STING is dispensable during KSHV infection of primary endothelial cells

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Abstract

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Viruses are obligate intracellular parasites requiring host cells to replicate and spread. To prevent this, cells utilize a variety of protein receptors in order to surveil for invading viruses, with detection resulting in a signaling cascade and the establishment of the antiviral state. Conversely, viruses employ several strategies to evade and suppress host innate immunity to facilitate replication and spread. Understanding the interplay between innate immunity and viral infection is critical for understanding factors that influence the course of infection and disease progression. In this thesis, I explored the interactions between innate immunity and Kaposi's Sarcoma-associated herpesvirus (KSHV), an oncogenic virus. KSHV encodes several genes that target and antagonize innate immune pathways, including the cGAS-STING pathway. Previous studies indicated that the DNA sensor cGAS, and the downstream adaptor protein STING, are important for activating innate immune pathways and controlling KSHV infection. However, the role of these proteins in primary endothelial cells, the main proliferating cells in the Kaposi's Sarcoma tumor, has not been explored. After giving background (Chapter 1) and

methods (Chapter 2), I show that STING is dispensable during KSHV infection of primary endothelial cells in Chapter 3. I characterize a STING-null batch of primary lymphatic endothelial cells, which led me to investigate the importance of STING during KSHV infection of primary endothelial cells. I found that KSHV minimally induces interferon (IFN)-β expression and does not detectably activate STING signaling. Furthermore, I find that genetic ablation of STING does not increase susceptibility to infection, nor does it increase the ability of KSHV to spread through the culture following lytic reaction. In Chapter 4, I investigate the requirement of the kinase CDC7 for survival of KSHV-infected cells. I find that pharmacologic inhibition of CDC7 leads to an increase in cell death in KSHV-infected cells compared to uninfected cells. Furthermore, I find that KSHV de novo infection decreases expression of CDC7 as well its substrate, MCM2. Additionally, I rule out either the requirement or the sufficiency of each gene in the latent locus. Overall, this thesis reveals the dispensability of STING in endothelial cells during KSHV infection and identifies CDC7 as required during KSHV latency. These experiments improve our understanding of host-virus interactions and provide new targets for therapy in KS patients.

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Chapter 1. Introduction

1.1 Herpesviruses

Herpesviridae is a family of large, DNA viruses infecting a wide variety of animals, including humans. Herpesviruses have infected animals for hundreds of millions of years, which implies a long history of coevolution between host and virus (1). Herpesvirus virions have a diameter ranging from 120-260 nm and have large, double-stranded (ds)DNA genomes ranging from 124-230 kB that encode 70-750 protein-coding genes. Herpesvirus virions are defined by several morphological characteristics, including an inner core housing viral genomic DNA, an icosahedral protein capsid, a tegument layer surrounding the capsid, and a lipid envelope from which glycoprotein protrude. In addition, herpesviruses share four major biological properties. The first is that herpesvirus genomes contain a large number of genes encoding enzymes involved in nucleic acid metabolism, DNA synthesis, and processing proteins. The second property is viral DNA replication and capsid assembly occur in the nucleus while final processing of the virion occurs in the cytoplasm. The third characteristic is that viral replication and virion production leads to host cell death. The final property is that herpesviruses have the ability to establish latency in host cells.

Herpesviruses undergo two replication strategies: latency and lytic replication. During latency, the viral genome is maintained as an extrachromosomal episome that is replicated by host machinery and very few viral genes are expressed. In contrast, during lytic replication, many genes are expressed temporally, and there is production of infectious virions. Lytic replication begins with the expression of immediate early (IE) genes, which do not require any new protein synthesis for expression (1). IE genes have functions that include activation of early gene transcription, innate and adaptive immune antagonism, disruption of cell cycle, and RNA transport and splicing. IE gene expression results in induction of early genes, which are expressed independently from viral DNA replication, but require IE gene expression and

translation. The function of early genes is varied, but they are usually involved in viral genome replication, including nucleotide metabolism (to support replication) and proteins and enzymes directly involved in the synthesis of new viral DNA. Lastly, late genes, the expression of which is generally dependent on viral DNA replication (but can be leaky), usually function as structural proteins for assembly of the virion, including proteins that make up the viral capsid, glycoproteins and tegument proteins. Following assembly and acquisition of the viral genome, herpesvirus capsids egress from the nucleus, and through a poorly understood mechanism, acquire tegument proteins and envelope and exit the cell as an infectious virion.

Within the *Herpesviridae* family, there are eight known species that infect humans as their primary host and are given the nomenclature Human Herpesvirus (HHV) 1-8. These human herpesviruses can be further divided into subfamilies based on host tropism, cell culture characteristics and phylogenetics: alphaherpesviruses, betaherpesviruses and gammaherpesviruses. The alphaherpesviruses include Herpes Simplex Virus 1 (HSV-1 or HHV-1) and Herpes Simplex Virus 2 (HSV-2 or HHV-2), both of which cause oral cold sores and genital sores. The third human alphaherpesvirus is Varicella-Zoster Virus (VZV or HHV-3), the etiological agent of chicken pox and shingles. Members of the betaherpesvirus subfamily include Human Cytomegalovirus (HCMV or HHV-5), HHV-6 and HHV-7. HCMV infection during pregnancy can cause birth defects. In addition, HCMV infection can cause severe disease in immunocompromised individuals, including pneumonia, retinitis and other diseases. HHV-6 is the causative agent of roseola, but no disease has yet been attributed to HHV-7. Finally, members of the gammaherpesviruses include Epstein-Barr Virus (EBV or HHV-4) and Kaposi's Sarcoma-associated Herpesvirus (KSHV or HHV-8). EBV causes mononucleosis and Burkitt's lymphoma while KSHV is the etiological agent of Kaposi's Sarcoma (KS), and two B-cell lymphoproliferative disorders, including Primary Effusion Lymphoma (PEL) and multicentric Castleman's disease (MCD).

1.2 KSHV and KS

KS was originally described in 1872 by Hungarian physician Moritz Kaposi as a disease primarily affecting elderly men with an unknown cause (2). Cases of KS increased during the human immunodeficiency virus (HIV) pandemic of the 1980s and it emerged as one of the most common AIDs-defining illnesses (1). KS is categorized into four different classes, including classic, endemic, AIDS-associated (epidemic) and iatrogenic (3). Classic KS generally occurs in elderly men of Mediterranean descent in the form of tumors on lower body extremities. Endemic KS is generally found in children in sub-Saharan Africa. The most serious class of KS is AIDS-associated, which afflicts patients infected with HIV who are immunocompromised and may be life-threatening. Those with AIDS-associated KS typically have lesions on their lungs or gastrointestinal tract. The last class of KS, iatrogenic, is rare but arises in transplant patients who are immunosuppressed and received a transplant from a KSHV-positive individual.

KS manifests as a highly vascularized, cutaneous tumor. The KS tumor is a heterogenous, inflammatory cellular environment consisting of many different cell types. However, the main proliferative agent is spindle cells of endothelial origin. While spindle cells express a variety of cellular markers, the expression profile of spindle cells aligns more closely with lymphatic endothelial cells (LECs) than with blood endothelial cells (BECs), leading to the hypothesis that KSHV infection of LECs leads to spindle cell formation and KS (4–6). Most spindle cells in the KS tumor are latently infected with KSHV, which is recapitulated in cell culture. However, a small proportion infected cells contain lytically replicating virus and there is evidence that both latent and lytic replication contribute to KS pathogenesis (7).

KSHV was identified as the etiological agent of KS in 1994 by representational difference analysis that identified KSHV genomic DNA in KS lesions, but not in normal tissue (8). Importantly, KSHV is required but not sufficient for the development of KS. KSHV, like all herpesviruses, has both latent and lytic replication programs. Latency is the default state in both infected cells taken from KS tumors as well as in cells infected in cell culture, with the virus

establishing a latent infection in >90% of infected cells (9). The viral genome is maintained as an extrachromosomal circular episome in the nucleus and is replicated by host machinery during cell division. While latency is the predominant state in tissue culture and KS tumor cells, there is a small population (between 1-5%) of infected cells where the virus is undergoing lytic replication. Both latency as well as lytic replication are important for the development of KS and subsequent disease progression (7).

Following the sequencing of the KSHV genome, KSHV was classified as a lymphotropic herpesvirus within the gammaherpesvirus subfamily. KSHV has a genome of 165-170 kB, with a central unique region of 140 kB containing all viral open reading frames (ORFs) (10). The central unique region is flanked by a series of GC-rich terminal repeats. The majority of genes encoded by KSHV are conserved among herpesviruses and include genes essential for replication, gene expression and structural proteins comprising the virion. KSHV does, however, encode more than 15 genes that are unique to the virus and not conserved among other herpesviruses. Many of these genes are homologs of cellular genes and function by altering cellular proliferation, signaling and immune activation (11).

During latency, viral gene expression is restricted to a small number of genes, including the latency-associated nuclear antigen (LANA), viral cyclin (vCyc), viral FLICE (FADD-like interleukin-1-β-converting enzyme), the Kaposins A, B and C, and about 12 microRNA (miRNAs) loci (12). LANA is highly expressed during latency and has numerous reported functions contributing to viral persistence and pathogenesis. For example, LANA tethers the viral episome to host chromosomes in order to ensure replication of the viral genome during mitosis as well as inheritance of the viral genome into daughter cells (13). LANA also modulates cell cycle and may promote tumorigenesis by binding to and inhibiting the function of the tumor suppressors p53 and pRB and by preventing the degradation of the proto-oncoprotein c-Myc. Finally, cytoplasmic isoforms of LANA have recently been shown to antagonize innate immune signaling during lytic replication (14).

vCyc shares a promoter with LANA and is spliced to form a distinct product from the same transcript. vCyc is a homolog of cellular cyclin D and modulates cell cycle by activating cyclin-dependent kinase 6 (15). Expression of vCyc can cause mitotic defects, including polyploidy, which may activate p53 and lead to apoptosis. Loss of p53, however, allows cells expressing vCyc to survive (16). In addition, vCyc overexpression activates the DNA damage response, which leads to autophagy and senescence of host cells (17).

vFLIP is a homolog of cellular FLIP and activates nuclear factor (NF)-κB by binding to the inhibitor of κB kinase-gamma (IKKγ). The vFLIP-IKKγ complex activates IKK, which phosphorylates IkB, leading to the release and activation of NF-κB (1). This likely inhibits cell death through the upregulation of antiapoptotic factors through NF-κB (15). vFLIP also plays a role in suppressing autophagy and in formation of the spindle cell phenotype (17, 18).

The kaposins consist of kaposin A, B and C transcribed from the same locus but are expressed as three separate proteins through differential initiation of translation. Overexpression of kaposin A in immortalized rat fibroblasts leads to transformation (19). Kaposin B may activate inflammatory pathways through the activation of mitogen-activated protein (MAP) kinase-associated protein kinase 2 (MK2) (20). The miRNAs are involved in a variety of functions, including deregulation of cell cycle, maintenance of latency and subversion of innate immune pathways, which will be discussed in detail later (21).

While latency is the predominant pathway following de novo infection, KSHV undergoes lytic reactivation in a small subset of infected cells. When the virus switches to the lytic program, there is ordered gene expression characteristic of herpesviruses, with expression of IE genes first. Many IE genes are transcription factors. One such IE gene is the replication and transcription activator (RTA/ORF50) protein, which is known as the lytic switch protein (22). RTA expression is sufficient and necessary to induce lytic replication through the activation of lytic gene promoters.

1.3 KSHV Latent and Lytic Cell Culture Systems

At present, there exist no robust or reliable animal models of KSHV infection that faithfully replicate the symptoms and pathology seen in human patients. Therefore, in order to study KSHV, we must rely on cell culture systems and in vitro infections. To date, several cell lines and cell systems have been developed to facilitate the study of KSHV. Several B cell lines are derived from PELs, including BCBL-1 and BC-1 cells. These cells stably maintain the KSHV episome in cell culture and can serve as a model for lytic reactivation (23). However, these cell-lines are not the most relevant tool for studying KS given that KS is an endothelial-based tumor. In addition, these PELs cell-lines cannot be used to study KSHV de novo infection since the cells are already infected with KSHV.

To study KS and de novo infection as well as latency, an endothelial cell model has been developed. A commonly used cell-line in this system is the Tert-immortalized microvascular endothelial (TIME) cell-line. To complement use of TIME cells, which are immortalized, we also use primary human neonatal microvascular BECs and LECs. These are cells isolated from human donors. KSHV infection of endothelial cells in cell culture leads to a predominantly latent phenotype, with 1-5% lytic, which supports what is observed in spindle cells in the KS tumor (9). While TIME cells are a useful model for KSHV infection, they are immortalized, which limits their relevance when studying certain cellular processes. Therefore, primary BECs and LECs are the most relevant cell types for studying KSHV and KS.

In order to study KSHV lytic replication, KSHV can be induced to reactivate following infection of endothelial cells. There are several methods to do this, including the use of chemical agents as well as through transgene expression of the KSHV lytic inducer RTA. For induction of KSHV lytic replication through chemicals, cells are first infected with KSHV, then treated with phorbol-12-myristate-13-acetate (PMA) (9). To induce lytic reactivation by transgene expression, KSHV-infected cells can be superinfected with a gutted Adenovirus expressing RTA but not Adenoviral genes (24, 25). This results in efficient lytic replication of the virus.

