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Theme Issue Article

The contribution of systems biology and reverse genetics to the understanding of Kaposi's sarcoma-associated herpesvirus pathogenesis in endothelial cells

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Summary

Kaposi's sarcoma-associated herpesvirus (KSHV) / human herpesvirus-8 is the causative agent of the endothelial cell-derived tumour Kaposi's sarcoma. Herpesviruses possess large complex genomes which provide many options to regulate cellular physiology during the viral life cycle and in the course of tumourigenicity. Novel techniques of systems biology and reverse genetics are increasingly applied to dissect the complex interaction of KSHV with endothelial cells. This review will outline novel results and pitfalls of these technologies in the elucidation of KSHV pathogenicity.

Keywords

Endothelial cells, angiogenesis, apoptosis, NF-kappaB, interferon

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Introduction

Kaposi's sarcoma (KS) is a tumour that occurs in four different epidemiologic forms (AIDS-KS, post-transplant KS, African KS and classic KS). Of these, AIDS-KS is best known due to its dramatically and unexpectedly increasing incidence in industrialised countries in the early 1980s, which heralded the outbreak of the pandemic AIDS. The apparent association of KS with infections by the human immunodeficiency virus-1 (HIV-1) suggested an infectious etiology of this tumour. This was confirmed in further studies. However, rather than HIV-1, a formerly unknown herpesvirus turned out to be the causative agent of KS (1). The respective herpesvirus was called Kaposi's sarcoma-associated herpesvirus (KSHV) or alternatively human herpesvirus 8 (HHV-8). It was found to be associated with all of the different epidemiologic forms of KS and also with the lymphoproliferative diseases primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (2–5). Today it is generally accepted that KSHV is required for KS development, but that, with the possible exception of classic KS, it is not sufficient to induce the disease (6). The requirement of specific co-factors, such as HIV-1 infection in the case of AIDS-KS, is illustrated by the significant decline of KS incidence after the introduction of highly active anti-retroviral therapy (HAART) for treatment of HIV-1 infection (7). Nevertheless, KS is still among the most commonly observed tumours in certain regions of central Africa and a clinical threat for organ transplant recipients (8, 9). Therefore, understanding the role of KSHV in KS pathogenesis is of clinical importance and the subject of intense research.

Unfortunately, KSHV research is severely hampered by the lack of an appropriate animal model system in which KSHV transmission causes KS. Moreover, the large KSHV genome, en-

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coding more than 86 genes, provides a myriad of possibilities to interfere with cellular signalling cascades in the course of the viral life cycle and thereby become tumourigenic. Modern technologies of systems biology and reverse genetics are increasingly applied in order to dissect the complex interaction of KSHV with infected cells. In the following we will summarise available results and potential pitfalls of these approaches with a focus on studies in endothelial cells, which are most relevant for KS pathogenesis.

Systems biology approaches to KSHV pathogenesis

In the past decade important progress has been made with regard to the availability of modern systems biology techniques to analyse the pathogenic activity of infectious agents in eukaryotic cells. This includes high throughput approaches which allow the analysis of the complete gene expression pattern of mammalian cells and tissues at the transcriptome level with array technology (10), methods to determine the complete proteome of mammalian cells using high throughput mass spectrometry, protein microarrays and differential 2D-gel electrophoretic procedures (11, 12). Furthermore, it is possible to perform high throughput analyses of protein interactions with yeast and mammalian two-hybrid analysis and the tandem affinity purification (TAP)-tag method (13–16) and high throughput transfection methods at the microarray level to analyse gene functions in living cells (17). All of these novel techniques have been applied in the past years in order to determine the pathogenic function of KSHV, both in endothelial and in lymphatic cells.

Systematic analyses of KSHV gene expression

The first systematic analysis of gene expression in the KSHV field aimed at the identification of the major viral genes expressed in KS and latently KSHV-infected BCBL-1 cells (18), which were obtained from PEL (19). To this end, a library of the KSHV genome consisting of nine overlapping genomic subfragments was cloned in the bacteriophage lambda. The DNA was digested and transferred to nylon filters and hybridised with radiolabelled cDNA probes obtained from reversely transcribed mRNA from KS and from BCBL-1 cells. This study indicated that KSHV gene expression in the non-replicating phase is restricted to few genes. A 1.1 kb transcript and a 0.7 kb transcript were both highly expressed in KS and BCBL-1 cells. T1.1 turned out to be a non-coding polyadenylated nuclear (PAN) RNA (also known as nut-1) encoded by the K7 gene. T1.1 is the most abundant transcript of KSHV both in latent and lytic infection (20). T0.7-mRNA was shown in subsequent studies to encode a splice variant of the K12 gene, which encodes kaposin, a major latent gene of KSHV that is expressed highly in KSHV-infected cells *in vivo* and *in vitro* (21, 22).

Another landmark paper before the microarray era investigated the transcription pattern of KSHV genes in another PEL cell line (BC-1), both after stimulation of the lytic replication cycle by tetradecanoylphorbol acetate (TPA) and in latently infected cells (23). Conventional northern blot analyses using large probes that in most cases were not specific for individual genes were used in this study (23). The work showed that the viral gene expression program can be categorised into three different classes: (i) class I genes, which are constitutively transcribed and not affected by TPA. These included v-cyclin (vCYC/orf72), latency associated nuclear antigen-1 (LANA-1/orf73) and the viral Fas-associated death-domain like IL-1β-converting enzyme (FLICE)-inhibitory protein (vFLIP/K13). (ii) Class II genes, which are transcribed prior to the activation of the lytic cycle but are induced to a higher transcription level during the lytic cycle (for example: nut-1/K7, T0.7/kaposin/K12, vIL-6/K2, vMIP-II/K4, vIRF-1/K9). (iii) Class III genes were primarily structural and replication genes (major capsid protein/orf25, minor capsid protein/orf26, orf6, orf21) and were transcribed only following TPA treatment (23).

Array technology was used for the first time to analyse the transcription program of KSHV in PEL cell lines (BC-3 and BCBL-1) after TPA induction in three studies (24–26). Several different time points (0–96 hours [h] [25] or 0–72 h [24], respectively) were used after TPA induction. Target sequences were generated by PCR from each single KSHV open reading frame (orf). These were either spotted as approximately 350 bp fragments on a nylon membrane (24) or as 200 – 1000 bp fragments on poly-L-lysine glass slides (25).

These approaches allowed the specific characterisation of the expression of each single KSHV-encoded gene. The results obtained in the three studies were largely in agreement with each other and with the results obtained by Sarid et al. (23). All studies confirmed the region between the K13 gene and orf73 as the major latently expressed locus of KSHV. Most genes with predicted immunomodulatory functions were grouped as immediate early or early genes (e.g. vIL-6/K2 and vMIP-II/K4) while most structural genes (e.g. the capsid proteins orf25 and orf26) were expressed late in the lytic cycle.

The first study investigating viral gene expression in endothelial cells found a concurrent expression of latent and a limited number of lytic genes shortly after the infection of the cells (27). Interestingly, genes with immunomodulatory (vIL-6/K2, vMIP-II/K4, vMIR-2/K5, vMIP-I/K6 and vIRF-2/K11) and antiapoptotic (vIAP/K7) functions were expressed early (2 h) after infection together with the latent genes. The viral modulator of immune recognition-2 (vMIR-2/K5) for example has been shown to inhibit MHC-I, ICAM-1, and B7–2 expression thus preventing elimination of infected cells by T- and NK-cells (28). The expression levels of these genes started to decline already at 8 h after infection (27). These findings suggested that the temporally limited expression of genes with immunomodulatory and antiapoptotic functions shortly after the virus has entered the cell may be crucial for the establishment of latent KSHV infection. In addition, differences between the expression pattern in KSHV-infected human microvascular endothelial cells (MVEC) and BCBL-1 cells in the presence of lytic induction were observed. Expression of the orfs 32, 44, and 49 was detected in MVEC but not in BCBL-1 cells, whereas expression of SOX/orf37 was detected in BCBL-1 cells but not in MVEC. These results suggested that the transcriptional program of KSHV may be different in endothelial cells as compared to lymphatic cells, as has been observed for the differential expression of the latency locus encoding vFLIP/K13, vCYC/orf72 and LANA-1/orf73 in

KS tissues and lymph nodes *in vivo* (29). In addition, the differential expression of SOX/orf37 may be meaningfull, because this gene regulates the shut-off of host transcription during the lytic cycle of KSHV (see below).

Impact of KSHV infection on the transcriptome of endothelial cells

The majority of transcriptome analyses investigated the impact of KSHV infection on the transcription pattern of the host cells. Since KSHV-related diseases affect endothelial cells or B-lymphocytes, these two cell types were mainly used in these studies, both in the presence of lytic and latent infection. However, it became evident that virus infection induces significantly different responses of cellular gene expression in both cell types, specifically in lytic infection. This may be partly due to the diffential expression of SOX/orf37 in both cell types. We will focus on studies performed in latently infected endothelial cells.

