed in the KSHV infected IκBSR HUVECs (lane 4) as compared to the infected control cells (lane 2), suggesting enhanced levels of apoptosis in these cells. This is not entirely surprising, as many lytic herpesviral gene products can trigger apoptosis. Additionally, since NF-κB is known to upregulate an anti-apoptotic cascade in endothelial cells (22), inhibition of this pathway in infected cells might be expected to trigger enhanced apoptosis.

KSHV-infected human fibroblasts do not display enhanced lytic replication or cytotoxicity in the presence of NFκB inhibition.

To determine whether the cell death associated with KSHV infection in the context of NFκB inhibition is common to all cell types, human foreskin fibroblasts (HFF) expressing the IκBSR were constructed. HFFs have been previously shown to be fully permissive for both latent and lytic KSHV infection (3, 24). As expected, IκBSR HFFs display strong inhibition of NFκB signaling in response to TNFα (Fig 3A), relative to control HFFs transduced with an empty retroviral vector. These cells were then infected with KSHV under conditions promoting latent infection of nearly all cells. Despite robust latent infection, as evaluated by staining for LANA (green, Fig 3C), no increase in cell death (Fig 3B) or ORF 59 staining (red, Fig 3C) was detected in IκBSR HFFs as compared to control cells. Likewise, no increase in lytic mRNA expression or virion production was observed in these cells (Fig 3D). Thus, neither lytic reactivation nor cell injury is an ineluctable consequence of KSHV infection in the absence of NFκB activation. Clearly, the relationship between NFκB signaling and spontaneous lytic reactivation is dependent on the cellular context.

NFκB signaling is activated in lytically infected cells of all lineages

Data showing that inhibition of NFκB induces lytic gene expression in HUVEC and PEL cells suggests that NFκB activation is inhibitory to lytic gene expression. Consistent with this, Brown et al (2003) reported that overexpression of recombinant p65 can extinguish gene expression from cotransfected reporter genes driven by lytic KSHV promoters. These data suggest that NFκB activation may be incompatible with lytic gene expression and lead to the prediction that the lytic cycle might actively suppress NFκB

activation. To test this prediction, we examined the state of NFκB activity at several time points during the progression of lytic KSHV infection. Contrary to the predictions of this model, we found robust NFκB activation in several cell types including HUVECs, HFFs and 293T. The NFκB activation state of HUVECs and HFFs was examined during both latent and lytic KSHV infections. Cells were infected with either KSHV alone or KSHV followed by an adenovirus encoding the lytic transactivator RTA (AdRTA). At 48 hpi nuclear extracts were prepared from the cells and NFκB DNA binding was assessed by EMSA (Fig 4A). Increased NFκB DNA binding could be seen in HUVECs upon latent infection (upper panel, lane 3) and was further enhanced upon reactivation with AdRTA (upper panel, lane 4). HFFs showed similarly increased NFκB DNA binding upon lytic KSHV infection (Fig 4A, lower panel). Moreover, this enhanced NFκB was functionally active, as judged by its ability to upregulate expression of an NFκB-dependent luciferase reporter. 293T cells were cotransfected with plasmids encoding CD8, RTA, and an NFκB-luciferase reporter. 36 hours post transfection, cells expressing CD8 (and therefore RTA) were enriched for by binding to magnetic beads bearing anti-CD8. The next day, these cells were either infected with KSHV or mock infected; at 48 hours post infection, luciferase levels were assayed (Fig 3B). KSHV-infected, RTA-expressing cells showed enhanced luciferase readings, indicating increased NFκB-dependent transcriptional activation under these conditions. (We note that the extent of upregulation of the reporter observed here is surely an underestimate of the degree of NFκB activation, owing to the global turnover of host mRNAs during lytic KSHV replication (7)).

As another indication of the activation state of the NFκB pathway during lytic growth, we assayed for phosphorylation of IκBα by the IKK complex in lytically infected HUVECs. Cells were infected with either KSHV alone or KSHV plus Ad-RTA; extracts were prepared at 48 and 72 hpi and assayed by immunoblotting for phospho-IκBα and

total levels of IκBα. Figure 4C shows increased phosphorylation of IκBα in lytically infected cells by 48hpi (top panel, lane 3, bottom band) and a corresponding decrease in steady state levels of total IκBα protein in these cells (middle panel, lane 3). By 72 hpi the intracellular pool of IkBα is virtually totally depleted, most likely as a result of proteasome action, and possibly exacerbated by KSHV-mediated mRNA turnover, which is very pronounced late in infection (7). These data affirm that the NFκB pathway is upregulated during lytic replication, and that this induction is occurring at or above the level of IKK activation.

DISCUSSION

Previous studies have described complicated interactions between the NFκB signaling pathway and KSHV infection in the context of PEL cell lines (4, 10-12, 19). These studies have shown increased lytic gene induction upon chemical inhibition of NFκB (4), enhanced apoptosis upon chemical inhibition of NFκB or knockdown of vFLIP, a potent NFκB inducer (10, 11), and even production of defective KSHV particles upon genetic inhibition of NFκB in TPA-induced PEL cells (19). Additionally, inhibition of RTA-mediated transactivation of lytic gene promoters was shown in the context of overexpression of the p65 subunit of NFκB (4). Taken together, these accounts have led some to propose a model of suppression of lytic gene expression and function by NFκB activation in PEL cells.

In our hands, inhibition of NFκB in BCBL-1 cells by treatment with Bay 11-7082 lead to increases in both lytic gene expression, in agreement with the work of Brown et al (2003), and enhanced apoptosis, in accord with the observations of Guasparri et al (2004). Results in HUVEC cells were largely congruent with these PEL observations; however, KSHV-infected human fibroblasts behaved completely differently – they displayed neither enhanced cytotoxicity nor increased spontaneous lytic gene reactivation. These results lead us to conclude that the relationship between NFκB and spontaneous reactivation of KSHV is complex, non-uniform and dependent on the cellular context. Cell specific differences in KSHV biology are not without precedent. For example, while many cell types can be latently infected *in vitro,* only a small subset of these can efficiently support full lytic replication following treatment with chemical inducers (3). Additionally, we and others have reported modifications in cellular morphology upon latent KSHV infection that occur only within the context of human

primary endothelium (5, 8, 9, 17, 23); differences in KSHV gene expression upon *de novo* infection and in stable latency have also been reported to differ between cell type (13, 18). Given these differences, which indicate intricate and complex interactions of the KSHV genome with the machinery of host gene expression, it is perhaps not surprising that the relationship of lytic induction to NFκB activation is not invariant in different cellular environments.