1.4 Innate immunity and DNA viruses

1.4.1 Pattern recognition receptors and innate immune activation

Innate immunity serves as a cell-intrinsic, first line of defense against invading pathogens. Many cell types, including immune and nonimmune cells, are capable of detecting and responding to microbes and other danger signals (26). Infection of cells leads to the presence and production of pathogen-associated molecular patterns (PAMPs) or host-derived danger-associated molecular patterns (DAMPs) (27). These are molecules with conserved molecular patterns not normally present in healthy cells, and they include bacterial flagellin, lipopolysaccharide, viral glycoproteins, and various nucleic acid species. These PAMPs are sensed by pattern recognition receptors (PRRs), which are specialized germline-encoded proteins that bind to and respond to specific PAMPs (28). PRRs respond by initiating a signal transduction pathway which leads to the production of antimicrobial and proinflammatory cytokines, alterations in host gene expression, and the recruitment of immune cells to the area of infection (29). A major cytokine that is produced during viral infection is type I interferon (IFN), which includes IFN-α and IFN-β (30). IFN-α is mainly produced by hematopoietic cells, including plasmacytoid dendritic cells whereas IFN-β can be produced by a wide variety of cell types (31). Type I IFN signals in autocrine and paracrine mechanisms to induce expression of IFN-stimulated genes (ISGs) (31). There are hundreds of putative ISGs, and many have direct antiviral functions (32, 33). Therefore, the induction of IFN-β is critical for establishing the antiviral state. Thus, PRRs represent an important first line of defense during microbial infection.

During viral infection, the major PAMP present during infection is nucleic acid (34). RNA viruses produce large amounts of double-stranded (ds)RNA during infection and in some cases, produce DNA species as well (as in the case of retroviruses) (35, 36). DNA viruses produce cytosolic DNA, which acts as both a PAMP and a DAMP (depending on the source) (37). DNA

viruses can also produce dsRNA species during infection (38). Therefore, cells employ a variety of PRRs dedicated to sensing dsRNA and cytosolic DNA.

There are two main cytosolic sensors of dsRNA expressed widely: retinoic acid induced gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (39). Both belong to the RIG-I-like receptor (RLR) family. RIG-I detects short, unmethylated 5' triphosphate dsRNA, whereas MDA5 senses long dsRNA with no end specificity (40). RIG-I and MDA5 signal through the adaptor protein mitochondrial antiviral signaling (MAVS), which activates and signals through TANK-binding kinase 1 (TBK1) and transcription factor interferon regulator factor 3 (IRF3) to induce IFN-β expression (41). While RNA sensors are thought to be more important for controlling RNA virus infections than DNA virus infections, there is mounting evidence that DNA viruses can be restricted by RLRs. Several herpesviruses, including KSHV, produce dsRNA species during infection through bidirectional transcription or through host RNA polymerase III (38, 42, 43). In support of this, genetic ablation of RIG-I leads to increased lytic gene expression and reactivation (42, 44). Finally, KSHV encodes a protein from ORF64 that directly antagonizes RIG-I, thus demonstrating the importance of RNA sensing pathways during a DNA virus infection (45).

During DNA virus infections, cytosolic DNA is a major PAMP and can be detected by a variety of PRRs, including absent in melanoma 2 (AIM2), interferon gamma inducible protein 16 (IFI16), toll-like receptor 9 (TLR9), and cyclic GMP-AMP synthase (cGAS) (46, 47). The expression of TLR9 is restricted to immune cells whereas AIM2 is involved in inflammasome activation rather than IFN induction, so they will not be discussed further (48). IFI16 has been proposed as a sensor of viral DNA (49). However, its exact role in the induction of IFN-β is controversial (50, 51). cGAS was identified as a DNA sensor during viral infection and is likely the primary sensor of cytosolic DNA in most cell types (50, 52, 53). cGAS binds cytosolic DNA and synthesizes the cyclic dinucleotide 2'3' cyclic GMP-AMP. cGAMP then binds the adaptor

proteins stimulator of interferon genes (STING), which is anchored at the ER (53, 54). STING then undergoes a conformational change and translocates to perinuclear vesicles (55). STING recruits TBK1, which autophosphorylates and then phosphorylates STING (56). This promotes the binding of STING to IRF3, allowing TBK1 to phosphorylate IRF3 (57). Phosphorylated IRF3 then translocates to the nucleus, where it induces transcription of IFN-β (58). Additionally, STING activates several other pathways, including NF-κB, STAT6 and autophagy, which may have antiviral functions (59–62).

Due to the importance of STING signaling in a variety of cellular processes, its activation is tightly regulated. Aberrant and constitutive activation can lead to autoimmune and autoinflammatory disorders whereas suppression of this pathway is implicated in cancer development (63–66). To this end, cells exhibit stringent control of STING activation and utilize negative regulation to prevent inappropriate STING activation. Upon binding to cGAMP, STING undergoes K48-linked polyubiquitination, which leads to degradation via the proteasome (67, 68). In addition, phosphorylation of STING by UNC-51-like kinase following binding of cGAMP leads to degradation of STING (69). In contrast, both K27-linked and K63-linked polyubiquitination, as well as phosphorylation of STING have been shown to promote and be required for innate immune signaling (56–58, 70–73). It is likely that these posttranslational modifications of STING first lead to activation, then lead to degradation as a means of preventing aberrant innate immune signaling.

Numerous studies have shown the importance of STING-mediated signaling during DNA virus infections. cGAS- and STING-deficient cells and mice are unable to activate innate immune pathways, including induction of IFN-β during infection with several DNA viruses, including HSV-1, Vaccinia virus, Adenovirus (Adv), HCMV and KSHV (14, 74–78). In addition, cGAS- and STING-deficient mice have higher viral titers and succumb to infection by DNA viruses more quickly than wild-type mice. Surprisingly, mice deficient in cGAS and STING are

more susceptible to RNA virus infections than wild type, despite normal IFN induction (79). Overall, the cGAS-STING pathway is critical for activating antiviral immunity and determining susceptibility to infection with a broad range of viruses.

1.4.2 Viral subversion of innate immune pathways

Due to the importance of innate immune pathways, including cGAS-STING and RLR-MAVS, viruses encode several proteins that directly antagonize activation of these pathways. Many DNA viruses have now been reported to encode proteins that antagonize the cGAS-STING pathway, including HSV-1, CMV, KSHV, MHV-68, Vaccinia virus and Adv (14, 77, 87, 78, 80–86). KSHV encodes at least three proteins that directly target cGAS or STING (14, 77, 78). ORF52 encodes a tegument protein that directly antagonizes cGAS during initial infection (78). ORF52 blocks the enzymatic activity of cGAS, which prevents cGAMP production. In addition, KSHV expresses at least two genes during lytic replication that antagonize either cGAS or STING. Cytoplasmic isoforms of LANA accumulate during lytic replication and block cGAS signaling (14). In addition, viral interferon regulatory factor 1 (vIRF1) is also expressed during lytic replication and antagonizes STING signaling by blocking its interaction with TBK1 (77).

In addition, several RNA viruses, including Dengue virus and Zika virus, antagonize the cGAS-STING pathway, suggesting it is broadly important for inhibiting viral infections (88, 89). There is some evidence that RNA virus infection may trigger the release of mitochondrial DNA, which may be sensed by cGAS (90). Alternatively, STING has been proposed to act as a restriction factor for RNA virus replication through inhibition of viral translation (91). Regardless of the mechanism, the fact that both DNA and RNA viruses target and antagonize the cGAS-STING pathway suggests that it exerts selective pressure on a wide variety of viruses. Several DNA viruses directly antagonize activation of RNA sensing pathways. HSV-1 blocks MAVS activation by targeting RIG-I and MDA5 (92). As mentioned previously, KSHV directly blocks RIG-I activation through ORF64, which deubiquitinates RIG-I to prevent signaling through MAVS (45).

In addition to targeting cGAS and STING directly, many DNA viruses, particularly KSHV, also block downstream activation of the pathway and subsequent signaling of IFN-β. KSHV LANA blocks expression of IFN-β by directly competing with IRF3 for binding to the *ifnb* promoter (93). In addition, KSHV antagonizes paracrine signaling of IFN-β by downregulating expression of the receptor, IFNAR1, through ORF54 (94). Finally, KSHV vIRF2 inhibits ISG expression by targeting STAT1 and IRF9 (95).

Some viruses, including herpesviruses, avoid activation of these pathways simply by virtue of their lifecycle. During herpesvirus infection, the viral DNA is not exposed to the cytosol under normal circumstances. Following entry, the genome, which is encapsidated, is shuttled to the nuclear envelope, where the capsid injects the DNA into the nucleus (1). In addition, KSHV infection results in a predominantly latent state, with the viral genome maintained as an episome tethered to host chromosomes. This limits the amount of viral DNA that can be sensed by host PRRs such as cGAS. Therefore, a major question in the field is how can a cytosolic DNA sensor detect viral DNA if the viral DNA is not predominantly in the cytosol? There have been several proposed sources of cytosolic DNA during herpesvirus infections. One hypothesis is that defective capsids release viral DNA into the cytosol prematurely. In addition, viral capsids may undergo ubiquitination, leading to degradation by the proteasome and subsequent release of viral DNA (96). Furthermore, there is some evidence that herpesvirus infections leads to the release of mitochondrial DNA, which activates antiviral immunity in a cGAS-dependent mechanism (97).

1.4.3 KSHV and the cGAS-STING pathway

The cGAS-STING pathway has been identified as playing an important role in restricting KSHV at different stages of infection. It has been reported that overexpression of cGAS leads to decreased susceptibility to KSHV (78). In addition, knockdown of cGAS or STING leads to increased lytic gene expression and reactivation suggesting that both cGAS and STING may be

important for restricting spontaneous lytic reactivation (77, 98). However, many of the studies demonstrating the importance of cGAS and STING in controlling KSHV rely on overexpression or cell-lines that are not relevant to KS (14, 77, 78). Several use iSLK cells, which are a renal cell carcinoma cell-line. Therefore, the exact role of endogenous STING during KSHV infection of primary endothelial cells has not been explored. Furthermore, it is unclear if STING restricts de novo KSHV infection or lytic reactivation (98). The use of appropriate cell systems for studying KSHV is extremely important.

1.5 Altered Cellular Replication and Cancer

1.5.1 Initiation of Cellular Replication

Eukaryotic cellular replication is a tightly controlled process beginning with the initiation of DNA replication. During this step, pre-replication complexes (pre-RCs) are assembled on DNA at origins of replication by binding of origin recognition complexes (ORCs) to the chromatin (99). ORCs are comprised of a stable core complex including subunits ORC2-ORC5 associated with ORC1 and ORC6, which are less stably bound (100). Assembly of the ORCs leads to the recruitment of two other proteins, CDC6 and Cdt1, which act by stabilizing the binding of ORCs and allows Cdt1 to load the replicative helicase, minichromosome maintenance (MCM) proteins 2-7, which is a heterohexamer (101). Loading of the MCM2-7 is the final step in pre-RC assembly, which is known as licensing of the replication origin. This process of Pre-RC formation is restricted to the G1 phase of the cell cycle. At the beginning of S-phase, replication origins are activated by the recruitment of protein kinases, CDC7 and CDK2. The helicase is activated by phosphorylation of the MCM complex and the loading of additional factors including CDC45 and the GINS complex (CDC45, MCM2-7 and GINS make up the CMG helicase) (102). While several phosphorylation events occur, the phosphorylation of MCM2 by both CDK2 and CDC7 has been identified as being critical for the initiation of replication. Phosphorylation of MCM2 allows for the interaction between Cdc45, MCM2-7 and GINS. Upon activation, MCM2-7

unwinds dsDNA bidirectionally from the origin to create the ssDNA template necessary to recruit the remaining DNA synthesis machinery (i.e. RPA, DNA polymerases) and to start the process of chain elongation. Following cell division, these pre-RC components are disassembled.

1.5.2 CDC7, MCM2 and Cancer

CDC7 and MCM2 have been identified as important therapeutic targets in cancer. Both CDC7 and MCM2 are overexpressed in many different cancers (103). In addition, levels of phosphorylated MCM2 may correlate with proliferation. Interestingly, knockdown of CDC7 and MCM2 leads to selective killing of many cancer cell lines (104, 105). This has led to the development of several CDC7 inhibitors, some of which are in clinical trials (106). The leading hypothesis for how CDC7 and MCM2 can be targeted during cancer is as follows: Depletion of CDC7 and/or MCM2 in “normal” cells leads to stalling of the replication fork and DNA damage (103). DNA damage is sensed by ataxia telangiectasia mutated (ATM) and ATM and RAD3-related (ATR), which activate different checkpoint proteins, including pRB, p53 and Chk1/Chk2. Activation of checkpoint proteins leads to cell cycle arrest in order to prevent further genomic instability. Cancer cells, on the other hand, have deregulated checkpoint proteins, so replication proceeds even in the absence of CDC7/MCM2. This leads to replication fork stalling and double-stranded DNA breaks. Accumulation of DNA damage activates ATM/ATR, which activate p38 and Caspase 3, leading to apoptosis.

1.5.3 KSHV and MCM proteins

The role of the MCM2-7 complex during KSHV infection has only recently been investigated. During latency, LANA interacts with MCM3, 4 and 6 in B cells (107). This interaction is only during the replicative phase (G1/S). Recruitment of MCM3 and 6 is required for replication of viral episome and depletion of MCM3 and 6 leads to decreased replication of the viral episome as well as decreased episome copy number. Additionally, depletion of MCM5 leads to

decreased replication of TR plasmids (108). In addition, ORC2 and MCM3 bind to TR and regions in the long unique region (LUR) of the KSHV genome (109).

During lytic reactivation, KSHV disrupts the MCM complex to promote viral replication. ORF59 interacts with MCM3, 4, 5 and 6 (110). Depletion of MCM6 in BCBL-1 and BC3 cells leads to increase in viral genome copies, viral late gene transcripts, and virion production following reactivation. KSHV lytic replication also leads to hypophosphorylation of MCM3 (which is associated with reduced chromatin loading). This leads to an inability of MCM3 to associate with histones in reactivated cells. Finally, ChIP showed lower levels of MCM3 and MCM4 association at cellular origins of replication and decreased levels of cellular DNA synthesis in cells undergoing reactivation.

While the MCM proteins have been identified as being important for both lytic and latent replication, a specific role for survival of latently infected cells has not been reported. In addition, the fate of MCM2 and CDC7 during de novo infection has not been explored. Recently, our lab used CRISPR/Cas9 to screen for host genes that are essential during KSHV latency. MCM2 was identified as being essential for KSHV-infected cells but not uninfected cells. To pharmacologically validate this finding, we used a CDC7 inhibitor and found that KSHV-infected cells are more sensitive to CDC7 inhibition than uninfected cells.