In a first report, KSHV-infected MVEC were compared to uninfected control cells (30). The authors used two commercially available cDNA arrays representing 2,350 and 9,182 human genes, respectively. In both arrays between 1.0 and 2.2% of all represented genes were up-regulated and between 1.2 and 1.8% were down-regulated more than two-fold. The most notable result arising from these studies was a significant up-regulation of a large number of interferon-induced genes (STAT1, IFI 6–16, MxA, IFI 9–27). In addition, regulated genes were involved in cytoskeleton arrangement, apoptosis, angiogenesis, and immune regulation. The authors observed a reasonable correlation of the most highly regulated genes on both arrays. Genes that were detected to be regulated in both arrays encoded a G-protein-coupled receptor (RDC1, up-regulated), bone morphogenic protein-1 (BMP1, down-regulated), endothelial plasminogen activator inhibitor-1 (PAI1, down), connexin 37 (down), bone marrow stromal antigen-1 (BMS-1, down), cystein-rich angiogenic inducer (IGFBP10/cyr61, down), BMP6 (down), and thrombin receptor (down), indicating that consistency was mostly due to down-regulated genes. However, significant differences between the arrays were also seen. This is most evident comparing the 20 most strongly up-regulated genes in both studies, which only overlapped in the expression of RDC1 (Table 1). However, this partly may be due to non-overlapping sets of genes used in both arrays.

In a similar approach Moses et al. used E6/E7-MVEC which were immortalised with the human papillomavirus (HPV) genes E6 and E7 (31) and infected these cells with KSHV (32). The authors controlled infection and showed that after four weeks more than 90% of the cells were latently infected. Using a cDNA microarray harboring 4,165 human genes two KSHV-infected E6/E7-MVEC lines were compared to uninfected control cells. Since differences in the two lines became apparent, only those genes were considered that were up- or down-regulated at least 1.8-fold in both lines. This resulted in 184 genes that were significantly regulated. In contrast to the study by Poole et al. (30), a massive induction of interferon-response genes was not observed in these experiments. A possible explanation for this difference could be that E6 and E7 may counteract interferon-inducing activity of KSHV. Genes up-regulated by KSHV in E6/E7-MVEC had functions in leukocyte recruitment (MCP-1, CCL14), cell shape regulation (neuritin, CDC42), angiogenesis (VEGF-R2, CD36, plasminogen activator inhibitor 2), cell growth (PCNA, CDC6 homologue) and tumourigenesis (PPARγ, c-Kit, c-Mer, Jun-D). The authors specifically focused on the up-regulated proto-oncogene c-Kit, a receptor tyrosine kinase for the ligand stem cell factor (SCF). They showed that treatment of infected E6/E7-MVEC with exogenous SCF increased proliferation as compared to uninfected E6/E7-MVEC. Inhibition of c-Kit activity with the inhibitor STI 571 decreased proliferation in the presence of SCF and ectopic expression of c-Kit in non-infected E6/E7-MVEC induced a similar morphological transformation as seen in E6/E7-MVEC after infection with KSHV. This finding suggested that c-Kit may have an essential role in KS tumourigenesis.

In a follow-up study the same group analysed the same cellular system of immortalised KSHV-infected E6/E7-MVEC with a different, commercially available microarray containing 12,626 probe sets in order to find additional genes involved in the spindle cell transformation process (33). Comparison of two KSHV-infected E6/E7-MVEC lines with uninfected control cells led to the identification of 97 up-regulated genes. Of these, 36 were found either by Poole et al. or by the authors themselves before (30, 32). In order to identify new genes involved in tumourigenesis the authors focused on six up-regulated genes [RDC1, c-Kit, Neuritin 1, Lim-domain only 2 (LMO2), Oseopontin (SPP1), c-Mer] and investigated whether the inhibition of these genes with antisense oligonucleotides may result in significant inhibition of focus formation in soft agar and of cell proliferation. Inhibition of c-Kit and Neuritin 1, which were both also found to be up-regulated by a previous study of these authors and of the G protein-coupled receptor RDC1 (also found to be up-regulated in the study by [30]) led to the strongest inhibition of focus formation and proliferation of KSHV-infected MVEC. Small interference RNA (siRNA) mediated knockdown of RDC1 and Neuritin 1 confirmed the antisense experiments. In addition, the authors showed that over-expression of Neuritin 1 and RDC1 increased the tumourigenicity of NIH 3T3 in nude mice. Finally, it was shown that RDC1 and Neuritin 1 expression levels were comparable to those in KSHV-infected MVEC. Altogether, these well controlled results suggested that RDC1, Neuritin 1 and c-Kit may be functional targets of the pathogenic activity of KSHV in KS.

It is known that KSHV enters target cells within minutes of infection and regulates pre-existing host cell signal pathways (34, 35). In order to characterise the early cellular response to KSHV infection Naranatt et al. used an oligonucleotide microarray representing 22,283 transcripts to screen for those host genes that are modulated immediately (2 and 4 h) after infection (36). The authors included MVEC, human foreskin fibroblasts (HFF) and a human B cell line (BJAB) in their study. The percentage of genes to be modulated at least two-fold was comparable (1.72%) to the previous studies (30). Of note, more genes were regulated in MVEC and HFF as compared to BJAB. Only 33 genes were found to be regulated across all cell types. Many more genes were regulated in a cell type specific manner (between 102 and 239). A total of 154 genes were modulated in the same way in at least two cell types. Interestingly, the correlation of KSHV-induced transcription profiles was 10 times higher be-

Table 1: Cellular transcripts up-regulated in KSHV-infected endothelial cells.

Rank order	Poole et al., 2002[1]	Poole et al., 2002[2]	Moses et al., 2002[3]	Naranatt et al., 2004[4]
1	Neurogranin	Incyte EST	CC-chemokine 14 (CCL14, SCYA14)	Prostaglandin-endoperoxide synthetase 2 (Cox2)
2	Calgranulin B (migration inhibitory protein 14	**RDC1 orphan G-protein-coupled receptor (CXCR7)**	CD36 (thrombospondin receptor)	*Interleukin 6*
3	CD14 monocyte differentiation protein	Antigen identified by monoclonal antibody MRC OX-2	Similar to ZN91 (H69048)	***SOCS-3 (suppressor of cytokine signalling-3)***
4	Fibrinogen B beta	*MxA*	Novel (R69677)	Stanniocalcin
5	Lipopolysaccharide binding protein	***SOCS-3 (suppressor of cytokine signalling-3)***	Similar to developmental protein (W02617)	Dual specificity phosphatase 5
6	*IRF7 (interferon regulatory factor 7)*	*Interferon-induced protein 56 (IFI-56)*	*MCP-1 (monocyte chemotactic protein-1, CCL2, SCYA2)*	Matrix metalloproteinase 1
7	**RDC1 G-protein-coupled receptor (CXCR7)**	*Interferon-induced transmembrane protein 3 (1–8U)*	Cytochrome P450, subfamily I	Solute carrier family 2
8	Homeobox protein HB24	PLAT (tissue plasminogen activator)	Coxsackie virus/ adenovirus receptor	*IFN-stimulated T-cell alpha chemoattractant (CXCL11, SCYB11)*
9	Ubiquitin-conjugating enzyme E2H10	Mal. T-cell differentiation protein	Endothelial cell multimerin precursor	Early growth response 1
10	Osteopontin	*Interferon alpha-inducible protein (IFI-6–16)*	von Willebrand factor	*Interleukin 1β*
11	Estrogen-related receptor alpha	IGFBP5 (insulin-like growth factor binding protein 5)	jun-D proto-oncogene	Jun B
12	TIMP1	Thromboinodulin	Novel (R63109)	*ICAM1*
13	Steroid 5-alpha reductase 1	Paired basic amino acid cleaving system 4	Neuritin	Early growth response 3
14	Calgizzarin	Complement component 1, r subcomponent	Apolipoprotein D	Urokinase plasminogen activator receptor precursor (UPAR)
15	*RIG-E (stem cell antigen 2)*	Nucleoside phosphorylase	Novel (H80158)	Sprouty (Drosophila) homologue 4
16	CBL-B	α2-Macroglobulin	*Interleukin 1 receptor, type 1*	Integrin, α2
17	Dual-specificity phosphatase-7	TGFβ3	LIM domain only 2 (rhombotin-like 1)	Dual specificity phosphatase 6
18	Leukocyte elastase inhibitor	γ-Tubulin complex protein 2	Solute carrier family 19, member 1	Hypothetical MGC:5618 (BF575213)
19	*MIP3b (macrophage inflammatory protein, CCL23, SCYA23)*	*Interferon-induced transmembrane protein 1 (9–27)*	Similar to nonmuscle myosin	*Superoxide dismutase*
20	HI05E3 protein	*IL-13*	Amyloid beta precursor protein	Phosphoprotein regulated by MAPK pathways

Genes detected in at least two different studies are given in bold. Genes involved in the interferon and/or cytokine response are given in italics. [1]Adult human dermal microvascular endothelial cells (MVEC) were infected for three weeks with KSHV (> 90% of cells latently infected) and compared to non infected cells using Clontech Human Array 1.2-II nylon filters which contains 2,350 probe sets. [2]MVEC were infected for two weeks by co-cultivation with one-tenth of infected cells described in footnote 1 (> 90% of cells latently infected) and compared to non-infected cells using Incyte Human UniGEM V2.0 microarrays containing 9,182 sequence-veryified human cDNA clones. [3]MVEC immortalized by retroviral expression of the E6 and E7 genes of HPV type 10 were infected for four weeks with KSHV (> 90% of cells latently infected) and compared to non-infected cells using arrays of 4,165 sequence verified IMAGE clones. Ranking was determined according to the mean of two independent experiments. Novel genes are given with gene ID. [4]MVEC were infected with KSHV (at five viral genome copies/cell) for 4 h and compared to non infected cells using the human genome HG-U133A (Affymetrix, Santa Clara, CA) chip which represents 22,283 human genes.

tween MVEC and HFF than between MVEC and BJAB. A significant difference between MVEC and HFF on one side and BJAB on the other side was noted by the finding that in the two first cell types a robust increase of IFN-induced genes including OAS2, MxA, IFN regulatory factor (IRF)-1 and –7, IFN-induced transmembrane protein-1, IFN-induced protein (IFI)-15 and guanylate binding protein (GBP)-1 was observed suggesting the induction of the innate immunity system. The increased expression of the large GTPase GBP-1 is of specific interest, because GBP-1 is induced in endothelial cells by the cytokines present in KS (37) and was found to be expressed in KS tissues *in vivo* (38). GBP-1 mediates the antiangiogenic effects of inflammatory cytokines on endothelial cells by inhibiting endothelial cell proliferation (39), migration (40) and invasion (38) *in vitro* and *in vivo* (41). Therefore, induction of GBP-1 may explain the highly significantly reduced proliferation rates of KSHV in-

fected as compared to non-infected endothelial cells in KS tissue sections (Zietz and Stürzl, unpublished observation).