Finally, despite the earlier finding (4) that p65-overexpression can impair reporter genes driven by KSHV lytic promoters (outside of the contect of viral infection), we find that lytic replication is associated with *increased* NFκB activity in endothelial, epithelial and fibroblast cell lines upon lytic infection. Increased NFκB activity has also been observed in PEL cell lines during lytic reactivation (19), though those studies were complicated by the use of TPA as an inducer (and the fact that isogenic KSHV-negative PEL cells are not available as controls). Therefore, NFκB activation and lytic gene expression cannot be strictly incompatible under all circumstances. Whatever inhibitory influence p65 activation may have on lytic promoters, it clearly can be bypassed or abrogated during full lytic infection. Exactly how this is accomplished will require further investigation.

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FIGURE LEGENDS

Figure 1. Inhibition of NFκB in BCBL-1 cells leads to increased lytic gene expression as well as increased apoptosis.

BCBL-1 cells were treated with the indicated amounts of Bay 11-7082 or vehicle (DMSO) for 30min at 37°C. Cells were resuspended in fresh media and total RNA and total protein were harvested at 48 hours post treatment. Taqman real time PCR was performed on cDNA made from total RNA. ddCT values (log based 2 indicator of fold change) for lytic genes Orf 50, Orf57 and gB are shown in panel (A). All values were normalized to GAPDH levels, and are expressed for each gene in BCBL-1 cells treated with Bay 1170-82 relative to those treated with the same amount of DMSO. Panel (B) shows a dose dependent increase in apoptosis as measured by PARP cleavage with increasing doses of the Bay 11-7082 compound. 20ug of total protein was blotted for PARP protein and β actin as a loading control.

Figure 2. Inhibition of NFκB in HUVEC cells leads to increased cellular toxicity, increased lytic gene expression, virion production and apoptosis upon *de novo* infection with KSHV.

(A) HUVECs were transduced with retroviruses encoding a degradation-resistant mutant of IκBα (IκBSR). NFκB DNA binding activity in these cells was blocked upon treatment with 10ng/ml TNFα for 2hrs, as compared to those expressing vector alone.

(B) HUVECs expressing IκBSR show increased cellular toxicity upon *de novo* latent KSHV infection. HUVECs expressing either vector or IκBSR were infected with KSHV for 6hrs, pictures were taken at 66 hours post infection.

(C) KSHV infected IκBSR HUVECs show increased staining for ORF 59 (red) as compared to HUVECs expressing vector alone. Cells were fixed and stained at 66 hours post infection.

(D) Taqman real time PCR ddCT values (log base 2) for ORF 50, ORF 57 and gB (upper panel) and PAN promoter values (lower panel) show increased lytic gene expression and enhanced virion production in IκBSR expressing HUVECs. All values were normalized to GAPDH or spike-in DNA levels, and are expressed for each gene in IκBSR HUVECs relative to vector HUVECs. (Please see Materials and Methods sections for details on virion DNA isolation)

(E) IκBSR HUVECs show increased apoptosis upon infection with KSHV. Total protein was isolated from all cells (floating and adherent to the dish) from vector and IκBSR HUVECs at 66 hours post infection. 20ug of total protein was blotted for PARP protein and β actin as a loading control.

Figure 3. Inhibition of NFkB in human foreskin fibroblasts (HFF) does not lead to increased cellular toxicity, increased lytic gene expression and virion production upon *de novo* infection with KSHV.

(A) HFFs were transduced with retrovirus encoding a degradation-resistant mutant of IκBα (IκBSR). NFκB DNA binding activity in these cells was blocked upon treatment with 10ng/ml TNFα for 2hrs, as compared to those expressing vector alone.

(B) HFFs expressing IκBSR show no increase in cellular toxicity upon *de novo* latent KSHV infection. HFFs expressing either vector or IκBSR were infected with KSHV for 6hrs, pictures were taken at 66 hours post infection.

(C) No increase in ORF 59 staining was seen in IκBSR HFFs as compared to vector despite extensive latent KSHV infection as determined by staining for LANA (green). Cells were fixed and stained at 66 hours post infection.

(D) Taqman real time PCR ddCT values for ORF 50, ORF 57 and gB (upper panel) and PAN promoter values (lower panel) show no increase in lytic gene expression and or virion production in IκBSR expressing HFFs. All values were normalized to GAPDH or spike-in DNA levels, and are expressed for each gene in IκBSR HFFs relative to vector HFFs.

Figure 4. **NFκB signaling is activated in lytically infected cells.**

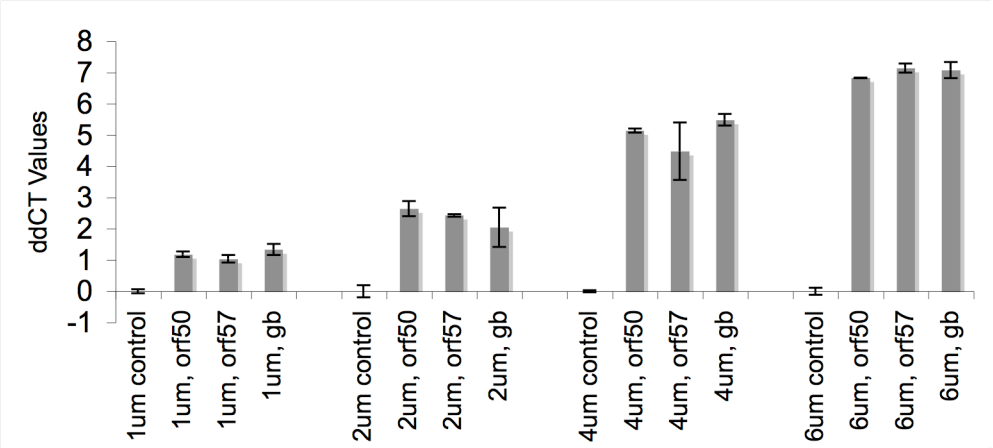
(A) Increased NFκB DNA binding activity is observed in lytically infected HUVECs (upper panel) and HFFs (lower panel), as compared to both uninfected, and latently infected cells. Nuclear fractions were harvested at 48 hours post infection, 5ug of protein was used in each binding reaction.

(B) Increased NFκB transcription is shown in 293T cells cotransfected with RTA and NFκB-luciferase reporter at 48 hours post infection. Both Mock and KSHV cells were transfected with CD8, RTA and luciferase plasmids for 36 hrs before infection, and enriched for CD8, and therefore RTA positive cells, using anti-CD8 coupled beads over magnetic columns.

(C) Lytically infected HUVECs show increased phosphorylation of IκBα (top blot, lower band) and corresponding decrease in levels of total IκBα (middle blot) demonstrating active NFkB signal transduction.