1.6 Hypothesis

Given the importance of innate immune signaling through the cGAS-STING pathway, we hypothesized that STING would be important for restricting de novo KSHV infection as well as spread following lytic reactivation in primary endothelial cells. For a comparison, we used another DNA virus, Adv, which potently activates cGAS-STING-dependent antiviral pathways. We hypothesized that STING would be required for restricting Adv replication and spread. However, we found that STING is dispensable for restricting susceptibility to infection as well as spreading through the culture following lytic reactivation during KSHV infection. In support of

this, KSHV minimally activates innate immune signaling. In contrast, STING is required for restricting Adv spread through the culture but not for blocking Adv replication. The finding that Adv replication is not restricted by STING is consistent with a previous report, but to our knowledge, our data showing spread of Adv is blocked by STING is novel (111).

It was surprising that MCM2 was identified in our screen as being essential for KSHV latency but not in uninfected cells given the function of MCM2. However, we hypothesized that KSHV alters and deregulates cell cycle checkpoint proteins. Therefore, when CDC7 is depleted/inhibited, cells proceed through abortive S phase, leading to DNA damage, which activates ATM/ATR, which in turn, activate p38 and caspase 3, resulting in cell death. We found that MCM2 may be required for survival of latently infected cells. Chemical inhibition of CDC7 resulted in an increase in cell death in KSHV-infected cells.

Chapter 2. Materials and Methods

2.1 Cell Culture Systems

Primary human neonatal microvascular BECs and LECs were obtained from Lonza and cultured in EBM-2 (Lonza) supplemented with a bullet kit containing 5% FBS, vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor 3, epidermal growth, and hydrocortisone. Numbers indicate that primary cells are from different donors (BEC1, BEC2, BEC3, LEC4, LEC6, and LEC8). Tert-Immortalized Microvascular Endothelial (TIME) cells (112) were grown under the same conditions as primary endothelial cells. HEK 293T and HEK 293 IN-β cells were cultured in DMEM (Corning) supplemented with 10% FBS, sodium pyruvate, L-glutamine, and penicillin/streptomycin. iSLK cells stably maintaining selectable GFP-expressing wild-type KSHV on a bacterial artificial chromosome (BAC16) were grown in DMEM. BCBL-1, BC-1, RAJI and BJAB cell lines were grown in RPMI supplemented with FBS, penicillin/streptomycin, and 2-Mercaptoethanol. Cells were cultured at 37° C, 5% CO_2

2.2 Viruses

KSHV-BAC16 was generated as previously described by inducing iSLK cells with doxycycline and harvesting the virus from the cellular supernatant by centrifugation (113). BCBL-1-derived KSHV (WT-KSHV) was generated as previously described (114). A gutted helper-dependent Adenovirus expressing the KSHV lytic and replication activator (HD-Ad-RTA) was obtained from D. Ganem (24) and generated as previously described (25).

A BAC vector (pKSB2HAdV64) containing the entire genome of HAdV-64 was created as follows: 1) the first 1475 bp and the last 1008 bp of the HAdV-64 genome (115) flanking a *galK* ORF from *pgalK* (116) were subcloned into pUC19; 2) the insert was then subcloned into *Eco* RI and *Hin* dIII digested pBluescript II SK(+); 3) the insert was then subcloned into *Not* I and *Hin* dIII digested pKSB2 (117); 4) the resulting vector was used to transform SW102 cells (116); 5)

HAdV-64 genomic DNA was isolated from virus and recombineered in place of the *galK* ORF (116). This vector was further modified by recombineering to replace the E3 region (bp 26215 to 30789) with a PCR product containing the CMV-eGFP ORF (bp 4709 to 1398) from pEGFP-N1 (Clontech Laboratories, Inc.) to create pKSB2HAdV64.eGFP. The sequence of this construct was verified by Sanger sequencing of the recombineered region and by restriction digest of the entire BAC vector. Release of the viral genome from pKSB2HAdV64.eGFP by flanking *Pac* I sites was followed by transfection of 293β5 (118) cells to produce virus. Genomic DNA was isolated from purified virus, and the sequence of the entire genome was verified by Illumina sequencing.

2.3 Latent KSHV Infections

Infections with KSHV were done as described (114). Briefly, virus stocks were diluted in serum free media containing 8 ug/mL of polybrene and incubated with cells for 4 hours with gentle rocking. The inoculum was then replaced with complete media and incubated for the indicated time post infection. For KSHV IFN-β induction experiments, cells were infected with KSHV-BAC16 at a MOI such that >75% of the cells were infected as indicated by immunofluorescence staining with an antibody to the KSHV LANA protein. For immunoblotting for p-STING, p-TBK1 and p-IRF3 following KSHV infection, cells were infected with WT-KSHV such that >80% of the cells were infected. For comparing infection rates after the establishment of latency in ΔSTING and scramble BECs, cells were infected with varying concentrations of KSHV-BAC16. All KSHV infections were done in serum-free media with polybrene.

2.4 Lytic KSHV Assay

For inducing lytic reactivation, cells were infected with either WT-KSHV or KSHV-BAC16 as previously described. After 4 hours, cells were rinsed with PBS and superinfected with HD-Ad-RTA for 1 hour in serum-free media, poly L:lysine (1μ/mL) and sodium butyrate (1 μM). Cells

were then rinsed twice with PBS and incubated in complete media for 24 hours. Cells were then harvested for RT-qPCR or immunoblotting.

2.5 Adenovirus Infections

For HAdV-64 replication titer, cells were infected at 100 genomes per cell for 45 minutes at 4° C, then rinsed with PBS and incubated for the indicated times. At 5 days post infection (dpi), cells were scraped, and the supernatant was frozen in liquid nitrogen. To titer HAdV-64 virions, the supernatant was then thawed and re-frozen in liquid nitrogen 3 more times and then added to 293T IN-β cells. Cells were incubated for 24 hours and then fluorescence was quantified using Typhoon fluorescent scanner. For measuring Adv spread, cells were infected with HAdV-64 at 10 genomes per cell and cells were incubated for 5 days. Plates were scanned on the Typhoon fluorescent scanner.

2.6 Immunofluorescence

To determine viral titers for comparing infection rates, aliquots of KSHV-BAC16-infected BECs and LECs were seeded on 4-well chamber slides and fixed with 4% (vol/vol) paraformaldehyde-phosphate-buffered saline. Infection rates were assessed using antibodies against the latent KSHV protein LANA (a kind gift from A. Polson and D. Ganem) as described previously (9). Cells were incubated with fluorophore-conjugated secondary antibodies goat anti-rabbit Alexa Fluor 488 (Molecular Probes/Invitrogen). Cells were mounted in medium containing DAPI (4’,6’-diamidino-2-phenylindole) before being viewed under a Nikon Eclipse E400 fluorescence microscope (Nikon, Inc.).

2.7 Antibodies and Reagents

Antibodies were purchased from the indicated source: anti-cGAS (catalog number 15102; Cell Signaling Technology), anti-STING (catalog number ab181125; Abcam) (catalog number 13647; Cell Signaling Technology), anti-p-STING (catalog number 85735; Cell Signaling Technology), anti-p-TBK1 (catalog number 5483; Cell Signaling Technology), anti-TBK1 (catalog number

3504; Cell Signaling Technology), anti-p-IRF3 (catalog number 4947; Cell Signaling Technology), anti-IRF3 (catalog number 4302; Cell Signaling Technology), anti-MCM2 (catalog number A300-191A; Bethyl Labs), anti-p-MCM2 (catalog number A300-756A; Bethyl Labs) and anti-β-actin (catalog number A5441; Sigma Aldrich). The anti-LANA antibody was obtained from Don Ganem (University California, San Francisco). Recombinant IFN-β was purchased from EMD Millipore (catalogue number: IF014).

2.8 Nucleic Acid and cGAMP Transfection

E. coli dsDNA (dsDNA-EC) (2.5 μg/mL) (Invivogen), high molecular weight Polyinosinic–polycytidylic acid [Poly(I:C)] (0.5 μg/mL or 1 μg/mL) (Invivogen), interferon stimulatory DNA (ISD) 100mer (10 μg/mL) [synthesized by heating equimolar sense and antisense oligonucleotides (sequences in Table 3.1) to 95° C and annealing at room temperature for 1 hour], calf thymus DNA (1 μg/mL) (Invitrogen), and 2'3' cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) (5 μg/mL) (Invivogen) were transfected into BECs or LECs using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

2.9 Quantitative RT-PCR

Total RNA was extracted using RNA isolation kits (Macherey-Nagel). RNA was reverse-transcribed using iScript Reverse Transcription Supermix (Biorad). Gene expression was measured by qPCR using SsoAdvanced Universal SYBR Green supermix (Biorad). Primers used are shown in Table 3.1. The relative levels of each transcript were normalized by the delta threshold cycle method to the abundance of Tubulin mRNA.

2.10 Western Blot Analysis

Cells were lysed in either RIPA lysis buffer or NP-40 lysis buffer supplemented with 1 mM sodium orthovanadate (Sigma Aldrich), 1 mM sodium fluoride (Sigma Aldrich), 40 mM 2-glycerophosphate (Sigma Aldrich), benzonase nuclease (Sigma Aldrich), and protease inhibitor cocktail tablets (Roche). Protein was quantified using bicinchoninic acid (BCA) assay (Pierce).

Lysate was run on a gradient 4-20% SDS-PAGE gel (Biorad) and then transferred to an Immobilon F polyvinylidene difluoride membrane (Millipore). The membranes were blocked using Odyssey blocking buffer (Li-COR) for at least 1 hour and then probed with the indicated primary antibodies overnight at 4° C with rocking. Blots were washed 3 times with Tris-buffered saline with Tween 20 (TBST) and then probed with IRDye secondary antibody (Li-COR) diluted in Li-COR buffer for at least 1 hour at room temperature. Blots were washed 3 times in TBST and then scanned with an Odyssey CLx infrared imaging system (Li-COR) for fluorescent blots.

2.11 CRISPR/Cas9 and stable targeting of STING (*TMEM173*) and MCM2

We obtained a pRRL plasmid expressing a gRNA targeting STING and Cas9-T2A cassette from Daniel Stetson (University of Washington), described here (50). Sequences for gRNAs targeting STING, MCM2 and the nontargeting scramble control are shown in Table 2.1. Lentivirus targeting STING, MCM2 or the non-targeting scramble control was generated by co-transfection of 293T cells with the plasmid and two packaging plasmids, pMD2.G and psPAX2 (Addgene) into HEK293T cells using Transit 293 transfection reagent (Mirus) per the manufacturer's instructions. For stable targeting of STING, BEC1-3 were transduced with the lentivirus for 6 hours and selected with puromycin (1 μg/mL) (VWR Scientific) for 3 days.

2.12 Flow Cytometry

ΔSTING and scramble BEC3 were infected with KSHV-BAC16 at low MOI as previously described. Immediately following infection, cells were treated with PMA (200 ng/mL) for 5 days. Cells were fixed in PFA and sorted by GFP fluorescence.

2.13 RNA sequencing of STING (TMEM173)

RNA was extracted from LEC4 and submitted for RNA sequencing to the Benaroya Genomics Core Facility and the results were analyzed by the Fred Hutchinson Cancer Research Center Genomics Shared Resource. LEC4 TMEM173 transcripts were aligned to human reference

genome 38 and human STING1 (TMEM173) transcript variant 1 (Accession NM_198282.4) using Integrative Genomics Viewer.

2.14 Cells Death Assay

TIME cells were infected with WT-KSHV or the indicated KSHV-BAC16 mutants (wt, ΔvCycΔvFLIP, ΔKaposins, or ΔmiR) or transduced with a lentivirus expressing mCherry or LANA. At 24 hpi, cells were treated with 1 μM PHA767491. Cells were then analyzed for cell death by trypan blue exclusion according to the manufacturer's protocol and by using the TC20 Automated Cell Counter 48 hours after drug treatment. For cell death assays using BJAB, BCBL-1, BC-1 and RAJI cell lines, cells were plated at 200,000 cells per mL and treated with various concentrations of PHA767491. Cell death was measured 48 hours later as previously described.

2.15 Statistical Analysis

Data are represented as the mean ± standard error of the mean (SEM) where indicated, and either Student's *t* test or Two-way ANOVA with Sidak posthoc test was used for statistical analyses with GraphPad Prism software.

Table 2.1: CRISPR/Cas9 oligonucleotide gRNA sequences

Gene name	gRNA
STING (TMEM173)	GGTGCCTGATAACCTGAGTATGG
MCM2 gRNA 1	GCAGGAAGTTCTTGAAGCGG
MCM2 gRNA 2	GCTGACGGCCAGTCAGAGGG
Scramble	GTTCGATCCGGCGACTCGAA

Chapter 3. STING is dispensable during KSHV infection of primary endothelial cells

Adapted from an article originally published in the scientific journal Virology

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3.1 Abstract

During DNA virus infections, detection of cytosolic DNA by the cGAS-STING pathway leads to activation of IFN-β. Kaposi's Sarcoma Herpesvirus (KSHV), an oncogenic DNA virus, is the etiological agent of Kaposi's Sarcoma, an endothelial cell (EC)-based tumor. To investigate the role of STING during KSHV infection of primary ECs we identified a primary lymphatic EC sample that is defective for STING activation and we also knocked out STING in blood ECs. Ablation of STING in EC does not increase susceptibility to KSHV latent infection nor does it increase KSHV spread after lytic reactivation indicating STING signaling does not restrict KSHV. In contrast, STING ablation increases Adenovirus spread at low MOI, but STING is dispensable for blocking replication. These experiments reveal that the importance of STING depends on the DNA virus and that STING appears more important for restricting spread to bystander cells than for inhibition of viral replication.