Interestingly, the increase of such genes was not seen in the lymphatic cell line BJAB, and the authors suggested that host gene modulation may be different compared to the investigated adherent cells. However, the observed differences in gene expression may be due to different susceptibility of these cells to infection, a parameter which has not been analysed in this study.

Angiogenic molecules induced by KSHV infection in MVEC included VEGF-A, -C, thrombomodulin, matrix metalloproteinase-1, angiopoietin-related protein 4 and urokinase-type plasminogen activator receptor; of these the latter and VEGF-A have been shown to be expressed in KS tissues (42, 43). A comparison with the data of Poole et al. (30) – in this study MVEC were infected with KSHV for several weeks instead of 2 or 4 h and a different microarray was used – showed that 10 genes were consistently up-regulated in both studies including the genes encoding suppressor of cytokine signalling (SOCS)-3, vEts, tissue plasminogen activator, IL-8, BCL-3, nucleoside phosphorylase and tissue inhibitors of metalloproteinase-1. In addition, there was agreement with respect to the activation of the IFN-response; however, different interferon-responsive genes were activated in both studies. A comparison of the 20 strongest up-regulated genes in the studies by Poole (30), Moses (32) and Naranatt (36) only revealed two genes which were overlappingly found in at least two studies, RCD1 and SOCS-3 (Table 1). This difference may reflect different modes of infection and non-overlapping sets of gene probes used in the different arrays. Considerating that numerous genes involved in inflammatory cytokine and interferon responses were increased in all studies (Table 1, genes given in italics), the up-regulation of SOCS-3 deserves specific attention. SOCS-3 is induced by various cytokines, including IL-6, IL-10, and IFNγ and known as a negative regulator of cytokine signalling (44). For example, SOCS-3 is essential in controlling the response to IL-6 (45) and G-CSF (46). In this framework it will be interesting to investigate whether SOCS-3 may partly counteract the antiangiogenic activities of certain cytokines in KSHV-infected endothelial cells in the pathogenesis of KS.

Several studies used microarray analyses with a focus on subsets of genes with biologically relevant activities in the framework of KS pathogenesis. These studies investigated the cellular differentiation and the angiogenic expression profile of KSHV-infected endothelial cells. The impact of KSHV infection on endothelial cell differentiation is of specific importance because immunohistochemical analyses investigating the cellular origin of KS spindle cells did not provide unequivocal answers as to whether KS may originate from lymph vessel or blood vessel endothelial cells (47–49). In order to address this question the gene expression of samples from KS, normal skin and various cell cultures including blood vessel endothelial cells and lymphatic endothelial cells was compared on oligonucleotide microarrays (22,283 probe sets) (50). A total of 1,482 genes differentiated KS from normal skin. Interestingly, this gene expression pattern was more related to lymphatic endothelial cells as compared to any other cell type tested. Of this signature 114 genes were selected, which discriminated lymphatic endothelial cells from blood vessel endothelial cells. Applying this signature to KSHV-infected lymphatic and blood vessel endothelial cells showed that the transcriptional program of the infected lymphatic endothelial cells moved closer to the program of uninfected blood vessel endothelial cells, whereas the transcription pattern of infected blood vessel endothelial cells moved closer to that of uninfected lymphatic endothelial cells. These findings were strongly supported by the fact that PROX1, the master regulatory transcription factor of lymphatic endothelial cell differentiation (51), was down-regulated in KSHV-infected lymphatic endothelial cells. These results were confirmed in a parallel study which used the same array technology to investigate gene expression in KSHV-infected MVEC (52). Infection of these blood vessel endothelial cells resulted in a significant up-regulation of lymphatic lineage specific genes including PROX1 (mean 2.15-fold), the lymphatic endothelial specific hyaluronan receptor LYVE-1 (mean 5.9-fold) and angiopoietin-2 (Ang-2). Ang-2 is involved in angio- and lymphangiogenesis and was also up-regulated by KSHV in MVEC (30). Altogether both studies demonstrated that KSHV can infect both lymphatic and blood vessel endothelial cells and drives the gene expression profile of each type closer to the other. This indicated that KSHV infection may either induce a transdifferentiated phenotype in endothelial cells or that KSHV may infect an undifferentiated endothelial precursor cell *in vivo* and may drive these cells toward a lymphatic endothelial cell phenotype.

In another study six KSHV-infected pools of lymphatic endothelial cells were compared to six uninfected pools of these cells with a commercially available microarray analysis (47,000 probe sets) (53). A specific focus was put on 239 angiogenesis-related genes. Of these genes, 33% were significantly affected upon KSHV infection in lymphatic endothelial cells. Again, Ang-2 was among the strongest up-regulated genes. Ang-2 is thought to destabilise endothelial cells interaction and to prime endothelial cells to respond to angiogenic stimuli, such as VEGF (54). It also plays an important function in lymphangiogenesis (54) and regulates the expression of inflammatory proteins in endothelial cells (55). In addition, the lymphatic angiogenesis factor VEGF-C, different VEGF receptors including VEGFR-3 (the receptor for VEGF-C [56, 57]), and members of the matrix metalloproteinase family were up-regulated in infected lymphatic endothelial cells. This indicated that both, autocrine and paracrine angiogenic activities, may be induced by KSHV infection of endothelial cells.

A recent study clearly underscored that specific care is required when the transcriptomes of lytically infected cells are analysed. This is based on the fact that shut-off of host transcription is a common phenomenon during lytic replication of herpesviridae (58). SOX/orf37, a homologue of a DNA exonuclease, is the viral protein responsible for shut-off of host transcription during the lytic cycle of KSHV (59). In order to detect cellular genes that may escape KSHV shut-off telomerase immortalised human microvascular endothelial cells were used as a model and infected with KSHV. Lytic replication was induced by infection with an adenovirus construct encoding KSHVRTA/orf50. Gene expression of non-infected cells and infected cells at different timepoints after lytic induction was compared with a microarray approach that identified 20,087 unique genes. In order to normalise for global changes that may occur in the transcriptome of

Table 2: Strongest up-regulated transcripts by vFLIP/K13 in HUVEC.

Gene symbol	Rank order[1] according to Sakakibara et al., 2009	Rank order (and fold induction) according to Thurau et al., 2009
CCL5 (CCL5)	1	1 (929)
TNF-α-induced p6	2	6 (212.3)
CCL3	3	8 (162)
CXCL5	4	21 (58.9)
CCL20	5	22 (58.1)
TNFR superfamily 11b	6	23 (56.9)
Kynureinase	7	24 (51.6)
CXCL10	8	30 (44)
Diubiquitin	9	33 (38.3)
C1S	10	37 (30.3)
MxA	11	38 (26.7)
CCL8	12	47 (19)
CXCL11	13	49 (18.3)
IFN-inducible p44-like	14	66 (11.3)
XIAP-associated factor-1	15	154 (4.3)
IL-27b	16	not detected
LOX1	17	not detected
IFN-ind. prot. with TRP1	18	not detected
MxB	19	not detected
Galectin 3-BP	20	not detected

[1] Ranking was determined according to the mean values of induction observed in two different array experiments. "not detected" indicates that the gene was not included in the list of the 173 most strongly up-regulated transcripts (< 4-fold) by vFLIP/K13 in HUVEC.

KSHV-infected cells, the hybridisation results were normalised to the signals of 10 different *in vitro*-synthesised transcripts added to an equal amount of total RNA of each sample (59). Using this stringent controls almost 98% of the host transcripts remained at or below pre-infection levels during KSHV lytic life cycle. Interestingly, about 2% of the host transcripts escaped the shut-off and were induced during lytic phase. Among the up-regulated transcripts were angiogenesis-related genes including Ang-2, angiopoietin-like polypeptide, ephrins A1 and B2, endothelin convertase and serpin B2 plasminogen activator inhibitor 2. Therefore, Ang-2 is not only expressed under latent conditions as shown by Vart et al. (53), but also during lytic infection. A second cluster of induced genes included several interferon-inducible genes like IFI 6–16, IFI 15, IFI 27, and GBP-2. In addition, a profound induction of IL-6 and CXCL2 cytokines was noted. Further analysis showed that escape from SOX-mediated degradation was not exclusively due to the presence of AU-rich elements (ARE) in the 3'-untranslated regions (UTR) (59), indicating that the mechanisms of the resistance to viral shut-off still have to be determined.