Figure 1.

A.


ddCT Values
8
7
6
5
4
3
2
1
0
-1
1um control
1um, orf50
1um, orf57
1um, gb
2um control
2um, orf50
2um, orf57
2um, gb
4um control
4um, orf50
4um, orf57
4um, gb
6um control
6um, orf50
6um, orf57
6um, gb

B.

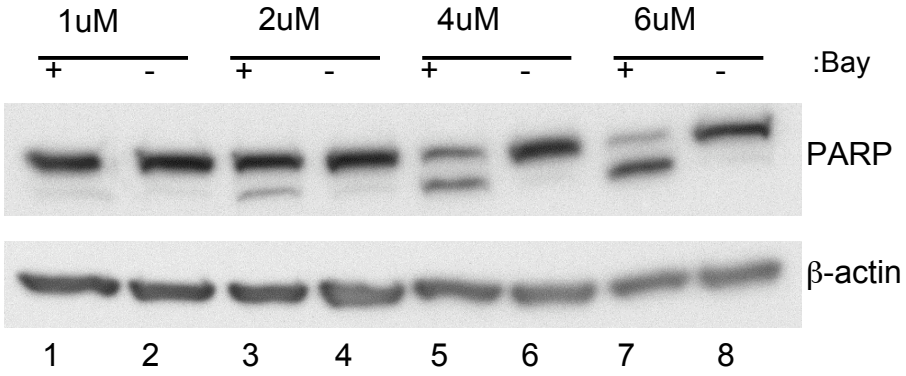

1uM
2uM
4uM
6uM
+
-
+
-
+
-
+
-
:Bay
PARP
β-actin
1
2
3
4
5
6
7
8

Figure 2.

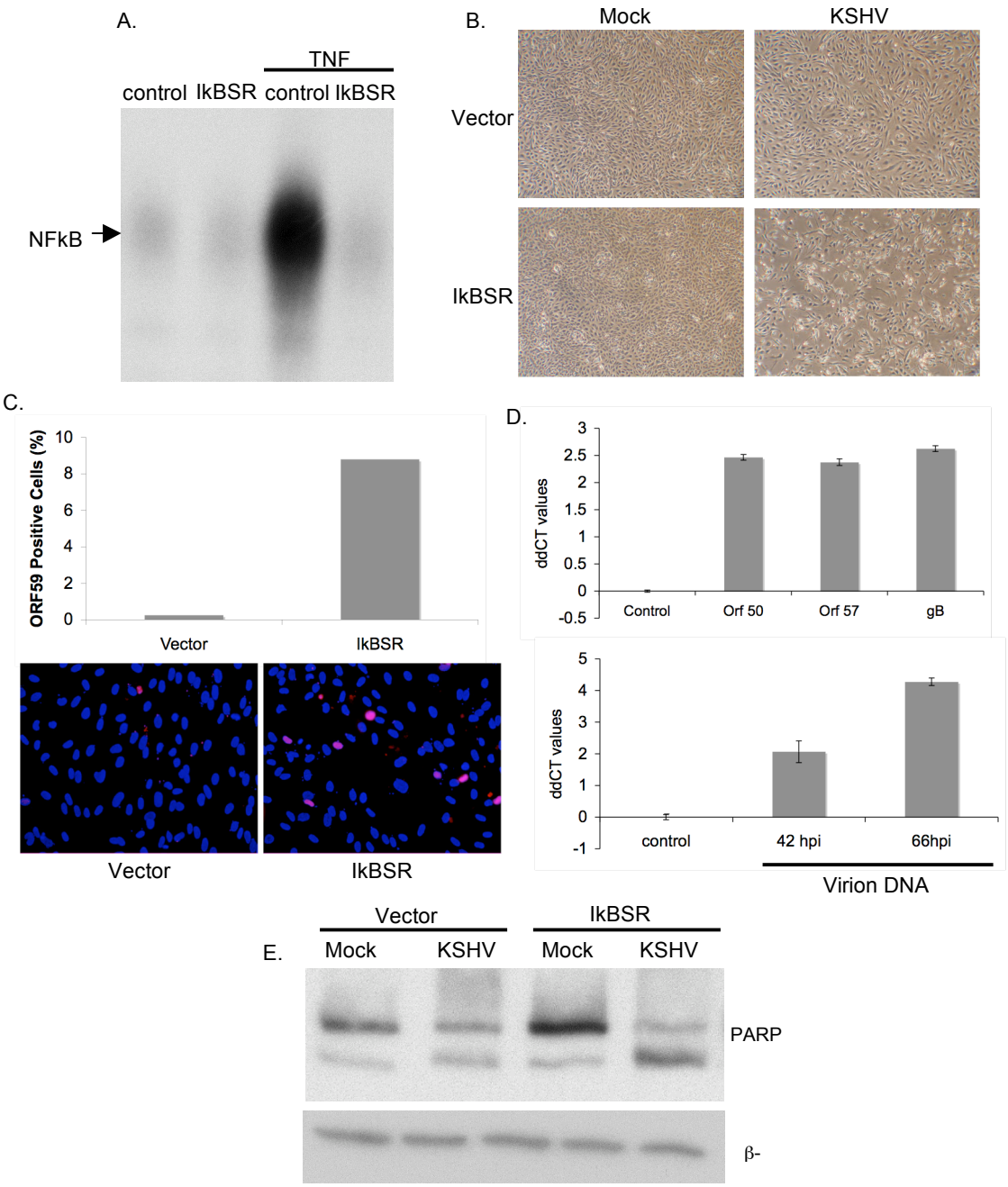

A.
TNF
control IkBSR control IkBSR
NFkB
B.
Mock
KSHV
Vector
IkBSR
C.
ORF59 Positive Cells (%)
10
8
6
4
2
0
Vector
IkBSR
Vector
IkBSR
D.
ddCT values
3
2.5
2
1.5
1
0.5
0
-0.5
Control
Orf 50
Orf 57
gB
ddCT values
5
4
3
2
1
0
-1
control
42 hpi
66hpi
Virion DNA
E.
Vector
IkBSR
Mock
KSHV
Mock
KSHV
PARP
β-

Figure 3.