3.2 Introduction

Innate immunity is critical for the detection and response to invading microbes. Detection relies on the sensing of pathogen-associated molecular patterns (PAMPs), which are structurally conserved molecules that are present or produced during infection (29). PAMPs are detected by pattern recognition receptors (PRRs), proteins that then signal to activate

production of antimicrobial factors, including cytokines such as type I interferon (IFN) (28). During viral infections, a major PAMP is nucleic acids, including double-stranded (ds)RNA and cytosolic DNA (34). Therefore, cells express a variety of PRRs dedicated to the detection of nucleic acids. For detection of dsRNA, major sensors include retinoic acid induced gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (119). For mislocalized and foreign DNA, major sensors include absent in melanoma 2, interferon gamma inducible protein 16, and cyclic AMP-GMP synthase (cGAS) (46, 47). While several DNA sensors have been described, cGAS is likely the primary PRR for sensing cytosolic DNA during DNA virus infections in most situations (50, 52, 120). Upon binding to DNA, cGAS synthesizes the cyclic dinucleotide 2'3' cyclic GMP-AMP (cGAMP), which binds to the ER adaptor stimulator of interferon genes (STING), leading to the ubiquitination of STING and recruitment of TANK-binding kinase 1 (TBK1) (53, 54, 70, 121–124). Activation of TBK1 leads to autophosphorylation and subsequent phosphorylation of STING and interferon regulator factor 3 (IRF3) (58, 125). Phosphorylated IRF3 then translocates to the nucleus where it acts as a transcription factor to induce expression of IFN-β and subsequent downstream IFN-stimulated genes (ISGs) (126). Despite extensive studies of these pathways, less is known about how STING affects the lifecycles of different DNA viruses.

Kaposi's Sarcoma-associated herpes virus (KSHV) is a gammaherpesvirus, with a large double-stranded DNA (dsDNA) genome (2). KSHV, like all herpesviruses, undergoes both lytic and latent replication, with latency being the predominant state in cell culture and its associated tumors (9, 127). KSHV is the etiological agent of Kaposi's Sarcoma, a common tumor of AIDS patients and one of the most common tumors in parts of sub-Saharan Africa (2, 128, 129). KS is a highly vascularized tumor composed primarily of spindle cells that express markers of endothelium (130). Endothelial cells are divided into two main categories: blood endothelial cells (BECs), which line blood vessels and lymphatic endothelial cells (LECs), which make up lymphatic vessels (131, 132). While KS spindle cells express markers of both BECs and LECs,

the expression profile of spindle cells from KS tumors aligns most closely with KSHV-infected LECs (5, 6, 130).

KSHV infects both BECs and LECs in cell culture and has been shown to both activate and antagonize innate immune pathways in these cells (133, 134). Recent studies have focused on KSHV genes that antagonize the cGAS-STING pathway. It has been reported that ORF52 encodes a tegument protein that directly antagonizes cGAS during initial infection (78). In addition, KSHV encodes at least two genes that are expressed during lytic replication that antagonize either cGAS or STING. Cytoplasmic isoforms of the latency-associated nuclear antigen (LANA) are reported to accumulate during lytic replication and block cGAS signaling (14). In addition, viral interferon regulatory factor 1 (vIRF1) is also expressed during lytic replication and antagonizes STING signaling by blocking its interaction with TBK1 (77). However, the role of STING during KSHV de novo infection remains unclear (14, 77, 78). Additionally, while some studies have suggested a role for STING in restricting lytic reactivation, it is unknown if STING is important for controlling this process in primary endothelial cells (14, 98). In addition, while numerous studies have reported on the importance of STING in controlling DNA virus infections, it is unclear whether STING plays a role in blocking initial infection or replication of DNA viruses (14, 77, 78, 98, 135–138).

Interestingly, we recently discovered a single-patient batch of primary neonatal LECs (LEC4) that are defective for activation of innate immune pathways following infection by DNA viruses, including KSHV and Adenovirus (Adv). We further characterized this defect and found that LEC4 are intrinsically defective in sensing cytosolic DNA and cGAMP, indicating a block in the cGAS-STING pathway. Further characterization reveals that STING, TBK1 and IRF3 are not phosphorylated in LEC4 following transfection with cGAS-STING agonists. Additionally, STING degradation, a hallmark of activation, fails to occur in LEC4. We show that pre-treatment of LEC4 with cGAMP fails to decrease susceptibility to KSHV while pre-treatment with IFN-β nearly completely block KSHV infection, thus demonstrating that other antiviral pathways are

not activated in LEC4 following exposure to cGAMP. Because mutations that inactivate the pathway are common across human populations (66), we sought to better understand the role of STING in KSHV infection. We investigated the role of STING during initial viral infection and spread. We first found that KSHV induces minimal IFN-β expression in BECs and STING competent LECs and does not detectably activate the STING-TBK1-IRF3 axis. To further interrogate the role of STING during viral infection, we used CRISPR/Cas9 to create STING knockout BECs. Surprisingly, genetic ablation of STING does not increase susceptibility to infection by KSHV nor does it enhance the ability of KSHV to spread through the culture following lytic reactivation. Additionally, STING is not required for the induction of IFN-β during KSHV infection. However, the role of STING during Adv infection is more nuanced, with STING restricting spread at low MOI but playing no role in blocking replication at high MOI. Overall, these experiments reveal that while we have identified a LEC isolate that is defective for STING, STING appears to be dispensable during infection with KSHV. STING is important for blocking infection in bystander cells for a different dsDNA virus, Adv, but not for controlling replication following initial infection by Adv. Therefore, it appears that KSHV maintains high-level control of the activation of STING-related DNA sensing pathways.

3.3 Results

3.3.1 DNA viruses fail to activate innate immune signaling in LEC4

To determine if there are differences in the innate immune response to KSHV, we infected primary blood (BEC) and lymphatic (LEC) endothelial cells from different patients, BEC1-2, LEC4 and LEC6, with KSHV-BAC16 and measured the induction of IFN-β. IFN-β transcripts were slightly elevated in all 3 batches of cells following KSHV infection, albeit to varying degrees, with the greatest induction observed in LEC6, and the smallest increase in LEC4 (Fig. 3.1A). Since latency is the primary state during KSHV infection, we wanted to determine if DNA virus replication would similarly stimulate a differential IFN-β response in BECs and LEC4. Adv

is a DNA virus that undergoes lytic replication in a wide variety of cell types. When infected with Adv, IFN-β was induced robustly in BEC1, but not in LEC4 (Fig. 3.1B). Overall, these results indicate modest increases in IFN-β expression following KSHV infection in cells from all donors tested, but defective innate immune activation to Adv viruses in LEC4.

3.3.2 cGAS-STING pathway agonists fail to activate innate immune signaling in LEC4

Since induction of IFN-β was minimal following infections with KSHV and Adv in LEC4, we next sought to determine if LEC4 are defective for innate immune activation of DNA sensing pathways. To test this, we transfected BEC2 and LEC4 with *E. coli* dsDNA (dsDNA-EC) and measured the induction of IFN-β. Surprisingly, IFN-β induction was completely absent in LEC4 but induced robustly in BEC2 (Fig. 3.2A). This trend was also observed for all other ISGs tested, including Mx1, Mx2, ISG15 and IFIT1 (Fig. S3.1A-D). Similarly, when cells were transfected with cGAMP, IFN-β induction was absent in LEC4 but robust in BEC2 (Fig. 3.2B). Examination of the expression levels of other ISGs followed a similar trend, including Mx1 and ISG15, with strong induction to cGAMP in BEC2, but no increase in expression in LEC4 (Fig. S3.1E and D). To determine if the defect in innate immune activation is a general feature of LECs, we transfected either dsDNA-EC or cGAMP into adult LECs as well as corresponding adult BECs and found that induction of IFN-β was robust in these cells, indicating that this defect is unique to LEC4 (Fig. S3.2A and B). To determine if this defect is a trait shared by other neonatal LECs, we transfected calf thymus (CT) DNA and cGAMP into two other primary neonatal LEC isolates (LEC6 and LEC8), and found a strong increase in expression of IFN-β in both LEC6 (Fig. S3.2C) and LEC8 (Fig. S3.2D) and were able to detect phosphorylated STING, TBK1 and IRF3 in both LEC6 (Fig. S3.2E) and LEC8 (Fig. S3.2F). Overall, these experiments show that other LECs patient isolates have intact cGAS-STING signaling, albeit with some heterogeneity in the magnitude of the response. The cGAS-STING pathway utilizes TBK1, which is also critical for signaling in dsRNA sensing pathways, including RIG-1/MDA5-MAVS. In order to determine if

the defect in innate immune signaling in LEC4 is upstream of TBK1, we transfected BEC2 and LEC4 with the dsRNA mimic, Polyinosinic-polycytidylic acid [Poly(I:C)], and found Poly(I:C) induced robust expression of IFN-β in both BEC2 and LEC4 (Fig. 3.2C), indicating that RNA sensing pathways are intact and that TBK1 is functional in LEC4. Overall, these experiments reveal that LEC4 have a unique defect in DNA sensing pathways leading to the induction of IFN-β not shared by other LEC isolates but RNA sensing pathways are functional.

3.3.3 Downstream STING signaling is blocked in LEC4 cells following treatment with cGAS-STING agonists

We next sought to characterize the block in STING-mediated innate immune activation. After activation by cGAMP, STING recruits TBK1, which autophosphorylates, and then phosphorylates STING, leading to the recruitment and phosphorylation of IRF3. To determine if TBK1, STING and IRF3 undergo phosphorylation in LEC4, we transfected BEC3 and LEC4 with interferon-stimulatory DNA (ISD) 100mer, cGAMP or Poly(I:C) and immunoblotted whole cell lysates. While Poly(I:C) led to the phosphorylation of TBK1 and IRF3 in BEC3 and LEC4, TBK1, STING and IRF3 were not phosphorylated in LEC4 in response to either ISD 100mer or cGAMP (Fig. 3.3A). In contrast, TBK1, STING and IRF3 were all phosphorylated following either ISD 100mer or cGAMP transfection in BEC3. Negative regulation of STING signaling is critical for controlling excessive and pathological innate immune signaling and inflammation (65). In order to achieve this, STING undergoes K48-linked polyubiquitination and phosphorylation following activation, leading to degradation of STING by the proteasome (67). To determine if STING is degraded in LEC4, we transfected BEC3 and LEC4 with cGAMP and immunoblotted for STING. Interestingly, while STING was rapidly degraded in BEC3, no such degradation occurred in LEC4 (Fig. 3.3B). STING protein expression remained stable through 4 hours post transfection. STING cDNA from LEC4s was sequenced and determined to have a wild-type sequence, indicating that LEC4s have an unknown defect in the STING signaling pathway (Fig. S3.3).

Together, these results indicate that downstream signaling events following STING activation are blocked in LEC4, preventing the phosphorylation of STING, TBK1 and IRF3 and the degradation of STING.

3.3.4 Pre-treatment with cGAMP decreases susceptibility to infection in BEC1, not LEC4

In addition to activating IRF3, STING also activates STAT6, NF-κB and autophagy pathways, which may have antiviral effects (59–62). To determine if STING is activating other antiviral pathways in LEC4, we pre-treated BEC1 and LEC4 with either IFN-β or cGAMP and infected with KSHV-BAC16 24 hours later. Pre-treatment with cGAMP led to significantly decreased infection of BEC1 with KSHV, as measured by GFP intensity (Fig. 3.4, left panel shows representative raw plate images, right panel shows quantification). However, there was no change in infection of LEC4s following the pre-treatment. However, pre-treatment of both BEC1 and LEC4 with IFN-β protected both from infection by KSHV, with reduced susceptibility to infection relative to the untreated controls. Therefore, these results indicate STING is not able to activate other antiviral pathways in LEC4 and that activation of downstream pathways can rescue cells from the defect in STING signaling.

3.3.5 KSHV does not activate STING-TBK1-IRF3 signaling to induce minimal innate immune signaling in primary endothelial cells

STING is reported to play an important role during KSHV infection (14, 77, 78, 98). However, it is unknown if KSHV activates the cGAS-STING pathway in endothelial cells. To determine if KSHV de novo infection of primary cells activates the cGAS-STING pathway, we infected primary BEC1-2 and LEC6 with KSHV-BAC16 and measured the induction of IFN-β over time. IFN-β expression peaks at 48 hours post infection (hpi), with minimal induction at 6 and 24 hpi (Fig. 3.5A). In addition, there was slight upregulation of the ISG IFITM1 at 48 hpi, following a similar trend as IFN-β (Fig. 3.5B). To determine if KSHV induces IFN-β through STING, we immunoblotted for p-STING, p-TBK1 and p-IRF3 at various time points up to 48 hpi with WT-

KSHV. Neither phosphorylated STING nor phosphorylated IRF3 was detected following KSHV infection, while phosphorylated TBK1 was detected up to 6 hpi but not at 24 or 48 hpi in BEC1 (Fig. 3.5C). However, when BEC1 were infected with KSHV first, then transfected with cGAMP, the pathway was activated as indicated by the presence of p-STING, p-TBK1 and p-IRF3 (Fig. S3.4). We next sought to determine if KSHV lytic replication activates innate immune signaling. To do this, we superinfected KSHV-BAC16-infected BEC1-2 and LEC6 with HD-Ad-RTA. This is a helper dependent adenovirus vector that expresses the KSHV lytic switch gene but no Adenovirus genes. While there was slight induction of IFN-β (Fig. 3.5D) and IFITM1 (Fig. 3.5E) in BEC1-2 and LEC6, the expression is far lower than what is observed during Adv infection (Fig. 3.1B). In addition, KSHV lytic reactivation did not activate STING signaling in BEC1, as induction of lytic WT-KSHV did not lead to detectable levels of p-STING, p-TBK1 or p-IRF3 (Fig. 3.5F). We confirmed lytic reactivation was occurring by measuring expression of the early gene K8.1 and late gene ORF10 and found robust induction in the KSHV+HD-Ad-RTA cells compared to KSHV-infected alone cells (Fig. S3.5A and B). We next investigated the activation of STING signaling in LEC6. Similar to BECs, we were unable to detect p-STING, p-TBK1 or p-IRF3 following de novo infection and lytic reactivation with WT-KSHV (Fig. 3.5G). Overall, these experiments reveal that while KSHV induces some expression of IFN-β in primary endothelial cells, the induction is minimal and correlates with undetectable or low levels of phosphorylated STING, TBK1 and IRF3.