Global effects of single KSHV genes on the endothelial cell transcriptome

In order to determine KSHV genes that may regulate the transcriptional responses of KSHV-infected cells, gene expression patterns in response to individual KSHV-encoded genes were investigated using array technology.

Among the first studies to analyse the effect of single KSHV genes in endothelial cells was the study of Polson et al. (60). They transiently expressed the viral G-protein coupled receptor (vGPCR/orf74) in the endothelial cell line SLK, which was established from an HIV-negative classical KS tumour (61). Microarray analysis was carried out 48 h after transfection with a chip containing 20,000 human cDNA probes. Comparison between transfected cells and untransfected cells showed that in vGPCR/orf74-expressing SLK cells 33 human genes were up-regulated between 1.8- and 4.3-fold. However, vGPCR/orf74 is a lytic gene of KSHV, and it was shown subsequently by the same group that, with the exception of IL-6, all of the other genes were subject to the transcriptional shut-off regulated by KSHV (62).

In order to overcome the pitfalls of virus-induced shut-off we focused on the transcriptome analysis of a major latent gene of KSHV, vFLIP/K13. vFLIP/K13 is an important antiapoptotic protein and the major nuclear factor (NF)-κB inducer of KSHV [63–67]. vFLIP/K13 was stably expressed in human umbilical vein endothalial cells (HUVEC). A microarray analysis with an oligonucleotide array covering more than 47,000 transcripts identified 173 genes to be up-regulated more than four-fold in vFLIP/K13-expressing cells. Many of the genes belonged to the group of IFN-response genes such as MxA, OAS2, IRF1 and GBP family, which have all been confirmed by others to be up-regulated in KSHV-infected endothelial cells (see above [30, 36]). Chemokines were found to be among the most highly induced genes including CCL3, CCL5, CCL20, CXCL5,

CXCL10, and CXCL11. Of note, the latter two are well known to have antiangiogenic activity (68). Surprisingly few genes involved in the regulation of apoptosis were detected. Altogether the vFLIP/K13-modulated host transcription pattern resembled more an inflammatory and defense state than an antiapoptotic response in endothelial cells. Of note, basically an identical study as ours was published shortly afterwards by Sakakibara et al. (69). Notably, a striking concordance of results was obtained in both studies. Of the 20 most highly up-regulated genes by vFLIP/K13 in the study (69), 15 were significantly up-regulated in endothelial cells in our study ([70], Table 2). In both studies the chemokine CCL5 (Rantes) was most strongly induced by vFLIP/K13. Rantes is a major chemoattractant for monocytes. In addition, it functions as one of the natural ligands for the chemokine receptor CCR5 and it suppresses replication of the monocytotrophic R5 strains of HIV-1, which use CCR5 as a co-receptor (71). This may explain why very high concentrations of monocyes are present in KS lesions (6, 72) and why despite this fact HIV-1 infection is rarely observed in AIDS-KS lesions (6, 73). Altogether, the concordance of both studies clearly indicates that a high reproducibility can be achieved in microarray analyses in case standardised technology of cell culture and gene transfer have been established.

Proteomic approaches to elucidate KSHV biology

Far fewer proteomic than genomic approaches have been used so far to elucidate the biology of KSHV. Zhu et al. (74) used 1D-gel electrophoretic separation technique with subsequent mass spectrometric analysis to identify all viral proteins that compose the virus particle of KSHV. The herpesviral virion consists of four morphologically distinct compartments: the inner nucleoprotein core containing the viral DNA, the icosahedral capsid covering the viral core, the outer lipid envelope containing glycomembrane proteins on the surface, and a layer between the envelope and caspid, called the tegument. The latter was only poorly characterised. It is believed that some tegument proteins are released into the infected cell during the *de novo* infection process of the host cell and therefore may trigger early cellular effects after infection. The authors isolated KSHV virions from the supernatant of TPA-induced PEL cells. These were lysed and subjected to polyacrylamide gel electrophoresis (PAGE) and subsequent Coomassie staining. Proteins of discrete bands were isolated and identified by mass spectrometric analyses. This led to the identification of 24 viral proteins that were contained in the KSHV virion. Five proteins were classified as capsid proteins (orf17.5, orf25, orf26, orf62, orf65) (75). Eight glycoproteins were detected to be members of the viral envelope (orf8, orf22, orf28, orf39, orf47, orf53, orf68, and K8.1). Based on sequence information only, five proteins of KSHV were predicted to be tegument proteins (orf19, orf63, orf64, orf67, orf75) (76). Of these, orf63, orf64, and orf75 were confirmed and three others (orf21, orf33, orf45) were biochemically classified as tegument proteins, because they were resistant to trypsin treatment and associated with the capsid. Finally, five proteins were newly found in the virion and remained uncategorised in this study (orf6, orf7, orf11, orf27, orf52). In a follow-up study of this group, two of these proteins, orf11 and orf27 were classified as tegument proteins (77).

A proteomic approach was also used to identify proteins binding to the terminal repeats (TR) of the KSHV DNA (78). TR are multiple GC-rich, 801-bp fragments, at both termini of the KSHV genome (76) and are important for segregation of the viral genome to the daughter cells and for virus production (79). KSHV TR DNA was used as affinity ligand on a sepharose column. Nuclear extracts from a KSHV positive PEL line (BC-3) or a KSHV negative B-cell line (BJAB) were loaded on the column. After elution of bound proteins the protein mixture was characterised as above by PAGE, Coomassie staining and mass spectrometry. In total 123 KSHV TR-binding proteins were identified. 116 proteins were identified in BC-3 extracts from which 95 were exclusively seen in BC-3. Twenty eight proteins were identified in BJAB extracts, only seven of them were exclusively seen in BJAB extracts. The authors selected four of the identified proteins (ATR, BRG1, NPM1 and PARP-1) for further investigation. Using a chromatin-immunoprecipitation assay, it was confirmed that these four proteins interacted with the TR elements. Finally, it was shown that all four proteins co-localised and associated with LANA-1. LANA-1 has been shown before to be crucial for the latent persistence of KSHV. It binds to elements in the TR and to chromosomal proteins of the host cells and regulates the segregation of the viral genome (80). In this way it assures the co-segregation of viral and cellular genomes to the daughter cells. In summary, this approach identified various proteins of the host that bind to the TR elements of KSHV and therefore may cooperate with LANA-1 in the regulation of viral persistance.

Using a similar proteomic approach, the same group screened for proteins that may associate with LANA-1 (81). A GST-tagged LANA-1 aminoterminal and a GST-tagged LANA-1 carboxyterminal domain were used as affinity ligands bound to glutathione-sepharose beads. Using this approach a total of 53 proteins were identified to bind the aminoterminus of LANA-1 and 56 to the carboxyterminus. The identified proteins could be grouped as DNA binding proteins, serine/threonine kinases, structural proteins, cytoskeleton proteins, transcriptional regulatory proteins and adapter and motor proteins. Of these, transcription factors were the largest group interacting with the amino-terminus of LANA-1, whereas DNA binding proteins made up a large proportion of proteins binding to the carboxyterminal region of LANA-1. The authors analysed the role of CENP-F in more detail. CENP-F is a large nuclear/kinetochor protein, which is involved in mitotic control, microtuble dynamics transcriptional control, and muscle cell differentiation (82). Interaction and co-localisation of CENP-F and LANA-1 was confirmed by immunoprecipitation and immunofluorescence analyses, respectively. Altogether several putative new binding partners of LANA-1 were detected by this proteomic approach. The biological relevance of these interactions remains to be elucidated.

Our group provided the first study characterising the impact of vFLIP/K13 of KSHV on the proteome of endothelial cells. To this end, 2D-difference gel electrophoresis analysis (2D-DIGE) was used. For this approach protein extracts of the cells to be compared are harvested and differentially labelled with two different fluorescent dyes. Subsequently, the lysates are combined, subjected to 2D-gel electrophoresis and the relative concen-

trations of the proteins in each spot are determined with a laser scanner. Although limited in resolution, 2D-DIGE is a powerful approach in order to identify the most strongly differentially expressed proteins between two samples. Using this approach, we compared the protein signals of primary human endothelial cells stably transduced with a retroviral vector encoding K13 (K13-EC) versus control vector transduced endothelial cells (Ctrl-EC). In total, 14 proteins were identified to be upregulated more than four-fold in K13-EC as compared to Ctrl-EC. Only the strongest up-regulated protein was isolated and identified by mass spectrometry. Thereby, we identified manganese superoxide dismutase (MnSOD), a mitochondrial antioxidant and an important antiapoptotic enzyme as the most strongly up-regulated protein by vFLIP/K13 in primary human endothelial cells. MnSOD expression was also up-regulated in endothelial cells upon infection with KSHV. The induction of MnSOD expression was dependent on vFLIP/K13-mediated activation of NF-κB, occurred in a cell-intrinsic manner, and correlated with decreased intracellular superoxide accumulation and increased resistance of endothelial cells against superoxide-induced death. MnSOD was also found to be up-regulated by vFLIP/K13 in endothelial cells at the RNA level in the two transcriptome analyses described above (69, 70). However, the transcriptional increase was only at intermediate strength. This clearly demonstrated that proteome analyses improve the insight into the effects of KSHV infection in endothelial cells. The up-regulation of MnSOD expression by vFLIP/K13 clearly may support survival of KSHV-infected cells in the inflammatory microenvironment in KS.