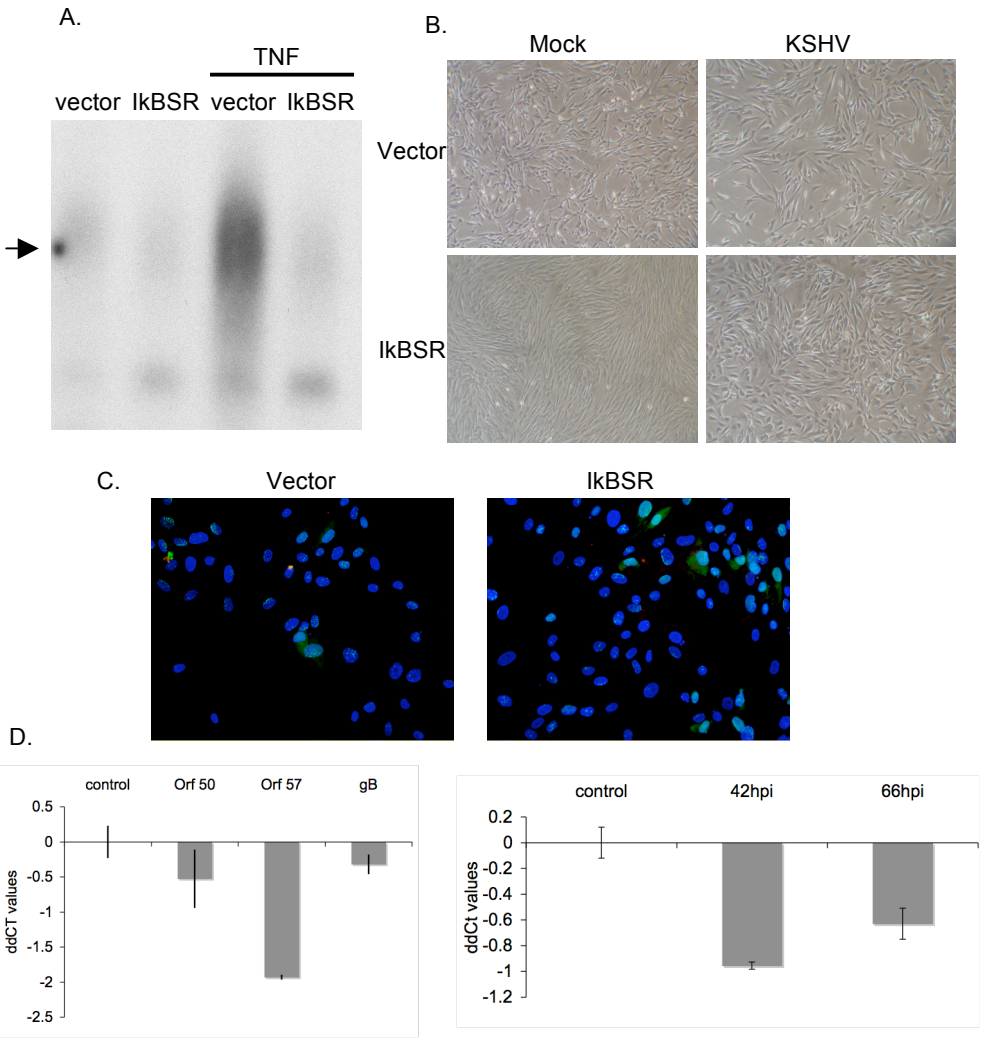
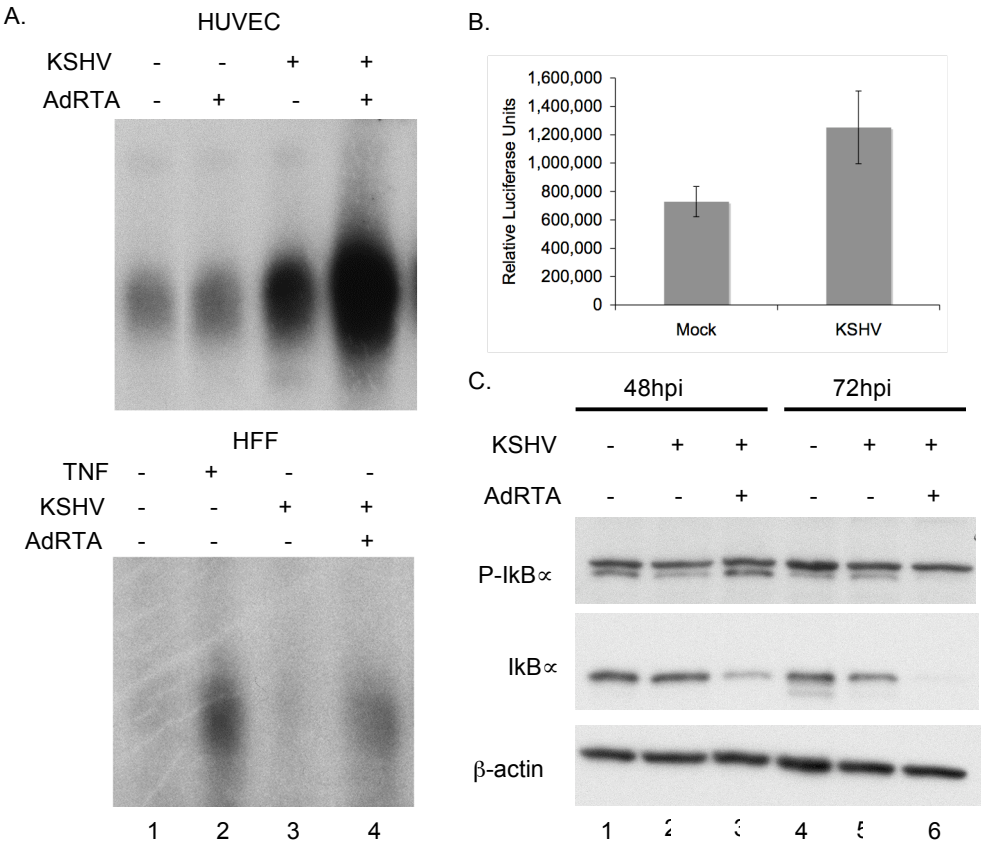

A.
TNF
vector
IkBSR
vector
IkBSR
B.
Mock
KSHV
Vector
IkBSR
C.
Vector
IkBSR
D.
control
Orf 50
Orf 57
gB
ddCT values
0.5
0
-0.5
-1
-1.5
-2
-2.5
control
42hpi
66hpi
ddCt values
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2

Figure 4.


A.
HUVEC
KSHV - - + +
AdRTA - + - +
HFF
TNF - + - -
KSHV - - + +
AdRTA - - - +
1 2 3 4
B.
Relative Luciferase Units
1,600,000
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
Mock
KSHV
C.
48hpi
72hpi
KSHV - + + - + +
AdRTA - - + - - +
P-IkBα
IkBα
β-actin
1 2 3 4 5 6

CHAPTER 4:
Characterization of vFLIP spindle cells using gene expression profiling and bioinformatics.