3.3.6 STING is dispensable during KSHV infection

Due to the importance of cGAS-STING signaling during DNA virus infection and the presence of STING response variants in the human population, we next sought to further determine the role of STING during KSHV infection. To determine if the defect in STING signaling in LEC4 increases susceptibility to infection by KSHV, we infected LEC4 and LEC8 with KSHV-BAC16 and measured infection rates by immunofluorescence microscopy and counting of LANA+ cells.

Surprisingly, we found no difference in infection rates when comparing LEC4 to LEC8, indicating that STING may not restrict initial infection (Fig. 3.6A). We next wanted to further characterize the role of STING during KSHV infection by genetic ablation of STING. To study this, we used a lentivirus expressing CRISPR/Cas9 to create STING knockout (ΔSTING) BEC1-3 (Fig. 3.6B). To determine if STING signaling is required for the induction of IFN-β following KSHV infection, we infected ΔSTING and scramble BEC3 with KSHV-BAC16 and measured IFN-β transcripts at 48 hpi. Interestingly, ablation of STING did not reduce IFN-β expression in ΔSTING BEC3 as transcript levels were equivalent when compared with scramble BEC3 (Fig. 3.6C). As described in the discussion, the induction of IFN-β without activation of STING could be due to low level, undetectable activation of STING, other DNA sensors (such as IFI16) or possibly from viral dsRNA activating IFN through RNA-dependent pathways. To determine if ablation of STING would increase susceptibility to KSHV, we infected ΔSTING BEC1-3 with KSHV-BAC16 and measured infection rates as described above. Consistent with our experiments comparing susceptibility of LEC4 to LEC8, when we compared infection rates of ΔSTING BEC1-3 to the scramble control with increasing doses of KSHV-BAC16, we found no difference in susceptibility to KSHV (Fig. 3.6D). STING is reported to restrict lytic reactivation of KSHV (14, 98). However, this role has not been demonstrated yet in primary endothelial cells and the importance of STING in preventing KSHV spread through the culture is unclear. To examine the role of STING in restricting KSHV spread following lytic reactivation, we infected ΔSTING and scramble BEC3 with KSHV-BAC16 at different multiplicities of infection and induced lytic reactivation with PMA. We then sorted by GFP at 5 dpi and found that while PMA increased the proportion of GFP+ cells in both ΔSTING and scramble BEC3, there was no difference in the increase in GFP+ cells in the ΔSTING cells compared to the scramble at either dilution of virus (6E). Therefore, STING has no effect on the spread of KSHV through a culture following lytic induction. Overall, these

experiments reveal that STING is dispensable for blocking latent infection and for restricting viral spread during lytic replication of KSHV.

3.3.7 STING is required for restricting Adv spread

To determine if the cGAS-STING pathway is important for controlling other DNA viruses, we tested the role of defective STING signaling during Adv infection. To do this, we infected scramble and ΔSTING BEC3 with Adv and found that induction of IFN-β was nearly completely ablated in the STING knockouts (Fig. 3.7A). We next sought to examine the role of STING during Adv infection by infecting at high and low MOI. We reasoned that infection at high MOI would investigate the role of STING in restricting initial infection and replication whereas low MOI infections would interrogate whether STING is important for blocking spread from cell to cell. At high MOI, when infectious Adv progeny from ΔSTING and scramble control BEC3 were titered on 293T IN-β cells, there was no difference in viral titer harvested from the two cells types (Fig. 3.7B). When infected at low MOI, initial infection was equivalent at 24 hpi (data not shown). However, Adv was able to spread through the culture more readily in the ΔSTING BEC3 compared to the scramble, as seen by an increase in integrated density (GFP intensity) in the ΔSTING BEC3 compared to the scramble control (Fig. 3.7C). Together, these results indicate that STING is required for restricting Adv spread through the culture but not replication.

3.4 Discussion

KSHV encodes multiple proteins that antagonize innate immune activation, including three that target cGAS or STING directly (14, 77, 78) . We wanted to determine if the cGAS-STING pathway was relevant for KSHV infection and the establishment of latency or if these inhibitors obviated the need for STING activation during infection. While studying the role of cGAS-STING in a relevant cell type for KS spindle cells, we fortuitously identified a lot of primary dermal microvascular lymphatic endothelial cells that were deficient for IFN-β activation by dsDNA or cGAMP. This primary cell lot did not induce significant IFN-β levels in response to KSHV

infection. We found there were not significant differences in infection rates between LEC4 and other lots of primary LEC cells indicating that the ability to activate STING in response to infection is not likely to be important for initial KSHV infection. To further analyze the role of STING in KSHV infection, we used CRISPR/Cas9 to knock out STING in primary dermal blood microvascular endothelial cells. Again, there was no difference between infection rates of cells with functional cGAS-STING pathway and cells where STING was knocked out. Therefore, we conclude that STING is negated through either active repression of the pathway or through avoidance of activation during initial infection of endothelial cells and the establishment of latency.

Spread of KSHV through the culture is also not inhibited by the presence of STING. Following lytic induction of wild-type and knockout blood endothelial cells infected with KSHV, we were able to identify spread of KSHV through the culture. There were no differences in the increases in KSHV-positive cells in the culture following lytic induction in the wild-type or STING knockout endothelial cells, indicating that STING signaling is not critical for controlling KSHV lytic replication and spread to new cells. Interestingly, while replication following infection of the endothelial cells with Adv is also not altered in the absence of STING, spread though the culture at low MOI is. This result is consistent with a previous report regarding infection, although the finding that STING restricts Adv spread has not been reported previously (111). Pre-treatment of endothelial cells with activators of the cGAS/STING pathway nearly completely blocked infection with KSHV. Since pre-treatment with IFN-β blocks initial infection but STING has little to no effect on KSHV spread, KSHV spread through the culture must rely upon efficient inhibition of the cGAS-STING pathway by KSHV. Thus, the KSHV latent and lytic proteins that inhibit the activation of the cGAS-STING pathway obviate the viral inhibition by STING signaling during lytic infection and spread. However, not all DNA viruses efficiently block the effects of STING activation as knockout of STING allowed more efficient spread of Adenovirus through the culture.

At 48 hours post infection, KSHV induced small amounts of IFN-β. However, we could still not detect activation of STING at this time point. It is possible that there are simply low levels of activation of STING. However, we showed that even in the STING pathway deficient cells, dsRNA was still able to induce interferon. Therefore, at 48 hours post infection there could be some activation of interferon through the formation of viral dsRNA. Alternatively, there could be other pathways that activate small amounts of interferon induction independent of the cGAS-STING pathways. While the induction of interferon was detectable in the KSHV-infected endothelial cells, it was greatly muted as compared to Adv infection or induction with dsDNA or cGAMP and it was only detected at 48 hours post infection. Therefore, we believe that this amount was not significant enough to prevent spread of KSHV through the culture.

This study examines the role of endogenous cGAS-STING activity during de novo infection and lytic reactivation of KSHV in primary endothelial cells and shows that the cGAS-STING pathway is not efficiently activated during KSHV infection of endothelial cells. While many other studies have made important contributions to our understanding of KSHV-innate immune interactions and have pointed to a role for STING in restricting lytic replication (14, 98), we have performed wild-type infection studies in endothelial cells. We show that STING does not play a significant role in KSHV infection, replication or spread in endothelial cells. As KS spindle cells appear to be most closely aligned with endothelial cells, these studies show the role of STING in a cell type that potentially provides relevance to in vivo KSHV-related tumors.

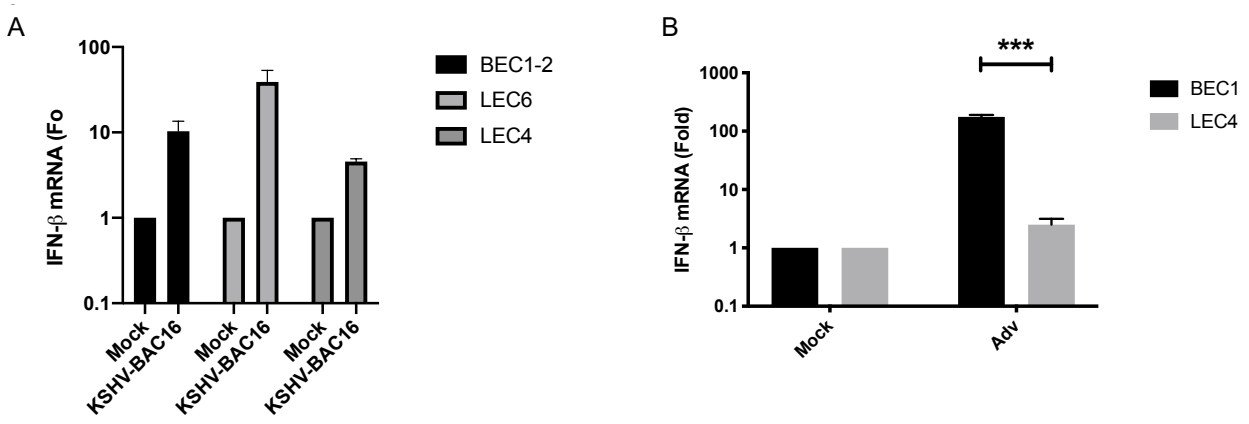

Figure 3.1: KSHV induces minimal IFN-β expression in primary endothelial cells but LEC4 are defective for innate immune activation during Adv infection. A) IFN-β mRNA was measured by RT-qPCR from BEC1-2, LEC4 and LEC6 that were infected with KSHV-BAC16 for 48 hours. The relative amount of mRNA was normalized to tubulin mRNA in each sample, and fold change relative to mock was calculated (ΔΔct). **B)** IFN-β was measured by RT-qPCR from BEC1 and LEC4 that were infected with Adv for 48 hours. The relative amount of mRNA was normalized as in (A). Data are shown as mean ± SEM from at least 3 biological replicates. *** $P < 0.001$; (Student's *t* test).

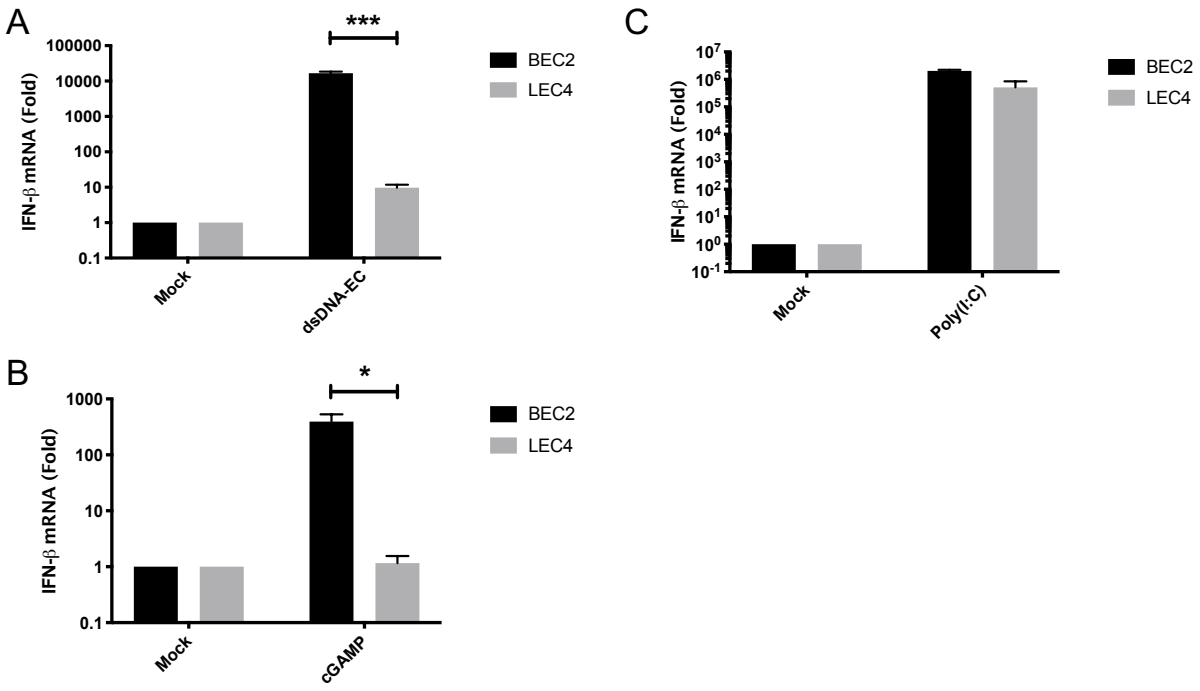

Figure 3.2: cGAS-STING pathway agonists fail to activate innate immune signaling in LEC4. IFN-β mRNA was measured by RT-qPCR from BEC2 and LEC4 that were transfected with **(A)** 2.5 µg/mL dsDNA-EC, **(B)** 5 µg/mL 2'3' cGAMP or **(C)** 0.5 µg/mL high molecular weight Poly(I:C) for 4 hours. The relative amount of mRNA was normalized as in Figure 3.1. Data are shown as mean ± SEM from at least 3 biological replicates. * $P < 0.05$; *** $P < 0.001$; (Student's *t* test).