Systematic analyses of KSHV gene functions

Only very recently systematic approaches have been carried out in order to investigate the functions of all KSHV-encoded genes in eukaryotic cells. These studies provided the complete interaction network and the intracellular localisation map of all KSHV proteins. In addition, systematic analyses of all binding sites of the KSHV-8-encoded transcriptional activator RTA/orf50 in the viral genome and of NF-κB activation by all KSHV-encoded proteins have been reported.

KSHV protein interaction

In a comprehensive study Uetz et al. (83) analysed the genome-wide protein interaction map of KSHV. In yeast-two-hybrid analyses more than 12,000 pairwise interactions of KSHV proteins and subfragments of these proteins were tested. One hundred twenty-three non-redundant interacting protein pairs have been identified by this work. As yeast-two-hybrid analyses provide a high rate of false positive hits, confirmation experiments by alternative methods were performed. Using beta-galactosidase and co-immunoprecipitation assays, about 50% of the protein interactions could be confirmed. The KSHV interactome exhibited distinct features as compared to cellular interaction networks. Specifically cellular interaction networks are dominated by single interactions, whereas KSHV interaction nodes commonly presented three neighbours. In this framework, the nuclear K10 protein was identified as a major interacting protein of HHV-8, which can bind to at least 15 different KSHV proteins (83). This may reflect that the viral particle is evolutionarily adapted to form a compact structure which may require multifunctional proteins and interactions between many different proteins. However, simulation of the interplay between the viral and the cellular proteomes showed that under these conditions the KSHV network changed completely from a highly coupled module to a more typical scale-free network of interacting submodules. This confirmed that viral proteins are involved in multiple interactions with host proteins and warrants further studies to determine those that are most relevant in the context of pathogenesis. Considering that most of the viral proteins are present only in lytic stages and that cellular proteins are subject to a significant shut-off mediated by SOX/orf37, it is clear that the effect of virus infection on the cellular proteome will be a key issue to address in the future.

Identification of RTA/orf50 binding sites in the KSHV genome

Recently, two studies used ChIP-on-chip analyses to investigate systematically the presence of RTA/orf50 binding sites in the KSHV genome (84, 85). RTA/orf50 is necessary and sufficient for the activation of the lytic replication cycle of KSHV and can activate its target genes through binding to RTA/orf50-responsive elements in the viral genome. ChIP-on-chip analysis combines chromatin immunoprecipitation with microarray technology and enables the identification of genome-wide interactions between transcription factors and DNA.

Both studies used BCBL-1 cells in which RTA/orf50 was ectopically over-expressed. In the first study, the immunoprecipited RTA/orf50-associated DNA was hybridised with microarrays covering the whole KSHV genomic sequences (84). This approach identified in total 19 direct RTA/orf50 binding sites located in the promoters, introns or exons of the KSHV genes K4.1, K5, K8, K10.1, K12 (containing two RTA/orf50 binding sites), K14/orf74, K15, PAN RNAs, orf8, orf16, orf29, orf45, orf50, orf59, orf71/72, the two origins of lytic replication (OriLyts) OriLyt-L and OriLyt-R, and the microRNA cluster. The authors confirmed all of the identified putative RTA/orf50 binding sites by performing conventional ChIP analysis with quantitative real-time PCR. Of note, six direct RTA/orf50 binding sites were located in the latent locus, suggesting that RTA/orf50 might also be involved in the regulation of latent gene expression. Finally, by performing sequence alignments, the authors identified the RTA/orf50 consensus binding sequence (5'-TTCCAGGAT$(N)_{0–16}$TTCCTGGGA-3'), which contains two imperfect tandem repeats, similar to that of p53 consensus binding elements.

The second study investigated binding and transcriptional effects of RTA/orf50 on 83 putative KSHV promoters (85). Through this approach 22 promoters were identified, which were bound directly by RTA/orf50. Of these, eight promoters were activated by RTA/orf50 in subsequent transfection studies: K2, K12, K15, K8, orf57, orf59, PAN, and Ori-R. Only 36% of the identified RTA/orf50 binding promoters were concordantly observed in the study of Chen et al. (84). The discrepancy in the identified target genes may reflect differences in the mode of recruitment of RTA/orf50 to promoters (either direct or indirect) and in the employed methodology.

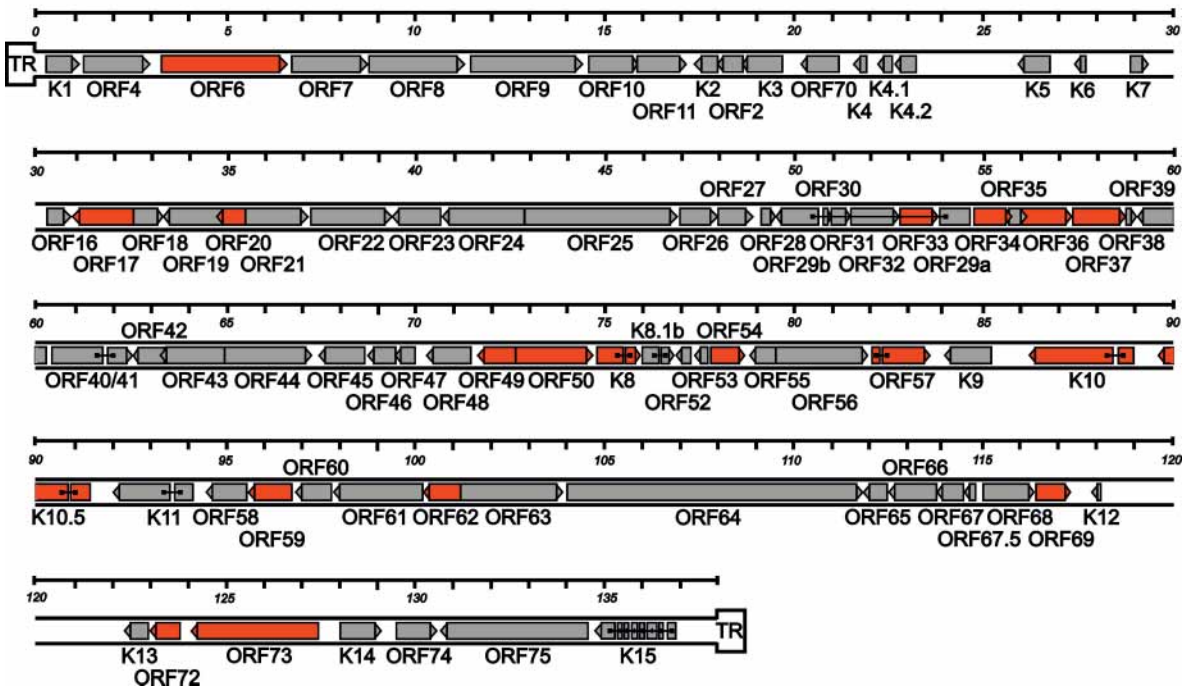

Figure 1: Gene map and nuclear localisation of KSHV proteins. Protein coding regions are indicated by arrows, and gene names are given (orfs: homologous to herpesvirus saimiri; K-genes: unique at time of sequencing). Orientations of the arrows indicate transcriptional orientations. Genes encoding proteins with exclusively nuclear localisation are labelled in red. Figure adopted with modifications from (86).

Intracellular localisation of KSHV proteins

A study by Sander et al. (86) presented a localisation map of all KSHV proteins in HeLa cells. To this end all individual viral open reading frames were expressed with an immunological tag (Myc tag) fused in frame at the 3' end of the coding sequences in HeLa cells. Classification of the proteins showed that 51% of all proteins localised in the cytoplasm, 22% in the nucleus, and 27% both in the nucleus and cytoplasm. It is of interest that 22% of the KSHV-encoded proteins were detected exclusively in the nucleus, whereas only 12% of randomly selected cellular proteins showed an exclusive nuclear localisation (87). Graphical depiction showed that proteins with exclusively nuclear localisation were mainly encoded by genes in the second half of the viral genome (Fig. 1, red). Comparing protein localisation with the expression state during the viral life cycle, it was noticed that all latency-associated proteins showed a nuclear or nucleo-cytoplasmic staining pattern whereas this was observed only for 47% of primary lytic, 43% of secondary lytic, and 43% of tertiary lytic proteins (86). A similar study investigated the localisation map of Epstein-Barr virus (EBV) and confirmed the nuclear preponderance (31%) of herpesviral proteins (88). This is in good agreement with the viral life cycle, which is preferentially associated with the nucleus. This argument specifically applies to the latent infection, which represents a regulatory state depending on the control of host cell and viral transcription in the nucleus.

Among the most surprising findings of the KSHV protein localisation study was the detection of vFLIP/K13 in the nucleus. Nuclear localisation is not observed for cellular FLIPs, which are resident exclusively in the cytoplasm (89, 90). This indicated that KSHV-encoded vFLIP/K13 may exert different and/or additional functions compared to cellular FLIPs. In fact, subsequent work confirmed the nuclear localisation and demonstrated that nuclear localisation of vFLIP/K13 correlates with its ability to activate NF-κB (91).

In reference to the study of (83), in which the nuclear K10 protein was identified as a major interacting protein of KSHV, it was surprising that only 33% (5 of 15) of the potential binding factors of K10 were also detected in the nucleus when expressed on their own (86). However, when K10 was co-expressed with its putative binding factors, co-localisation was observed in 87%. These findings confirmed that K10 is a major interacting protein of KSHV and demonstrated that mutual protein interactions in an infected cell affect subcellular localisation. In agreement with this, other investigators have described significant relocations of KSHV proteins in latently rather than lytically infected primary effusion lymphoma cells (92, 93). The localisation map of individually expressed KSHV proteins provides a useful reference to detect positional effects on KSHV proteins in virus-infected cells.