INTRODUCTION

Infection of human endothelial cells with KSHV has emerged as a widely used system to study Kaposi Sarcoma development *in vitro*. Several groups have shown that human endothelial cells support authentic latent and lytic KSHV infection and demonstrate many of the characteristics of the KS lesion, including production of inflammatory cytokines and angiogenic factors. Additionally, there have been several reports in the literature of human endothelial cells undergoing dramatic morphological changes upon infection with KSHV, reminiscent of the spindle cells found in KS lesions (4, 6, 7, 14, 15). In a previous report, we showed that the morphological changes seen in endothelial cells upon latent KSHV infection could be reproduced by the expression of a single latent KSHV protein, vFLIP (6).

In order to better characterize these spindle-shaped, vFLIP expressing endothelial cells, we used microarray gene expression profiling. This analysis provided us with a list of differentially expressed genes in vFLIP expressing spindle cells. To highlight the dominant biological pathways involved in the changes in gene expression upon vFLIP expression and spindle cell formation, we employed the Gene Ontology (GO) database, a database of structured controlled vocabularies that describe gene products in terms of their associated biological processes, cellular components and molecular functions (2). Additionally, using the PromoSer (9) and PAINT (17) programs we examined the promoters of modulated genes for overrepresentation of transcription factor binding sites. This allowed us to assess the dominant contributing transcription factors in vFLIP expressing spindle cells. Together these analyses allowed us to address the global transcriptional profile of these vFLIP spindle cells, and reaffirmed the central role of NFκB in their development.

MATERIALS AND METHODS

Cells and retrovirus production and infection

HUVECs were purchased from Clonetics and cultured in EGM-2 media supplemented with the microvascular supplement pack (Clonetics). Retroviruses were produced using the amphotropic Phoenix packaging cell line transfected with the Moloney Murine Leukemia Virus based vector pMX(Puro-IRES-EGFP). Phoenix cells were transfected using FuGENE 6 (Roche) according to the manufacturer's specifications. 36 hours after transfection supernatants were collected and concentrated at 5,000rpm for 16hrs. Concentrated retroviruses were resuspended in EGM-2 media with 6μg/ml polybrene and filtered through a 0.2μm filter. Concentrated retrovirus was diluted in EGM-2 media with 6μg/ml polybrene and applied to recipient HUVECs. These cultures were spun at 2,000rpm for 1.5 hours after which virus-containing media was removed and regular culture media was added back. 24 hours after transduction media containing 0.5μg/ml of puromycin was added to the HUVECs. Transductions of both vFLIP and vector were done in triplicate.

RNA preparation, labeling, and microarray hybridization

Total RNA was prepared from cells at 5 days post transduction using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reference RNA was a mixture of RNAs purified from dividing untransduced human foreskin fibroblast, TIME cells and human umbilical vein endothelial cells (HUVEC). The integrity of the purified RNA's was analyzed using the 2100 Bioanalyzer (Agilent). RNAs were quantified using the ND1000 spectrophotometer (Nanodrop). The Low RNA Input Linear Amplification Kit (Agilent) was used according to the manufacturer's protocol to generate labeled cRNA. Experimental samples (labeled with Cy5) and reference samples (labeled with

Cy3) were competitively hybridized to Whole Human Genome Oligo Microarrays (Agilent) according to the manufacturer's protocol. These microarrays have 41,000 probes that map to 20,087 unique unigene cluster IDs (Unigene Build #188). Cyanine 3-CTP and Cyanine 5-CTP were obtained from Perkin Elmer. Hybridizations and washes were performed according to the manufacturer's protocol. Washed arrays were scanned using the 48-slide DNA Microarray Scanner (Agilent) and feature intensities extracted using Feature Extraction Software version 8.5 (Agilent). All raw data were stored in the MIAME (minimum information about a microarray experiment)-compliant Princeton University MicroArray Database (PUMAdb) and can be accessed at http://puma.princeton.edu/.

Microarray Data Analysis

Only features passing quality filters requiring signal intensity to be well above background in both Cy5 and Cy3 readings were considered. Lowess normalized $\log_2$-ratios of these features were then downloaded from the PUMA database. We then used SAM (Significance Analysis of Microarrays) to generate a list of features that underwent statistically significantly changes between the three control arrays and the three vFLIP arrays. Additionally, probes were only accepted if they met the criteria of having 80% good data between the 6 scanned arrays while allowing for a false discovery rate of 0.1%. The differentially expressed probes identified by this analysis were then grouped into 10 discrete bins via K means clustering, grouping together those probes that showed similar expression trends.

Genes represented by the probes in each bin were then submitted for analysis by the Gene Ontology (GO) TermFinder, a statistical tool used to measure the overrepresentation of GO terms in clusters of similarly responding genes. Additionally, upstream promoters (defined as 3kb upstream of the transcriptional start site) of the

genes in each bin were recovered from the Promoser database. These promoters were then analyzed using PAINT, a program that automates promoter analysis of a set of genes for the presence of transcription factor binding sites, to look for statistically significant overrepresentation of canonical transcription factor binding sites suggestive of a dominant transcriptional program.

RESULTS AND DISCUSSION

Three cultures each of HUVECs expressing either empty vector or vFLIP were maintained until the vFLIP cultures were maximally spindled. At this point RNA was harvested and global gene expression was assayed by hybridization of each culture (three control, three vFLIP) against a common reference. Six individual microarrays were hybridized, and the data was extracted and analyzed as explained in the Methods section.

Analysis of gene expression in vFLIP expressing spindled HUVECS yielded differential expression of 3400 genes, 2000 upregulated genes and 1400 downregulated genes, as compare to HUVECs expressing vector alone and maintaining their typical cobblestone morphology. K means clustering was used to segregate genes into 10 discrete bins based on similar patterns of expression. This is displayed in a heat map cluster in Figure 1 where each column represents an individual microarray and each row represents an individual probe. Clusters 1,3,5,9 contain probes corresponding to genes downregulated in the context of vFLIP expression, while clusters 2,4,6,7,8,10 contain probes upregulated in the context of vFLIP expression.

Analysis of the common upstream biological processes represented in each cluster was done using the GO TermFinder. The results of these analyses for each cluster are summarized in column 4 of Table 1. Only GO terms that were significantly overrepresented in a cluster (p value $\leq$ 0.01), as compared to the human genome, were considered. Tables listing the overrepresented GO terms for each cluster are listed in Tables 2-9, clusters 1 and 5 did not have any overrepresented GO terms that met our statistical criteria, and therefore are not included.