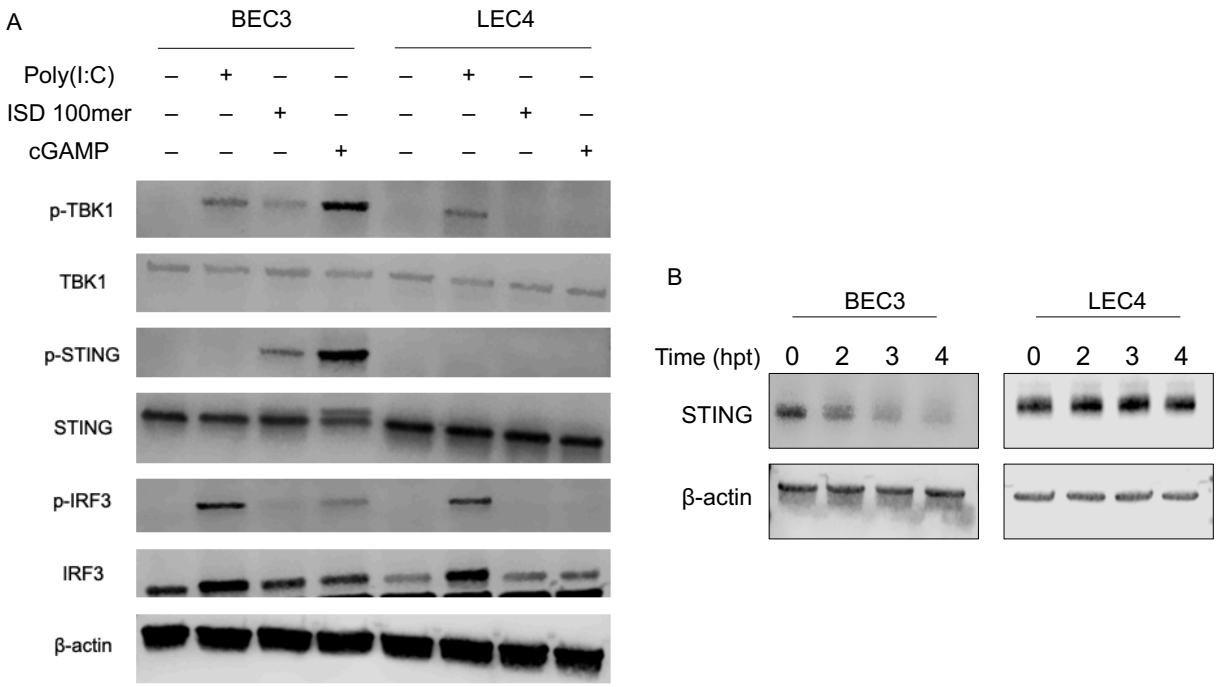

Figure 3.3: Downstream STING signaling is blocked in LEC4 cells. (A) BEC3 and LEC4 were transfected with either 1 μg/mL Poly I:C, 10 μg/mL ISD 100mer or 5 μg/mL 2'3' cGAMP for 3 hours and whole cell lysates were immunoblotted with the indicated phospho-specific or total protein antibodies. **(B)** BEC3 and LEC4 were transfected with 5 μg/mL 2'3' cGAMP and cells were harvested at the indicated time points and whole cell lysates were immunoblotted in native (nonreducing) sample buffer for the indicated antibodies.

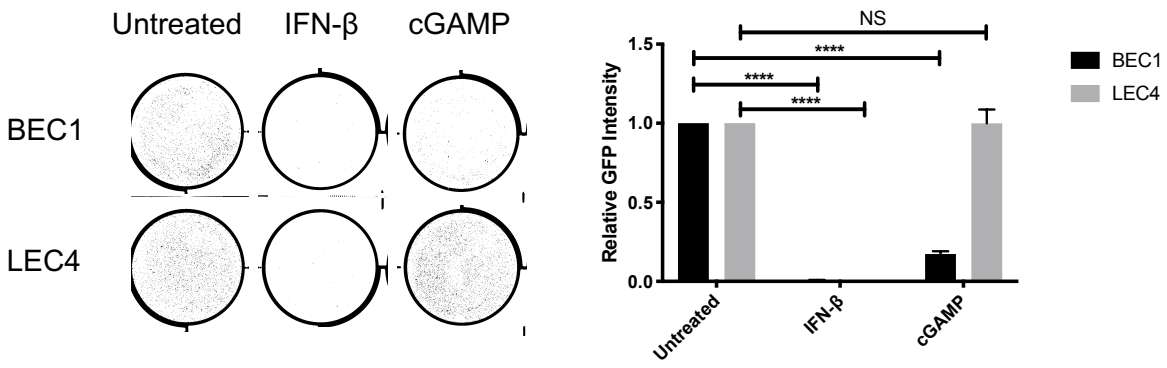

Figure 3.4: cGAMP pre-treatment fails to reduce susceptibility to KSHV infection in LEC4. BEC1 and LEC4 were transfected with 5 μg/mL 2'3' cGAMP or treated with 1000 IU/mL IFN-β for 24 hours. Cells were infected with KSHV-BAC16 and the integrated density of the fluorescence (relative GFP intensity) was quantified by Typhoon 48 hours post infection (hpi). The left panel show representative raw plate staining and the right panel shows the mean ± SEM from at least 3 biological replicates. **** $P < 0.0001$ (Two-way ANOVA with Sidak posthoc test).

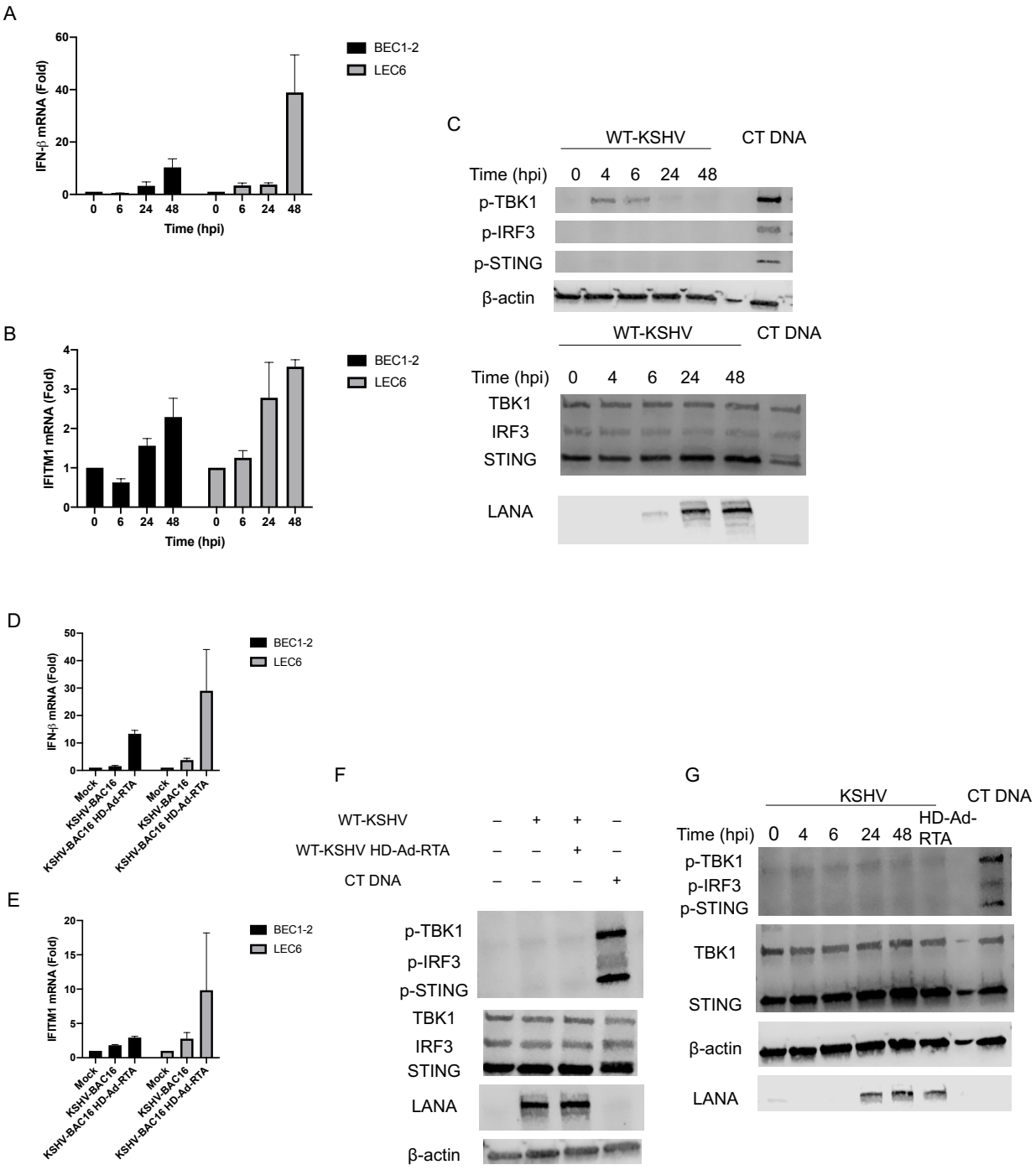

Figure 3.5: KSHV induces minimal innate immune signaling and does not strongly activate STING-TBK1-IRF3 in primary endothelial cells. (A) IFN-β or **(B)** IFITM1 mRNA was measured by RT-qPCR from BEC1-2 and LEC6 that were infected with KSHV-BAC16 and harvested at the indicated timepoints. The relative amount of mRNA for each gene was

normalized as in Figure 3.1. **(C)** BEC1 were either infected with KSHV or transfected with 1 μg/mL CT DNA and whole cell lysates were harvested at the indicated timepoints (3 hours post transfection for cells transfected with CT DNA) and immunoblotted with the indicated antibodies. The top and bottom panels are the same lysate, run on different gels. **(D)** IFN-β or **(E)** IFITM1 mRNA was measured by RT-qPCR from BEC1-2 and LEC6 that were infected with either KSHV-BAC16 or KSHV-BAC16+HD-Ad-RTA and harvested at 24 hpi. The relative amount of mRNA for each gene was normalized as in Figure 3.1. **(F)** BEC1 cells were infected with WT-KSHV alone or WT-KSHV+HD-Ad-RTA and harvested at 24 hpi or transfected with 1 μg/mL CT DNA (3-hour transfection). Whole cell lysates were immunoblotted with the indicated antibodies. **(G)** LEC6 were infected with WT-KSHV, WT-KSHV+HD-Ad-RTA or transfected with CT DNA and whole cell lysates were harvested at the indicated time points (24 hours for WT-KSHV+HD-Ad-RTA cells and 3 hours for CT DNA transfection) and immunoblotted for the indicated antibodies. Data are shown as mean ± SEM from at least 3 biological replicates.

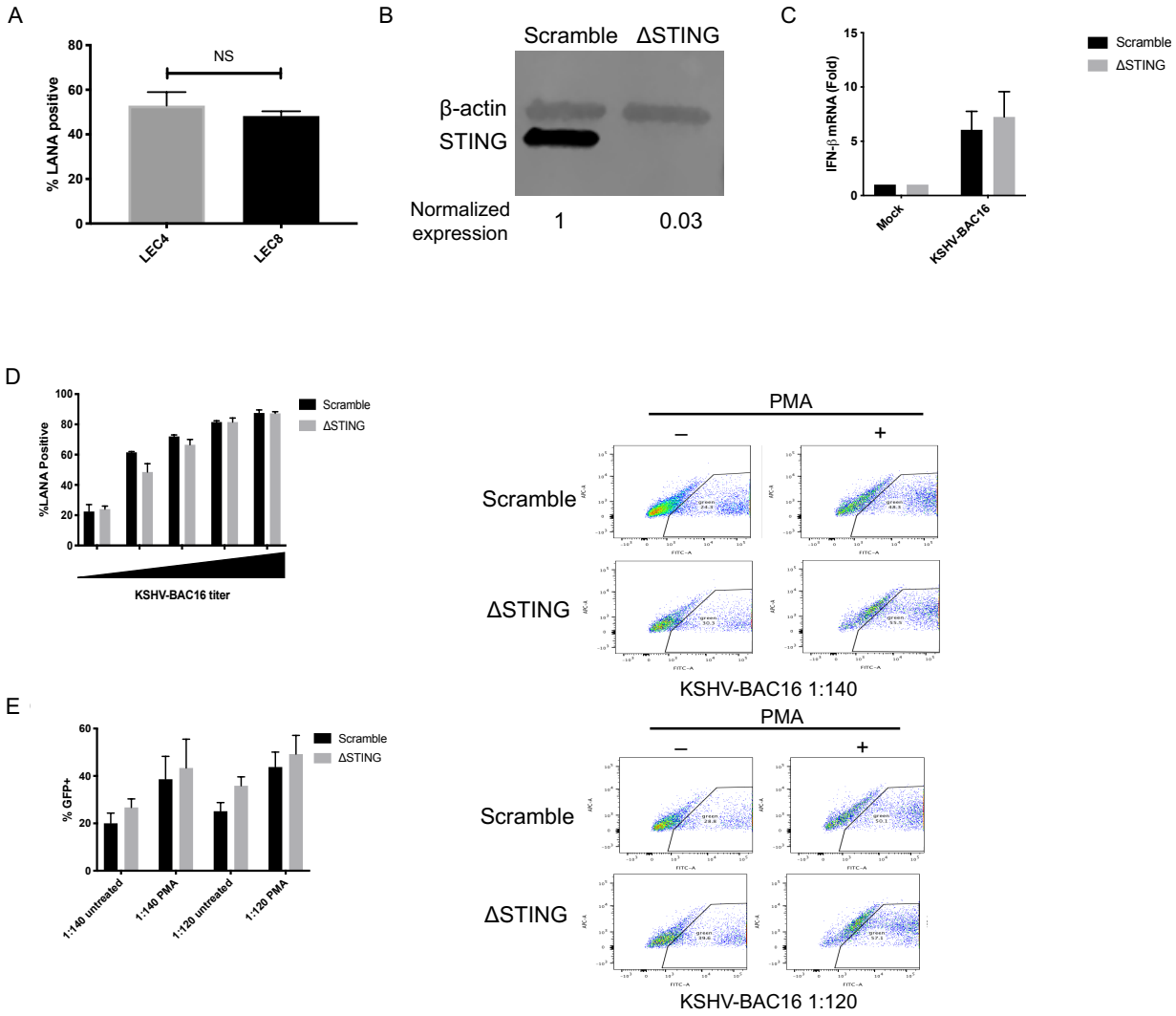

Figure 3.6: STING does not reduce susceptibility during de novo infection or restrict spread of KSHV during lytic replication. (A) LEC4 and LEC8 cells were infected with KSHV-BAC16 and infection rates were measured by immunofluorescence microscopy and quantifying the percent of LANA+ cells in the culture at 48 hpi. **(B)** BEC1-3 were transduced with a lentivirus expressing Cas9 and a guide RNA targeting STING (ΔSTING) or a nontargeting (scramble) control and whole cell lysates were immunoblotted with the indicated antibodies. **(C)** Scramble and ΔSTING BEC3 from (B) were infected with KSHV-BAC16 and IFN-β mRNA was measured by RT-qPCR at 48 hpi as described earlier. **(D)** Scramble and ΔSTING BEC1-3 were infected

with KSHV-BAC16 at different dilutions of virus and infection rates were measured by immunofluorescence microscopy and quantifying the percent of LANA+ cells in the culture at 48 hpi. **(E)** Scramble and ΔSTING BEC3 were infected with KSHV-BAC16 at 2 different dilutions (1:140 and 1:120) for 4 hours. Immediately following infection, cells were treated with PMA for 5 days. Cells were sorted by GFP by flow cytometry. Quantitation is shown in the left panel and representative flow plots are shown in the right panel. Data are shown as mean ± SEM from at least 2 biological replicates.