Activation of signal transduction pathways by KSHV

The first systems biology approach to investigate the effect of KSHV on cellular signal transduction was published just recently (66). Infection with KSHV constitutively activates the transcription factor NF-κB in endothelial cells and lymphocytes (94, 95). Activation of NF-κB is crucial for the development and progression of KSHV-associated diseases. It protects KSHV-in-

fected cells against spontaneous apoptosis (94) and maintains the latent viral life cycle (96, 97). The latter is mandatory for the establishment of viral persistence. In agreement with this, the inhibition of NF-κB signalling delays the growth of KSHV-associated lymphomas in a mouse model (98). Paradoxically, NF-κB signalling has been reported to both inhibit γ-herpesvirus lytic replication (96) and to be required for the production of replication-competent KSHV virions (99).

Only a few KSHV-encoded genes have been studied for their impact on NF-κB signalling. The genes vFLIP/K13 (63), K15 (100) and orf74 (101) were described as activators and K10.5 (102) as an inhibitor of NF-κB, whereas conflicting results were obtained on the activity of K1 (103, 104). However, it was not known whether these KSHV proteins are the only ones to act on NF-κB, or whether different KSHV proteins cooperate positively or negatively in the regulation of this important signalling pathway. A systematic analysis of all single and pairwise combination effects of KSHV genes on NF-κB requires almost 4,000 transfection experiments. Therefore, this approach demanded high throughput transfection technology, which has only recently become available.

In 2001 Ziauddin and Sabatini succeeded in scaling down high throughput gene function analysis to the microarray level (17). Different cDNA expression plasmids were spotted onto slides using a microarray robot. The dried slides were exposed to a transfection reagent, placed in a culture dish and covered with adherent mammalian cells in medium. Alternatively, DNA and transfection reagent can be mixed at once and applied onto the slide (17). Both methods create microarrays of simultaneously transfected cell clusters with different plasmids in distinct and defined areas in a lawn of cells. The process of creating a microarray of clusters of transfected cells was called transfected cell microarrays. The transfection method was named reverse transfection, because, in contrast to conventional transfection protocols, DNA was "seeded" first and the cells were added subsequently. Reversely transfected cell microarrays (RTCM), also called "cell chip analyses", allow the carrying out of several hundred to thousand transfection experiments in parallel in eukaryotic cells on a single glass slide. Cotransfections of appropriate reporter plasmids can be used to establish quantitative measures of gene effects on signalling pathways (Fig. 2, for review see Stürzl et al. [105]).

RTCM was used to analyse systematically the effects of KSHV genes on the NF-κB signalling pathway (66). All KSHV-encoded genes were investigated individually (n=86) and additionally all K- and latent genes were examined in pairwise combinations (n=231). Statistical analyses of more than 14,000 transfections identified orf75 as a novel, and confirmed vFLIP/K13 as a known KSHV-encoded activator of NF-κB. vFLIP/K13 and orf75 showed cooperative NF-κB activation. siRNA-mediated knockdown of orf75 expression demonstrated that this gene significantly contributes to NF-κB activation in KSHV-infected cells. Furthermore, the approach confirmed K10.5 as an NF-κB inhibitor and identified K1 as an inhibitor of both vFLIP/K13- and orf75-mediated NF-κB activation. All results obtained with RTCM could be confirmed with classical transfection experiments. This work described the first successful application of RTCM for the systematic functional analysis of genes encoded by an infectious agent. The finding that KSHV encodes several activators of the NF-κB signal transduction pathway supports the importance of this pathway in the viral life cycle. It has to be investigated in further studies, whether these genes may cooperate or act in different phases of the viral life cycle in order to regulate the fine-tuning of the balance between latency and lytic replication since this depends critically on the state of NF-κB activity.

Model systems to study the role of KSHV in endothelial cell pathology

Non-transgenic animal models

A variety of animal models have so far been tried for KSHV. In the course of attempting to infect different mammalian species with KSHV it has been found that KSHV infects macaques (106), NOD-SCID mice (107) and humanised SCID mice (108, 109). None of these infection experiments led to the appearance of KS-like tumours. Recently, engraftment into mice of a murine endothelial cell line that had previously been transfected with a recombinant KSHV genome in a bacterial artificial chromosome (BAC) vector was reported to induce the growth of a vascularised spindle cell sarcoma; this appeared to involve the viral G-protein coupled receptor homologue (orf74), as siRNA-mediated suppression of this gene inhibited the appearance of these tumours (110).

Many mammalian species harbour KSHV-related viruses. Thus, virtually all Old World primates harbour at least one, if not two, KSHV-like rhadinovirus. Phylogenetically, these fall into two lineages, RV1 and RV2, and many Old World primates are hosts to both lineages (111, 112). The majority of these primate viruses have not yet been isolated in culture but have been discovered using degenerate consensus oligonucleotide primers of conserved genomic regions, such as the herpesviral DNA polymerase (111–118). KSHV is a representative of the RV1 lineage, while Rhesus Rhadinovirus (RRV), which has been isolated in culture, fully sequenced, and is amenable to experimentation (113, 119, 120), belongs to the RV2 lineage. No KS-like disease has so far been published in primates infected either with KSHV or RRV, even in animals that had been previously or simultaneously infected with SIV (simian immunodeficiency virus). However, cases of MCD-like lymphoproliferations and endothelial cell hyperplasia have been reported in RRV/SIV co-infected macaques (121, 122).

Murine herpesvirus-68 (MHV-68), originally isolated from bank voles, is also, like KSHV, a γ_2-herpesvirus, although more distantly related to KSHV than the primate rhadinoviruses. It has been widely used to study the immune response to a γ_2-herpesvirus in a small animal model and to analyse the function of individual viral genes *in vivo*. However, its value as a model for the role of KSHV in the pathogenesis of KS is limited, as no KS-like disease has been seen in MHV-68 infected mice.

Multiple tumour graft models of KS and PEL have been established (110, 123–126).

Transgenic mice expressing individual KSHV genes

Several KSHV genes have been engineered into transgenic mice, often under the control of endothelial or B-cell specific promoters, to study the role of individual KSHV proteins in an *in vivo* model of tumourigenesis. These models are limited in several respects: (i) whereas lymphoproliferative lesions and lymphomas in mice are easily classified on the basis of histology and marker gene expression, this is not the case for endothelial cell tumours, which are often reported as KS-like lesions, but may represent fibrosarcomas with a spindle cell morphology; (ii) by definition, they examine the impact of individual viral genes outwith the context of the entire viral genome. As discussed elsewhere in this chapter, the impact of individually expressed viral proteins, in particular those expressed during the lytic viral replication cycle, on the cellular gene expression profile may differ from their role in the context of the entire viral genome because of the effect of the viral SOX homologue on the stability of cellular mRNAs. However, the latter concern does not apply to the latent viral proteins that are known to be expressed in tumour cells. It may also not apply to viral genes that, beyond their lytic gene expression pattern in induced PEL cells (24–26) may be expressed in substantial proportion of tumour cells *in vivo* (e.g. K1, vIL-6/K2).

Mice transgenic for KSHV latent genes

LANA-1/orf73. The KSHV latent promoter (LANA-1p) showed B-cell lineage specificity in transgenic mice (127). KSHV LANA-1 expression in transgenic mice resulted in 100% B-cell hyperplasia, and lymphomas at about twice the rate of background in the C57/BL6 strain of mice (128).

vCYC/orf72. Whereas vCYC single transgenic mice did not develop tumours, lymphomas developed rapidly in a p53null background (129, 130). Presumably, loss of p53 counteracted the proapoptotic signals that are associated with forced vCYC expression.

vFLIP/K13. The vFLIP/K13 transgenic mice exhibited increased incidence of lymphoma (131).

Mice transgenic for KSHV lytic genes.

vGPCR/orf74. vGPCR transgenic mice activated the same signalling pathways as predicted from human culture studies and exhibited KS-like lesions (132–136). The vGPCR was required for lesion initiation, though it was not essential once a fully malignant tumour had formed (132).

K1. K1 transgenic mice also exhibited KS-like lesions and lymphoma (104, 137)].