Overwhelmingly, we found overrepresentation of GO terms involved in the immune response upregulated in vFLIP expressing spindle cells. This included GO

terms such as immune response, defense response, response to pest, pathogen or parasite, antigen processing and presentation, inflammatory response and chemotaxis, among others. Significantly, cluster 2 also showed a specific overrepresentation of genes involved in the NFκB pathway (Table 1). Cluster 3 was the only cluster of downregulated genes that corresponded to significant GO terms. These terms included mitotic cell cycle, M phase, cytoskeleton organization and biogenesis, cell proliferation and several metabolic systems. These analyses suggest that the global gene expression program of vFLIP expressing spindle cells is one of increased immune responsiveness and inflammation and decreased proliferation and metabolism.

In order to determine the dominant transcription factors involved in the gene expression profile of these spindle cells, we looked for overrepresentation of transcription factor binding site in the upstream promoters of genes in the 10 clusters. Upstream promoters from genes included in each cluster were analyzed for the overrepresentation of canonical transcription factor binding sites as compared to the human genome. The results of this analysis are summarized in Table 1, column 3. We found a highly significant overrepresentation of NFκB binding sites in the majority of clusters containing genes upregulated during vFLIP expression. This result is not surprising, as vFLIP is a known inducer of NFκB signaling (3, 11-13) through it's interactions with TRAF 2 and 3 (8) and the NEMO subunit of IKK (5).

Besides the several members of the NFκB family, OLF-1 and CDP transcription factor binding sites were also found to be overrepresented in clusters showing genes upregulated by vFLIP expression. OLF-1 also known as Early B cell Factor, is a transcription factor important for B cell and neuronal development and a promoter of adipogenesis (1). CDP, which stands for CCAAT displacement protein, is a transcription factor involved in passive transcriptional repression by competing with transcriptional

activators for binding of CCAAT sites. This protein is found in various precursor cells and is though to act to repress transcription of genes that are upregulated upon terminal differentiation of the cell (16). These results may point to additional functions of vFLIP activity, or more likely, are secondary targets of vFLIP induction of the NFκB transcriptional program.

Cluster 3 is the only cluster of downregulated genes to show evidence of significant transcription factor binding site overrepresentation. As shown in Table 1, binding sites for the AP-1 transcription factor are overrepresented in this cluster. AP-1 is generally characterized as activating a proliferative transcriptional program and is downstream of the MAPK signaling pathway (10). Inclusion of genes with AP-1 sites in their promoters within this cluster of repressed genes correlates with the GO terms associated with this cluster, which includes mitotic and metabolic processes. Together these results suggest an anti-proliferative phenotype for vFLIP expressing spindle cells. This is in accordance with our earlier study where we reported observing no increase in proliferation or cell lifespan in these spindle cells.

In conclusion, using microarray technology and informatics tools we have shown that the driving factor in the formation of spindle cells upon expression of the KSHV vFLIP gene is the activation of the NFκB signaling pathway, and it's downstream transcriptional targets. This is in agreement with our previous finding that inhibition of NFκB activity, by the treatment of cells with Bay 11-7082 or overexpression of the IκB-super repressor, blocks the development of a spindled morphology upon expression of vFLIP in HUVEC cells.

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FIGURE LEGENDS

Figure 1. **Heat map representation of control and vFLIP microarrays**.

Differentially expressed probes were identified and organized into 10 clusters as described in Methods section. Columns correspond to individual microarrays and rows correspond to gene probes. Green color denotes downregulation, red color denotes upregulation.

Table 1. **Summary of significantly overrepresented transcription factors and GO terms in individual clusters.**

Significant: p value ≤ 0.01

Tables 2-9. **List of statistically significant GO terms associated with individual clusters.**

Cluster 2 (Fig 2), Cluster 3 (Fig 3), Cluster 4 (Fig 4), Cluster 6 (Fig 5), Cluster 7 (Fig 6), Cluster 8 (Fig 7), Cluster 9 (Fig 8), Cluster 10 (Fig 9).

Figure 1.

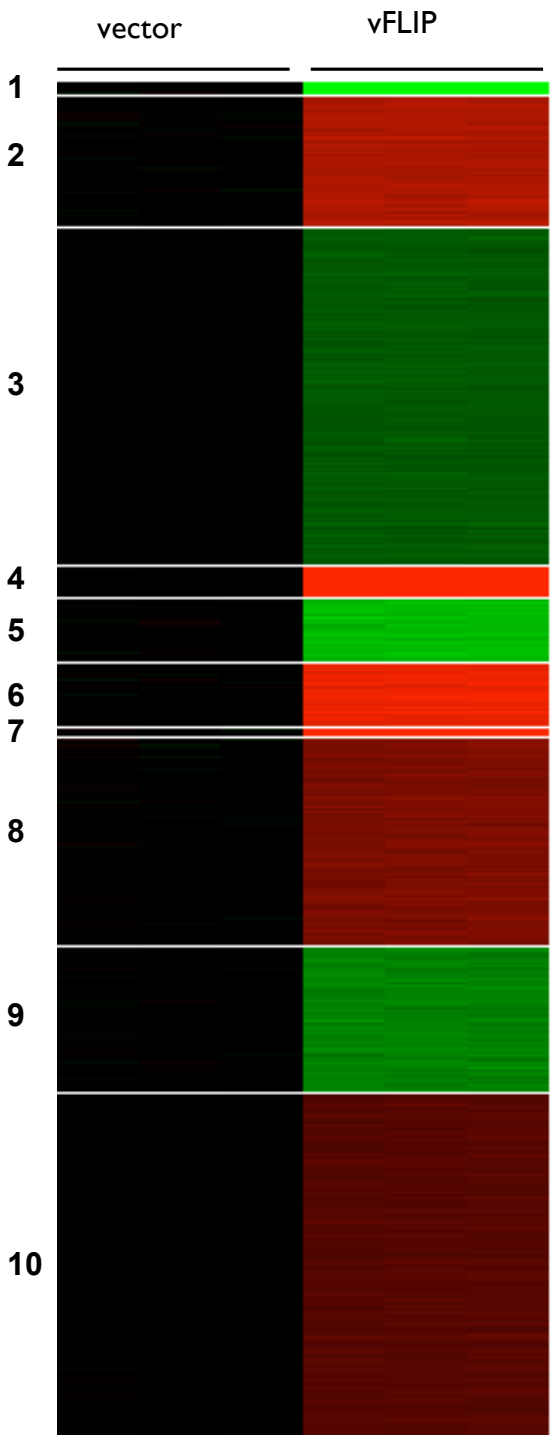

Table 1.