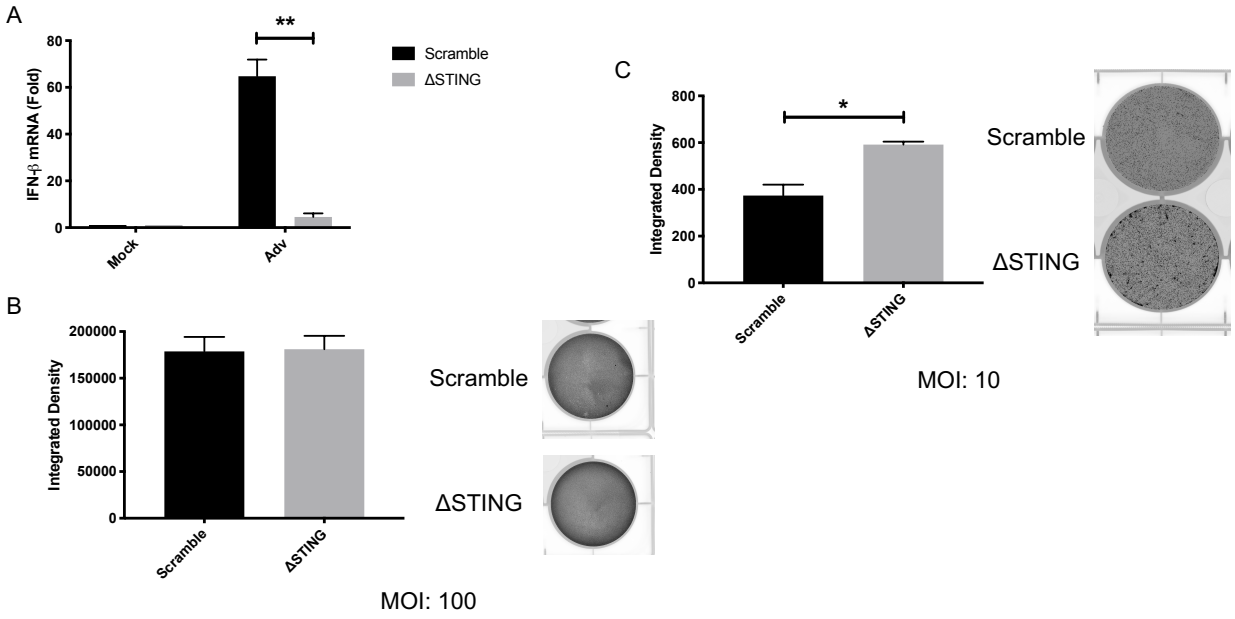

Figure 3.7: STING restricts Adv spread at low MOI. (A) IFN-β mRNA was measured by RT-qPCR from scramble and ΔSTING BEC3 that were infected with Adv for 48 hours. The relative amount of mRNA was normalized as in Figure 3.1. **(B)** Scramble and ΔSTING BEC3 were infected with Adv at 100 genomes/cell and supernatant and cells were harvested at 5 days post infection (dpi). Viral progeny were titered on HEK 293T-Integrin-β cells and integrated density of the fluorescent signal was measured by Typhoon. Representative raw plate staining is shown to the right **(C)** Scramble and ΔSTING BEC3 were infected with Adv at 10 genomes/cell and integrated density of the fluorescent signal was measured by Typhoon at 5 dpi. Representative raw plate staining is shown to the right. Data are shown as mean ± SEM from at least 3 biological replicates. * P < 0.05; ** *P* < 0.01; (Student's *t* test).

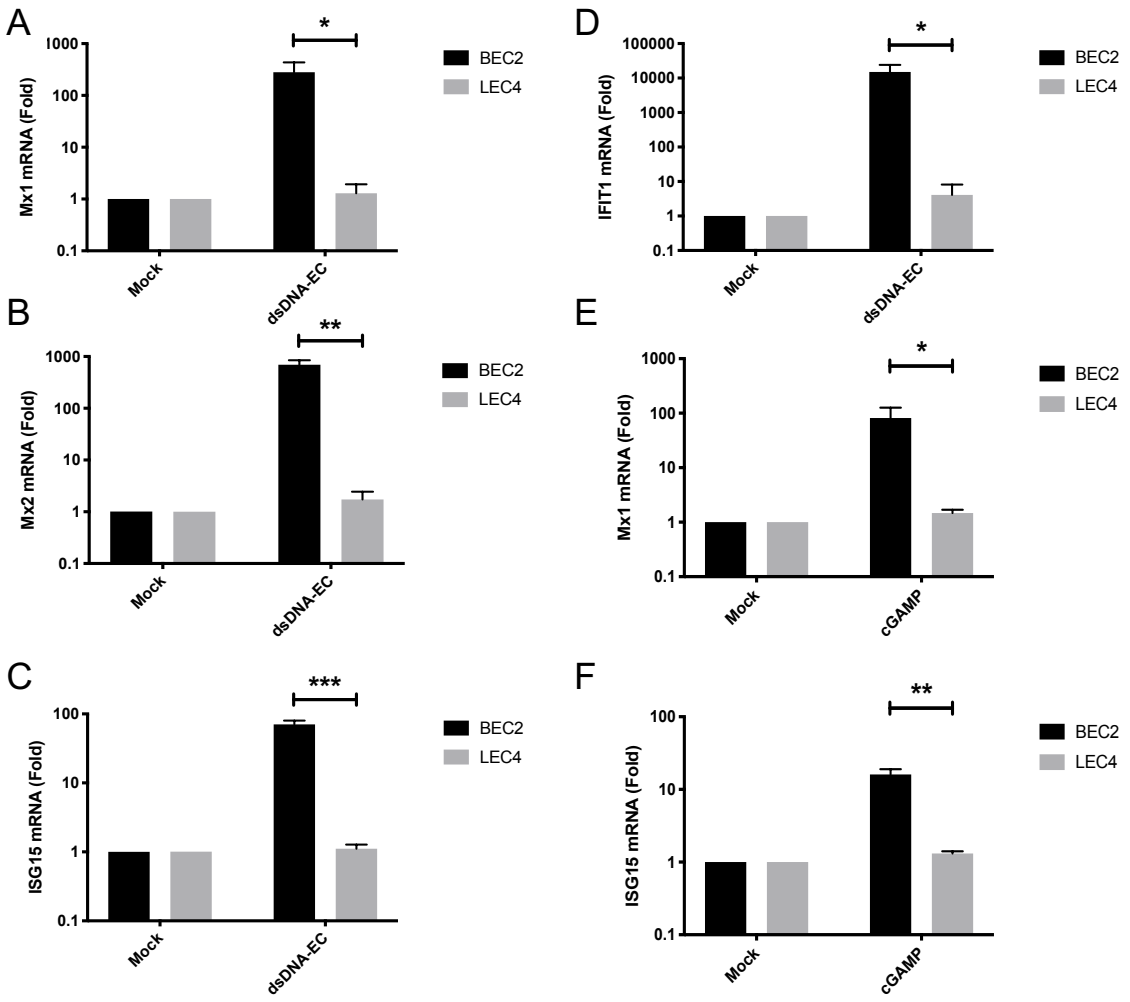

Figure S3.1: Cytosolic DNA and cGAMP fail to induce expression of other innate immune genes. (A) Mx1, **(B)** Mx2, **(C)** ISG15 and **(D)** IFIT1 mRNA was measured by RT-qPCR from BEC2 and LEC4 that were transfected with 2.5 µg/mL dsDNA-EC for 4 hours. **(E)** Mx1 and **(F)** ISG15 mRNA was measured by RT-qPCR from BEC2 and LEC4 that were transfected with 5 µg/mL cGAMP for 4 hours. The relative amount of mRNA for each gene was normalized to tubulin mRNA in each sample, and fold change was calculated as described previously. Data are shown as mean ± SEM from at least 3 biological replicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test).

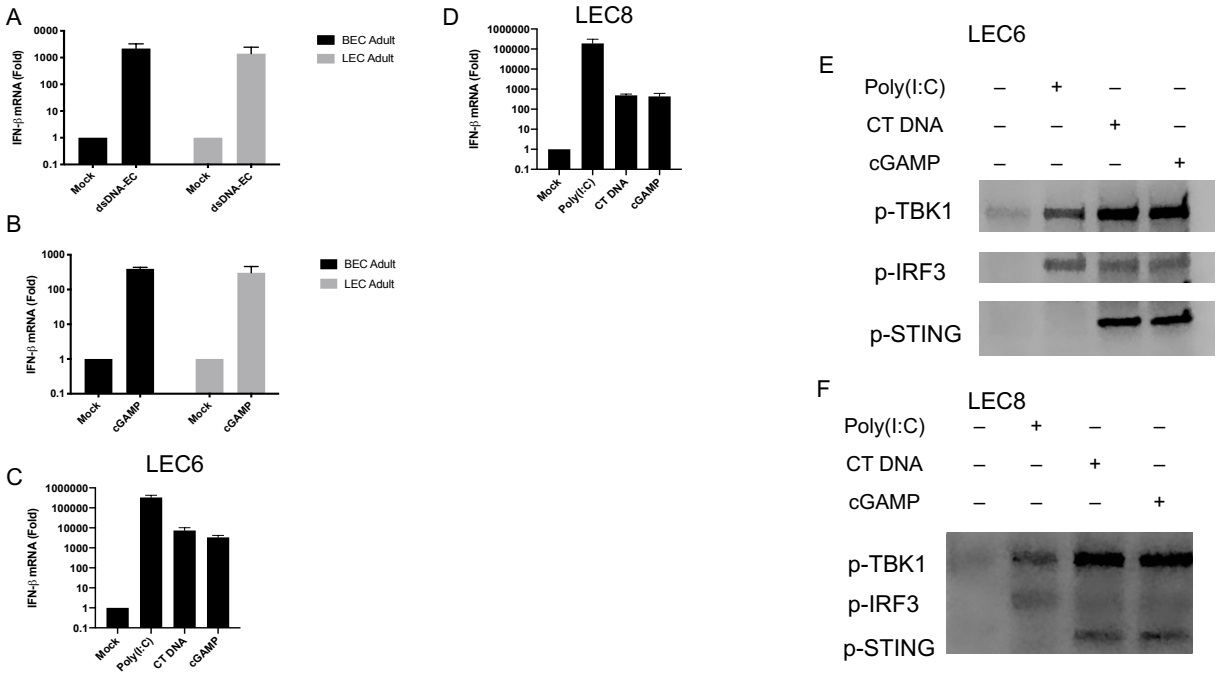

Figure S3.2: Cytosolic DNA and cGAMP induce IFN-β expression via activation of the cGAS-STING pathway in other LECs. IFN-β mRNA was measured by RT-qPCR from Adult BECs and LECs that were transfected with **(A)** 2.5 μg/mL dsDNA-EC or **(B)** 5 μg/mL cGAMP for 4 hours. IFN-β mRNA was measured by RT-qPCR from neonatal **(C)** LEC6 or **(D)** LEC8 transfected with either 1 μg/mL Poly(I:C), 1 μg/mL CT DNA or 5 μg/mL cGAMP for 4 hours. The relative amount of mRNA was normalized to tubulin mRNA in each sample, and fold change was calculated as described previously. Neonatal **(E)** LEC6 or **(F)** LEC8 were transfected with either 1 μg/mL Poly(I:C), 1 μg/mL CT DNA or 5 μg/mL cGAMP for 3 hours and the whole cell lysate was immunoblotted for the indicated antibodies. Data are shown as mean ± SEM from at least 3 biological replicates.

A

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NM_198282.4   61 GCAGCCTTGGTTCTGCTGAGTGCCTGCCTGGTGACCCTTTGGGGGCTAGGAGAGCCACCA
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NM_198282.4  121 GAGCACACTCTCCGGTACCTGGTGCTCCACCTAGCCTCCCTGCAGCTGGGACTGCTGTTA
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NM_198282.4  421 CCAGCTGAGATCTCTGCAGTGTGTGAAAAAGGGAATTTCAACGTGGCCCATGGGCTGGCA
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NM_198282.4  721 AGCAACAGCATCTATGAGCTTCTGGAGAACGGGCAGCGGGCGGGCACCTGTGTCCTGGAG
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B

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NM_198282.4  361 PELLISGMEKPLPLRTDFS
LEC4         361 PELLISGMEKPLPLRTDFS
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Figure S3.3: TMEM173 sequence from LEC4 isolates. RNA was extracted from LEC4 and submitted for RNA sequencing to the Benaroya Genomics Core Facility and the results were analyzed by the Fred Hutchinson Cancer Research Center Genomics Shared Resource. LEC4 TMEM173 transcripts were aligned to human reference genome 38 and human STING1

transcript variant 1 (Accession NM_198282.4) using Integrative Genomics Viewer. **(A)** Nucleotide sequences and **(B)** amino acid sequences of LEC4 aligned to NM_198282.4 are shown.