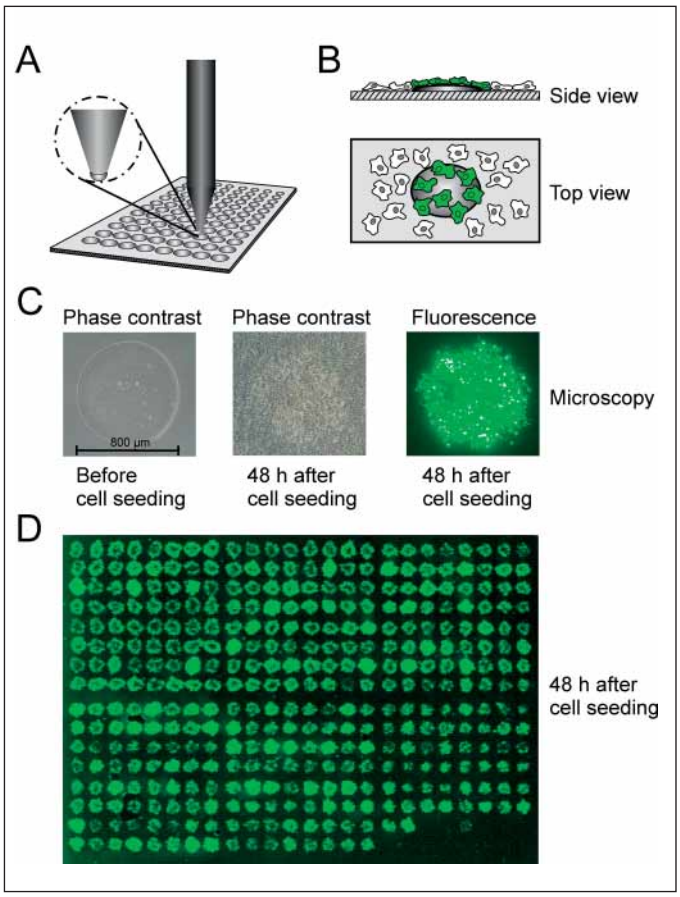

Figure 2: Reversely transfected cell array to determine signalling activity of over-expressed KSHV proteins. A) Transfection mixtures containing a reporter plasmid expressing GFP under the control of signal transduction pathway sensitive promoters (e.g. NF-κB) in combination with one or two different plasmids expressing the HHV-8-encoded genes are spotted onto a microarray slide. B) Subsequently the slide is overlayed with HEK 293T cells, which leads to transfection of the cells over the application spots. GFP-expression indicates activation of the respective signalling pathway. C) Phase contrast pictures of the transfection spot without cells (left) and 48 h after cell seeding (middle). Epifluorescence detection of GFP expression in the cells on the respective transfection spot (right). D) Representative laser scanning photograph of a reversely transfected cell array with 371 transfection spots.

Reverse genetics approaches to KSHV pathogenesis

Since the discovery of KSHV in 1994 (1), a substantial body of evidence on the biochemical and cell biology function of individual viral proteins and their role in the pathogenesis of one of the three KSHV-associated tumours (KS, PEL and the plasma cell variant of MCD) has accumulated. However, given the known interactions of different viral proteins, as well as the involvement of several viral proteins in the same cellular pathways, an important task will be to investigate their functional role in the context of the entire viral genome/proteome. The availability of a viral genome cloned into a BAC vector (138) has enabled KSHV researchers to approach this issue. Several viral genes have since been deleted from the viral genome and the resulting phenotype studied, mostly in stable cell lines transfected with the wild-type and mutant viral genome. Because of the technical difficulties in rescuing infective KSHV from cloned viral genomes, only a few studies have so far analysed the phenotype associated with cell-free infection, of e.g. endothelial cells with mutant viruses. Table 3 summarises the results obtained so far.

Table 3: Phenotypes associated with infection with mutant KSHV.

Deleted viral gene	Test system	Phenotype	Reference
K1	Stable HEK 293 transfectants	Essential for virus production; alteration of pAKT levels	Hartmann et al. (in prep.)
vIL-6/K2	Stable transfectants	No effect on virus production	[154]
Orf45	Stable HEK 293 transfectants	Reduced virus production, lytic gene expression maintained	[156]
RTA/orf50	Stable HEK 293 transfectants	Essential for activation of the lytic replication cycle	[166]
K-bZIP/K8	Stable Vero transfectants	Essential for induction of the lytic cycle by TPA or sodium buyrate; not required for steps downstream ofRTA/orf50	[172]
vFLIP/K13	Stable HEK 293 transfectants; infection of endothelial cells with free virus	Increased lytic replication, loss of spindle cell formation in primary endothelial cells, decreased virus persistence in endothelial cells; changes in virus-induced cellular transcriptome pattern	[97] Al-Kharsah et al. (in prep.)
LANA-1/orf73	Transient and stable transfectants	Loss of viral episome persistence; increased lytic replication	[199, 200]; Al-Kharsah et al. (in prep.)
LANA-1 promoter (cohesin binding element)	Stable transfectants	Deregulation of vGPCR/orf74, vOX2/K14 expression	[202, 203]
Orf57	Stable HEK 293 transfectants	Inhibition of lytic gene expression	[175]
K15	Stable HEK 293 transfectants; infection of endothelial cells with free virus	Decreased migration/ invasion of KSHV-infected cells, changes in virus-induced cellular transcriptome pattern	Hillenbrand et al. (in prep.)

Deletion of K1

K1 is a viral type I transmembrane protein, featuring two hypervariable domains in its extracellular region, and an ITAM motif in its carboxyterminal, cytoplasmic region (139, 140). The K1 protein is expressed during the lytic (productive) replication cycle (24–26). Wang et al. (141) reported that, while expression of K1 is not a consistent feature of KS tumours, some KS biopsies show marked K1 expression both at the transcript and protein level. In MCD, K1 expression was documented by immunohistochemistry on a small subpopulation of mantle zone lymphocytes of KSHV-positive MCD (142). K1 transfectants in rodent fibroblasts showed focus formation and tumour formation in nude mice (140) and K1 transgenic mice develop sarcomatoid tumours and plasmablastic lymphoma (104). Retroviral transduction of primary endothelial cells extends their life span (141). Biochemically, K1 activates several intracellular signalling cascades, leading to increased Ca-influx, increased phosphorylation of syk, vav, cbl, and the p85 subunit of PI3K, activation of NFAT, AP1 (143, 144). Activation of PI3K leads to activation of AKT by K1 (145). K1 induces the expression of angiogenic cytokines, including VEGF, and may therefore play a paracrine role in the pathogenesis of KS or PEL (141, 146).

In addition to these findings, suggesting a contribution of K1 to KSHV-induced tumourigenesis, K1 has also been reported to be involved in the regulation of the lytic replication cycle. Depending on the induction model and cell line employed, K1 appeared to either support or repress the activation of the lytic replication cycle (103, 144). This may be due to the variable effect of K1 on NF-κB activation, which is a matter of ongoing debate (103, 104)

Deletion of K1 from a recombinant KSHV genome completely abolished the reactivation of KSHV from stably transfected HEK 293 cells and altered the levels of pAKT, thus supporting a role for K1 in the activation of the lytic replication cycle (Hartmann et al., in preparation).

Deletion of vIL-6/K2

The viral IL-6 homologue, vIL-6, is expressed in a subpopulation of PEL cells *in vivo* and in many KSHV-infected B cells in MCD lymphoid follicles (92, 147, 148). It induces proliferation, angiogenesis and haematopoesis in IL-6-dependent cell lineages (147, 149, 150) and serves as an essential autocrine factor in PEL cell lines (151); it also induces vascular endothelial growth factor (VEGF), which has been implicated in the pathogenesis of PEL and KS (152). A single chain antibody to vIL-6 blocking its interaction with the IL-6 receptor complex was found to inhibit the proliferation of a PEL cell line and to inhibit vIL-6-induced signal transducer activator of transcription 3 (STAT3) phosphorylation in vIL-6-transfected cells (153). Therefore, vIL-6 may contribute to PEL cell proliferation and to the angiogenesis noted in patients with this lymphoma.

A KSHV gene deletion mutant lacking vIL-6 has been reported to reactivate normally and to improve the growth of a human lymphoma cell line (BJAB) in low serum to the same extent as a KSHV wild-type genome (154).

Deletion of orf45

Orf45 encodes a KSHV tegument protein, which interacts with KIF3A, a kinesin-2 motor protein that transports cargo along microtubules (155). An orf45 deletion mutant showed a defect in virus production and infectivity, suggesting a role for orf45 in viral egress and possibly entry (156). Interestingly, the stability of orf45 protein is controlled by ubiquitination mediated by the E3 ligase family seven in absentia homologue (SIAH) (157). In addition, the orf45 protein interacts with, and inhibits phosphorylation of IRF-7, thus inhibiting its nuclear translocation and the IRF-7-mediated induction of the type I interferon response (158).

Deletion of RTA/orf50

The viral immediate-early transactivator RTA, encoded by orf50, is the key element in the activation cascade. Ectopic expression of RTA/orf50 in PEL cells triggers the lytic cycle, leading to the production of infectious virus (159, 160). RTA/orf50 acts through binding to specific DNA sequences (161), and by interacting with cellular transcription factors (162–164). RTA/orf50 can also activate its own promoter (165) and cellular genes (163). The suppression of p53-mediated apoptosis and the interaction with STAT3 leading to transcription of STAT-responsive genes suggest the implication of RTA/orf50 in promoting cell proliferation (162).

Deletion of the RTA/orf50 gene from a recombinant KSHV genome leads to a complete suppression of lytic cycle reactivation, confirming the essential role of RTA/orf50 in the activation of the lytic replication cycle (166).

Deletion of K-bZIP/K8

The product of KSHV gene K8, K-bZIP (167), represses or enhances RTA/orf50-mediated gene activation (168, 169). K-bZIP also binds to p53, thereby repressing p53-mediated apoptosis, promotes both CCAAT/enhancer binding protein alpha (C/EBPa) and p21/CIP-1 expression and, through interaction with C/EBPa, is able to promote p21/CIP-1-mediated inhibition of entry into S phase (170, 171). Together, these features of K-bZIP probably contribute to the creation of a suitable environment for lytic viral replication by antagonising apoptosis and preventing competition with host-cell DNA synthesis for limited resources. Deletion of K-bZIP from the viral genome eliminated the activation of the lytic replication cycle by treatment with sodium butyrate or TPA; however, overexpression of RTA/orf50 in cells carrying the K-bZIP deletion mutant resulted in increased lytic gene expression (172). In the absence of K-bZIP, overexpressed RTA/orf50 binds to the lytic origin of replication, ori lyt, suggesting that K-bZIP may serve to modulate the interaction of RTA/orf50 with ori lyt (172).