Cluster	Up/Down-regulation	Significant Transcription Factor(s)	Examples of Significant GO Terms
2	Up	NF-kappaB (p50) Olf-1	Immune response, Defense response, NF-kappaB
3	Down	AP-1	Mitotic cycle, Metabolism, Cytoskeletal organization
4	Up	NF-kappaB NF-kappaB (p50) c-Rel NF-kappaB (p65) NF-kappaB NF-kappaB	Immune response, Defense response, Antigen processing
6	Up	NF-kappaB NF-kappaB NF-kappaB (p65)	Immune response, Defense response, Antigen processing
7	Up	NF-kappaB (p65) CDP CR3+HD NF-kappaB NF-kappaB	Immune response, Defense response, Chemotaxis

Table 2

GO term description	Percent of query set	P-value
physiological process	143 of 281 (51%)	0.00E+00
biological_process	159 of 281 (57%)	0.00E+00
response to biotic stimulus	31 of 281 (11%)	0.00E+00
cellular physiological process	128 of 281 (46%)	0.00E+00
cellular process	148 of 281 (53%)	4.14E-09
primary metabolism	92 of 281 (33%)	4.32E-08
immune response	27 of 281 (10%)	6.34E-08
response to stimulus	43 of 281 (15%)	7.86E-08
metabolism	97 of 281 (35%)	1.41E-07
defense response	28 of 281 (10%)	1.98E-07
cellular metabolism	93 of 281 (33%)	2.66E-07
cell communication	59 of 281 (21%)	7.34E-07
regulation of biological process	58 of 281 (21%)	7.78E-07
protein metabolism	51 of 281 (18%)	2.67E-06
cellular macromolecule metabolism	51 of 281 (18%)	3.53E-06
cellular protein metabolism	50 of 281 (18%)	5.58E-06
regulation of cellular process	52 of 281 (19%)	2.02E-05
macromolecule metabolism	60 of 281 (21%)	2.21E-05
signal transduction	47 of 281 (17%)	5.03E-05
I-kappaB kinase/NF-kappaB cascade	9 of 281 (3%)	8.73E-05
organismal physiological process	36 of 281 (13%)	1.62E-04
positive regulation of I-kappaB kinase/NF-kappaB cascade	7 of 281 (2%)	7.66E-04
protein kinase cascade	12 of 281 (4%)	7.79E-04
regulation of cellular physiological process	46 of 281 (16%)	8.56E-04
positive regulation of biological process	17 of 281 (6%)	9.22E-04
regulation of physiological process	47 of 281 (17%)	9.46E-04
regulation of I-kappaB kinase/NF-kappaB cascade	7 of 281 (2%)	1.06E-03
positive regulation of signal transduction	7 of 281 (2%)	2.11E-03
apoptosis	15 of 281 (5%)	2.29E-03
programmed cell death	15 of 281 (5%)	2.51E-03
cell death	15 of 281 (5%)	4.79E-03
death	15 of 281 (5%)	5.07E-03
cell adhesion	16 of 281 (6%)	5.86E-03

Table 3.

GO term description	Percent of query set	P-value
mitotic cell cycle	30 of 750 (4%)	0.00E+00
M phase	27 of 750 (4%)	0.00E+00
cell cycle	54 of 750 (7%)	0.00E+00
physiological process	312 of 750 (42%)	0.00E+00
biological_process	359 of 750 (48%)	0.00E+00
metabolism	226 of 750 (30%)	0.00E+00
macromolecule metabolism	145 of 750 (19%)	0.00E+00
cellular metabolism	215 of 750 (29%)	0.00E+00
primary metabolism	208 of 750 (28%)	0.00E+00
cellular physiological process	298 of 750 (40%)	0.00E+00
cell division	20 of 750 (3%)	0.00E+00
cellular process	329 of 750 (44%)	3.82E-09
M phase of mitotic cell cycle	26 of 750 (3%)	5.75E-09
mitosis	26 of 750 (3%)	8.56E-09
cell organization and biogenesis	70 of 750 (9%)	9.47E-09
biopolymer metabolism	100 of 750 (13%)	1.12E-08
organelle organization and biogenesis	45 of 750 (6%)	6.63E-08
cytoskeleton organization and biogenesis	27 of 750 (4%)	1.67E-06
spindle organization and biogenesis	8 of 750 (1%)	1.86E-06
microtubule-based process	16 of 750 (2%)	4.98E-06
DNA metabolism	33 of 750 (4%)	1.15E-05
cell proliferation	33 of 750 (4%)	3.27E-05
regulation of cellular physiological process	101 of 750 (13%)	8.18E-05
regulation of physiological process	103 of 750 (14%)	1.27E-04
regulation of progression through cell cycle	28 of 750 (4%)	1.28E-04
cellular macromolecule metabolism	98 of 750 (13%)	1.34E-04
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	101 of 750 (13%)	1.41E-04
regulation of cellular process	104 of 750 (14%)	1.76E-04
microtubule cytoskeleton organization and biogenesis	9 of 750 (1%)	2.60E-04
regulation of biological process	109 of 750 (15%)	3.14E-04
protein metabolism	95 of 750 (13%)	5.57E-04
cellular protein metabolism	94 of 750 (13%)	7.07E-04
response to DNA damage stimulus	17 of 750 (2%)	1.26E-03
response to endogenous stimulus	17 of 750 (2%)	3.40E-03

Table 4.

GO term description	Percent of query set	P-value
defense response	19 of 66 (29%)	0.00E+00
immune response	19 of 66 (29%)	0.00E+00
response to biotic stimulus	22 of 66 (33%)	0.00E+00
response to stimulus	23 of 66 (35%)	0.00E+00
organismal physiological process	20 of 66 (30%)	3.03E-10
response to pest, pathogen or parasite	11 of 66 (17%)	5.20E-07
response to external biotic stimulus	11 of 66 (17%)	9.14E-07
response to external stimulus	12 of 66 (18%)	1.44E-06
physiological process	40 of 66 (61%)	4.05E-06
biological_process	43 of 66 (65%)	7.24E-06
antigen presentation, endogenous antigen	3 of 66 (5%)	1.91E-04
antigen processing, endogenous antigen via MHC class I	3 of 66 (5%)	1.91E-04
response to stress	11 of 66 (17%)	2.49E-04
antigen processing	3 of 66 (5%)	1.75E-03
antigen presentation	3 of 66 (5%)	5.05E-03
cellular process	35 of 66 (53%)	5.53E-03
cell communication	17 of 66 (26%)	8.73E-03

Table 5.