Deletion of orf57

The orf57 protein of KSHV promotes viral gene expression by mediating the nuclear export of viral RNAs in an exportin-1/chromosomal region maintenance-1 (CRM1) independent manner, most likely through its interaction with the cellular export factor Aly/REF (173, 174). A transposon mutant of the orf57 gene, which also affected expression of the neighbouring orf56 gene, showed a defect in the expression of early and late lytic genes (K8, K8.1, orf59) (175).

Deletion of K8.1

Orf K8.1 is alternatively spliced and encodes two highly glycosylated type I transmembrane proteins that interact with cell surface heparan sulfate. Inhibition studies using soluble extracellular domains of the K8.1 protein have been reported to inhibit, or not to inhibit, viral infectivity (176, 177). After deleting the K8.1 gene from a recombinant viral genome in BAC vectors the mutant virus was still infectious for HEK 293 cells, suggesting that K8.1 is not required for infection of at least this cell line or, more broadly, epithelial cells (178).

Deletion of the lytic replication origins

The KSHV genome contains three origins of DNA replication. One, located in the terminal repeat subunits, is used for the replication of the viral episome, while two others, oriLyt-L and oriLyt-R, are used during the lytic replication cycle. OriLyt-L is located between orfs K4.2 and K5, while oriLyt-R is located between orf69 and vFLIP/K13. Deletion of one or the other oriLyt revealed that oriLyt-L is sufficient for lytic viral replication while oriLyt-R on its own is not able to propagate the viral genome (179).

Deletion of vFLIP/K13

vFLIP/K13 is expressed on the same bi-cistronic transcript as vCYC (29, 180, 181). It can block Fas-induced and superoxide-induced apoptosis and has been postulated to act as a tumour progression factor by counteracting apoptotic signals induced by virus-specific T killer cells (64, 70, 182). It has also been proposed to contribute to the continuous NF-κB activation observed in PEL cells (183). Inhibition of NF-κB and down-modulation of vFLIP/K13 by RNA interference induces apoptosis in PEL cell lines, indicating that vFLIP/K13-induced NF-κB activation may be important for their survival (184). Transduction of vFLIP into primary endothelial cell cultures induces spindling, suggesting that the vFLIP/K13-induced NF-κB activation may also play an important role in the formation of spindle cells, the histological hallmark of KS lesions (185).

Deletion of vFLIP/K13 from the viral genome leads to an increase in lytic reactivation (97) (Al-Kharsah et al., in preparation), due to the fact that the strong induction of the NF-κB pathway by vFLIP/K13 represses lytic reactivation (97). In addition, loss of vFLIP/K13 from the viral genome leads to a more rapid clearance of latently infected primary endothelial cells and a loss of spindle cell formation in primary endothelial cell cultures infected at low m.o.i. (Al-Kharsah et al., in preparation). In primary endothelial cells, infection with a vFLIP/K13 deletion mutant of KSHV leads to a reduced increase in the expression of several interferon-regulated cellular genes that have been reported to be activated by over-expressed vFLIP/K13 (see above), confirming that, in the context of the entire viral genome, vFLIP/K13 plays a role in the activation of interferon-regulated cellular genes (Al-Kharsah et al., in preparation). Together, these

observations suggest that vFLIP/K13, which is expressed at very low levels in the context of its physiological bi-cistronic mRNA also containing the vCYC gene, plays an important role in the maintenance of latency and survival of latently infected cells.

Deletion of LANA-1/orf73 and control elements in the LANA-1 promoter

Among the KSHV proteins expressed during latent infection, only LANA-1 has been detected in virtually all KSHV-infected cells by immunofluorescence or immunohistochemical analyses (92, 148, 181). LANA-1 is required for the persistence of the KSHV episomal genome (186, 187). LANA-1 binds to mitotic chromosomes through its aminoterminal region and to two short motifs of 16 nucleotides (LANA-1-binding sites, LBS-1 and –2) in the terminal repeat subunit (TR) of the KSHV genome via its carboxyterminal region (188–191). In this way, LANA-1 tethers viral episomes to mitotic chromosomes and it is assumed that this ensures the distribution of KSHV genomes to daughter cells upon mitosis. LANA-1 also mediates the replication of TR-containing plasmid DNA in transfected cells, thus illustrating its role in the replication of latent episomes (160, 187, 189). Among the many cellular proteins interacting with LANA-1 are the p53 and retinoblastoma (pRb) proteins, which results, respectively, in the inhibition of p53-dependent transcription, the activation of E2F-dependent genes and the transformation of rodent fibroblasts in cooperation with oncogenic H-ras (192, 193). Furthermore LANA-1 acts as a transcriptional activator and/or repressor, associates with the mSin3 repressor complex as well as cellular factors involved in the methylation of DNA (DNMT3a) and interaction with methylated DNA (MeCP2) (194–196). Further cellular interaction partners of LANA-1 include brd2 (RING3), brd3, brd4 (HUNK), members of the BET protein family involved in transcriptional regulation (197, 198). In addition, LANA-1 acts as a transcriptional repressor of the viral TR promoter/enhancer and represses the activation of the lytic replication cycle (189, 191).

Deletion of orf73, the viral gene encoding LANA-1, from the viral genome results in a complete loss of episomal maintenance, i.e. no stable cell clones containing the KSHV genome can be established (199). Upon transient transfection, a KSHV genome lacking LANA-1 shows increased lytic reactivation (200). These results confirm two important roles of LANA-1 during viral latency, i.e. its requirement for episomal persistence and its role in maintaining latency by inhibiting the activation of the lytic replication cycle.

The viral promoter directing the expression of LANA-1/orf73 during latency is located in the immediate vicinity of a promoter directing the expression of the bi-cistronic mRNA for vGPCR/orf74 and vOX2/K14 (201). Functional separation of these two promoters is ensured by a CTCF/cohesin binding site (202). Deletion of this CTCF/cohesin binding site from a complete viral genome results in instability of cell clones carrying the mutated KSHV genome and increased expression of lytic cycle gene products; in addition, the cell cycle dependent expression of the vGPCR/orf74 and vOX2/K14 genes is deregulated in the absence of the CTCF/cohesin binding site, suggesting a critical role for the CTCF/cohesin complex in regulating cell cycle control of viral gene expression during latency (203).

Deletion of K15

The K15 gene encodes, through alternative splicing, a family of transmembrane proteins with up to 12 transmembrane domains and a c-terminal cytoplasmic domain (204, 205). The longest of these proteins activates several intracellular signalling pathways, including NF-κB, Mek/Erk and JNK and induces the expression of inflammatory and angiogenic cytokines (100, 206). Deletion of the K15 gene from the KSHV genome reduces the invasion into matrigel and migration of KSHV-infected cells, suggesting that K15 may contribute to the migration and/or invasion of KSHV-expressing cells into surrounding tissue (Hillenbrand et al., in preparation).

Conclusion

A systematic analysis of viral gene expression patterns indicated consistently that, following cell entry, predominantly immunomodulatory and antiapoptotic genes are increased. Analyses of the cellular transcriptome of KSHV-infected cells were connected with several pitfalls which impaired the consistence of results: (i) use of none overlapping probe sets, (ii) different conditions of infection (time points after infection, control of infection) and cell stimulation (absence and presence of lytic stimulation), (iii) differential responses of different cell types to infection, and (iv) shut-off of cellular gene expression by SOX/orf37 in lytically infected cells. Lack of concordance of results likely is not due to technical problems of the microarray analyses, which showed high reproducibility in studies investigating vFLIP/K13 regulated gene expression in endothelial cells by two different independent laboratories. As yet the most concordant findings reported from the majority of transcriptome analyses are that viral infection is associated with a strong IFN-associated cell response and induces transdifferentiation of endothelial cells to a phenotype in between lymphatic and blood vessel endothelial cells. Interestingly, the induction of an IFN-response was often associated with the induction of members of the GBP family, of which GBP-1 has been shown to act antiangiogenic in endothelial cells. In addition, the paracrinely active angiogenic factor Ang-2 was commonly observed to be up-regulated in KSHV-infected cells, both in latent and also lytic infection. Both findings togther may indicate that the major proliferative capability in KS may not be due to the KSHV-infected cells but due to paracrine activation of non-infected cells in the vicinity. At first glance this suggests an unusual pathogenesis of KS. However, these observations may simply point to the importance of tumour cell (KSHV-infected endothelial cell) and stromal cell (non-infected endothelial cells) interactions, which generally are regarded to be key in the development of many other tumours.

Proteomics studies are still few and due to low numbers require confirmation by independent laboratories. However, it is clear that these approaches are closer to the functional understanding of KSHV pathogenicity as compared to transcriptome analyses. In addition, complete expression libraries of all KSHV-encoded genes and high throughput transfection technologies are available which will help to decipher the complex interaction of KSHV with eukaryotic cells.

Also reverse genetic studies using recombinant KSHV mutants are at the very beginning. First results confirmed RTA/

orf50 as the major regulator of viral reactivation and lytic replication and also showed that the effect of this molecule is modulated at least by two co-activators (K1, K8/K-bZIP), and by vFLIP/K13 and LANA-1 which are acting adversely and regulate the manifestation of latency. In the context of the viral genome, vFLIP/K13 also appears to play a role in the regulation of interferon-regulated genes and K15 makes a major contribution to the release of inflammatory and angiogenic cytokines released from KSHV-infected cells and their ability to invade surrounding tissue.

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