GO term description	Percent of query set	P-value
defense response	27 of 139 (19%)	0.00E+00
immune response	25 of 139 (18%)	0.00E+00
response to biotic stimulus	28 of 139 (20%)	0.00E+00
antigen presentation, endogenous antigen	6 of 139 (4%)	0.00E+00
antigen processing, endogenous antigen via MHC class I	7 of 139 (5%)	0.00E+00
response to stimulus	33 of 139 (24%)	0.00E+00
antigen processing	7 of 139 (5%)	3.65E-12
organismal physiological process	29 of 139 (21%)	7.67E-09
biological_process	81 of 139 (58%)	9.18E-08
physiological process	73 of 139 (53%)	2.49E-07
antigen presentation	6 of 139 (4%)	1.47E-06
response to external stimulus	15 of 139 (11%)	6.24E-05
response to pest, pathogen or parasite	12 of 139 (9%)	3.39E-04
cellular process	67 of 139 (48%)	5.17E-04
response to external biotic stimulus	12 of 139 (9%)	5.97E-04
cellular physiological process	59 of 139 (42%)	1.69E-03
response to stress	15 of 139 (11%)	2.12E-03

Table 6.

GO term description	Percent of query set	P-value
biological_process	24 of 24 (100%)	0.00E+00
organismal physiological process	14 of 24 (58%)	0.00E+00
response to stimulus	14 of 24 (58%)	0.00E+00
defense response	14 of 24 (58%)	1.50E-09
immune response	14 of 24 (58%)	1.58E-09
response to biotic stimulus	14 of 24 (58%)	3.14E-09
response to pest, pathogen or parasite	9 of 24 (38%)	5.49E-09
response to external biotic stimulus	9 of 24 (38%)	8.69E-09
response to external stimulus	9 of 24 (38%)	8.15E-08
physiological process	21 of 24 (88%)	2.04E-07
response to wounding	7 of 24 (29%)	6.05E-07
inflammatory response	6 of 24 (25%)	6.44E-07
response to stress	9 of 24 (38%)	9.79E-07
chemotaxis	5 of 24 (21%)	2.40E-06
taxis	5 of 24 (21%)	2.40E-06
cell surface receptor linked signal transduction	9 of 24 (38%)	2.98E-06
cellular process	20 of 24 (83%)	6.56E-06
response to chemical stimulus	6 of 24 (25%)	8.30E-06
response to abiotic stimulus	6 of 24 (25%)	1.94E-05
cell communication	11 of 24 (46%)	3.31E-04
signal transduction	10 of 24 (42%)	3.60E-04
regulation of inflammatory response	2 of 24 (8%)	9.14E-04
regulation of physiological process	10 of 24 (42%)	9.17E-04
regulation of biological process	10 of 24 (42%)	2.08E-03
G-protein coupled receptor protein signaling pathway	5 of 24 (21%)	5.39E-03
regulation of cellular process	9 of 24 (38%)	8.07E-03

Table 7.

Cluster

GO term description	Percent of query set	P-value
physiological process	204 of 459 (44%)	0.00E+00
biological_process	236 of 459 (51%)	0.00E+00
cellular physiological process	182 of 459 (40%)	0.00E+00
cellular process	210 of 459 (46%)	2.03E-09
cell communication	75 of 459 (16%)	3.52E-04
positive regulation of biological process	23 of 459 (5%)	4.23E-04
positive regulation of cellular process	21 of 459 (5%)	4.67E-04
signal transduction	63 of 459 (14%)	6.22E-04
regulation of biological process	72 of 459 (16%)	1.05E-03
response to stress	32 of 459 (7%)	1.10E-03
protein kinase cascade	15 of 459 (3%)	1.11E-03
regulation of cell proliferation	15 of 459 (3%)	1.78E-03
establishment of localization	60 of 459 (13%)	2.22E-03
response to external stimulus	26 of 459 (6%)	2.25E-03
regulation of cellular process	67 of 459 (15%)	2.32E-03
localization	60 of 459 (13%)	2.46E-03
regulation of physiological process	64 of 459 (14%)	8.46E-03
transport	54 of 459 (12%)	8.77E-03
cell proliferation	20 of 459 (4%)	9.07E-03

Table 8.

GO term description	Percent of query set	P-value
biological_process	152 of 327 (46%)	3.65E-05
cell communication	54 of 327 (17%)	6.29E-03
cellular process	131 of 327 (40%)	6.70E-03
phosphorus metabolism	20 of 327 (6%)	1.00E-02

Table 9.

GO term description	Percent of query set	P-value
biological_process	334 of 747 (45%)	0.00E+00
protein metabolism	107 of 747 (14%)	1.34E-07
cellular physiological process	264 of 747 (35%)	2.75E-07
cellular protein metabolism	105 of 747 (14%)	3.58E-07
physiological process	285 of 747 (38%)	6.70E-07
cellular macromolecule metabolism	105 of 747 (14%)	8.90E-07
cellular process	294 of 747 (39%)	1.52E-06
regulation of apoptosis	24 of 747 (3%)	8.11E-06
regulation of programmed cell death	24 of 747 (3%)	1.03E-05
biopolymer modification	65 of 747 (9%)	1.26E-05
protein modification	63 of 747 (8%)	2.29E-05
primary metabolism	183 of 747 (24%)	1.01E-04
macromolecule metabolism	123 of 747 (16%)	1.18E-04
cell death	30 of 747 (4%)	1.90E-04
death	30 of 747 (4%)	2.10E-04
metabolism	195 of 747 (26%)	3.21E-04
positive regulation of biological process	31 of 747 (4%)	5.07E-04
cellular metabolism	186 of 747 (25%)	5.22E-04
establishment of cellular localization	32 of 747 (4%)	5.98E-04
establishment of protein localization	32 of 747 (4%)	6.25E-04
positive regulation of cellular process	28 of 747 (4%)	6.27E-04
cellular localization	32 of 747 (4%)	7.45E-04
positive regulation of signal transduction	11 of 747 (1%)	9.23E-04
protein localization	32 of 747 (4%)	9.64E-04
intracellular protein transport	24 of 747 (3%)	1.33E-03
intracellular transport	31 of 747 (4%)	1.40E-03
apoptosis	27 of 747 (4%)	1.56E-03
programmed cell death	27 of 747 (4%)	1.80E-03
establishment of localization	88 of 747 (12%)	2.07E-03
protein amino acid phosphorylation	29 of 747 (4%)	2.20E-03
localization	88 of 747 (12%)	2.36E-03
response to unfolded protein	8 of 747 (1%)	2.80E-03
protein kinase cascade	19 of 747 (3%)	2.91E-03
signal transduction	89 of 747 (12%)	3.29E-03
intracellular signaling cascade	44 of 747 (6%)	5.09E-03
phosphorylation	31 of 747 (4%)	5.92E-03
protein transport	29 of 747 (4%)	6.46E-03