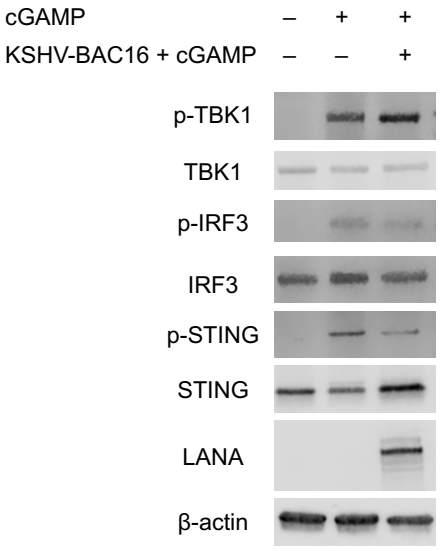

Figure S3.4: The STING-TBK1-IRF3 axis can be activated by cGAMP in KSHV-infected BECs. BEC1 were infected with KSHV-BAC16 and incubated for 72 hours and transfected with 5 μg/mL cGAMP for 3 hours. Whole cell lysate was immunoblotted for the indicated antibodies.

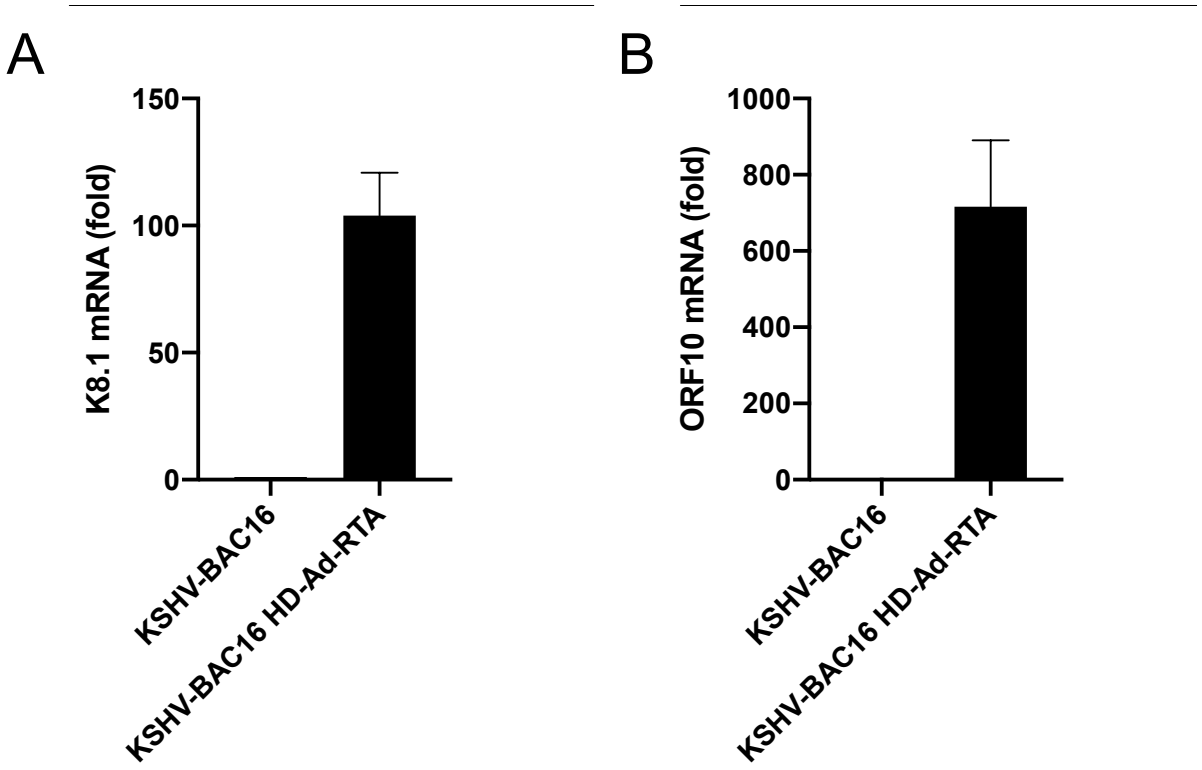

Figure S3.5: K8.1 and ORF10 expression is induced during lytic reactivation in BECs. (A) K8.1 or **(B)** ORF10 mRNA was measured by RT-qPCR from BECs that were infected with either KSHV or KSHV+Ad-RTA and harvested at 24 hpi. The relative amount of mRNA for each gene was normalized to tubulin mRNA in each sample, and fold change was calculated as described previously. Data are shown as mean ± SEM from at least 3 biological replicates.

Table 3.1: Oligonucleotide sequences for RT-qPCR and ISD 100mer.

Gene/oligo name	Sense (S)	Antisense (AS)
IFN-β	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG
Mx1	GACATTCGGCTGTTTACC	CTTCCAGTGCCTTGATTT
Mx2	ACCGCCATTCGGCACAGT	TGCCCTTGGTTGGCTCCT
ISG15	TGGACAAATGCGACGAACC	CCCGCTCACTTGCTGCTT
IFIT1	CACCCACTTCTGTCTTACT	ACATTCTTGCCAGGTCTA
IFITM1	GGATTTCGGCTTGTCCCGAG	CCATGTGGAAGGGAGGGCTC
K8.1	AAAGCGTCCAGGCCACCACAG	GGCAGAAAATGGCACACGGTT
ORF10	GTCCTGTCCCGCTCTCTTTTTTG	CAATAAGGTGTTCGTGCTTGCCC
Tubulin	TCCAGATTGGCAATGCCTG	GGCCATCGGGCTGGAT
ISD 100mer	GGATGAGTCCATGTCTAGATAATCACTAGA TACTGACTAGACATGTACTAGATGTATGTCT AGATAATCACTAGATACTGACTAGACATGTA CTAGATGT	ACATCTAGTACATGTCTAGTCAGTATCTAGTGATT ATCTAGACATACATCTAGTACATGTCTAGTCAGTA TCTAGTGATTATCTAGACATGGACTCATCC

Chapter 4. CDC7 is required for survival of KSHV-infected cells

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4.1 Abstract

A CRISPR/Cas9 screen for host genes that are essential for survival of KSHV-infected cells identified MCM2, which is a subunit of the replicative helicase during cellular replication. MCM2 is phosphorylated by the kinase CDC7 to initiate DNA replication. Further experiments revealed that chemical inhibition of CDC7 leads to cell death in KSHV-infected cells but not uninfected cells. De novo infection with WT-KSHV, but not KSHV-BAC16, leads to downregulation of MCM2. Cells infected with both WT-KSHV and KSHV-BAC16 are sensitive to CDC7 inhibition. Infections with mutant viruses for each latent gene failed to identify a specific latent gene that is required for sensitivity to CDC7 inhibition. Interestingly, treatment of uninfected cells with IFN-β also confers sensitivity to CDC7 inhibition. However, de novo infection of IFNAR mutant cells with WT-KSHV does not rescue cells from MCM2 depletion indicating that KSHV activation of IFN is not necessary for induction of sensitivity to CDC7 inhibition. Overall, we show that KSHV infection leads to sensitivity to CDC7 inhibition, which therefore may be a promising target for antiviral therapy against KSHV and the treatment of KS.

4.2 Introduction

Initiation of cellular replication is a tightly regulated process that begins with the loading of the ORC complex onto origins of replication. Ctd1 and Cdc6 are then recruited to ORC complex proteins, which leads to the recruitment and loading of the heterohexamer MCM2-7 onto the origin of replication (99). MCM2 is then phosphorylated first by Cdk2, then by CDC7. This allows for the recruitment of Cdc45 and GINS. MCM2-7-Cdc45-GINS then moves outward

bidirectionally from the origin of replication and unwinds the DNA, allowing for replication by DNA polymerases (101). Initiation of this process is carefully regulated as defects can lead to cancer.

Viruses must alter cellular replication during infection in order to promote replication of viral genomes. KSHV, a herpesvirus undergoing both latent and lytic lifecycles, both requires and disrupts the MCM proteins at various points during replication. During latent replication, KSHV LANA interacts with MCM3, 4 and 6, presumably recruiting these proteins to viral origins of replication so that the viral genome can be replicated by host machinery (107). Depletion of MCM6 leads to decreased replication of the episome and decreased genome copy number. During lytic replication, ORF59 disrupts the MCM complex. ORF59 interacts with MCM3, 4, 5 and 6 (110). Depletion of MCM6 results in an increase in viral genome copies, viral late gene transcripts, and virion production following reactivation. KSHV lytic replication also leads to hypophosphorylation of MCM3 (which is associated with reduced chromatin loading). This leads to an inability of MCM3 to associate with histones in reactivated cells. Finally, lower levels of MCM3 and MCM4 association at cellular origins of replication and decreased levels of cellular DNA synthesis was observed in cells undergoing reactivation. Overall, KSHV interacts with the MCM proteins during both latent and lytic replication to maintain latency and promote viral replication, respectively. While the MCM proteins have been studied in the context of latent and lytic replication of the virus, their role in the survival of latently infected cells has not been studied.

Recently, our lab used CRISPR/Cas9 to screen for host genes that are essential for survival of latently infected cells but not uninfected cells. MCM2 was identified in this screen and we sought to characterize this dependency. We found that knocking out MCM2 may lead to an increase in cell death in infected cells compared to uninfected cells. KSHV-infected endothelial cells and B cells are sensitive to chemical inhibition of CDC7, with increased cell death compared to uninfected cells. Interestingly, KSHV de novo infection downregulates expression

of both CDC7 and MCM2, which is independent of innate immune signaling. Use of mutant viruses failed to identify a particular latent gene that is required for this sensitivity, and overexpression of LANA was not sufficient for sensitivity to CDC7 inhibition. Finally, CDC7 inhibition may lead to an increase in lytic gene expression, but not necessarily an increase in lytic reactivation. Overall, we identify CDC7 as a novel target for antiviral therapy during KSHV infection.

4.3 Results

4.3.1 MCM2 may be required for survival of KSHV-infected cells

In order to validate the results from the screen, we infected TIME cells with WT-KSHV, then transduced these cells with lentiviruses expressing CRISPR/Cas9 and gRNAs targeting MCM2 (two different gRNAs) or a nontargeting scramble control, resulting in MCM2 knockout cells (Fig. 4.1A). We then measured cell death and found there was a slight increase in cell death in cells transduced with gRNA 2 relative to the scramble control (Fig. 4.1B). Overall, these experiments show that endothelial cells latently infected with KSHV are more sensitive to loss of MCM2 than their mock counterparts. However, given that gRNA 1 did not show increased cell death during KSHV infection, more experiments are needed to determine if MCM2 is required for survival of latently infected cells.

4.3.2 γ-herpesvirus-infected cells are sensitive to CDC7 inhibition

In order to complement the genetic experiments, we next sought to test the requirement of CDC7 by pharmacological inhibition. PHA767491 is a potent CDC7 inhibitor that acts by blocking the active site of the kinase (106). We used this inhibitor to test the requirement of CDC7 activity for survival of latently infected cells. Interestingly, we found that KSHV-BAC16-infected TIME cells displayed an increase in cell death when treated with PHA767491 relative to both untreated infected cells as well as PHA767491-treated uninfected cells (Fig. 4.2A). This effect appears to be dose dependent, as the rates of cell death increase as the concentration of

the inhibitor increases. We next wanted to determine if other cell lines infected with KSHV would be similarly sensitive to treatment with a CDC7 inhibitor. To test this, we treated BCBL-1 (KSHV+/EBV-), BC-1 (KSHV+/EBV+), RAJI (KSHV-/EBV+) and BJAB (KSHV-/EBV-) cell lines with different concentrations of PHA767491 and measured cell death. Similar to the TIME cell experiments, we found that KSHV infection resulted in increased sensitivity to CDC7 inhibition, with BCBL-1 and BC-1 cells exhibited increased cell death when exposed to PHA767491 relative to BJAB cells (Fig. 4.2B). This effect was again dose-dependent, with increasing cell death rates corresponding to increasing levels of inhibitor. Interestingly, RAJI cells, which are KSHV-/EBV+, were similarly sensitive to CDC7 inhibition, suggesting that γ-herpesviruses may alter similar pathways, leading to conserved vulnerabilities. Overall, these experiments show that inhibition of CDC7 leads to increased cell death in KSHV and EBV-infected cells.

4.3.3 WT-KSHV de novo infection downregulates CDC7 and MCM2 expression

In order to determine if KSHV infection has an effect on the expression of CDC7, we infected TIME cells and immunoblotted for CDC7. Intriguingly, we found that WT-KSHV de novo infection downregulates CDC7 expression relative to uninfected cells (Fig. 4.3A). Similarly, we found that WT-KSHV infection led to downregulation of MCM2 (Fig. 4.3B, quantification to the right). To ensure that this downregulation of MCM2 is not an artifact of immortalization, we next wanted to determine if KSHV infection of primary BECs would display the same phenotype. After infecting with WT-KSHV, we found that MCM2 is similarly downregulated in primary BECs (Fig. S4.1). To determine if treatment with the CDC7 inhibitor would further deplete phosphorylated MCM2, we infected TIME cells with WT-KSHV and then treated them with PHA767491. Consistent with our previous results, levels of both p-MCM2 and total MCM2 were decreased with KSHV infection, with further depletion when cells were exposed to the CDC7 inhibitor at the highest dose (Fig. 4.3C, quantification of p-MCM2 and total MCM2 shown as upper and lower panels, respectively). Interestingly, levels of p-MCM2 in uninfected cells were

largely unaffected by the presence of the inhibitor except at the highest concentration tested. We next wanted to determine if a specific latent gene could be identified as required for downregulation of MCM2. To test this, we infected TIME cells with different KSHV-BAC16 mutants and selected these cells with hygromycin B to ensure all cells were infected. Surprisingly, we found that infection with KSHV-BAC16 (wt and all mutants tested) led to a much more modest downregulation of MCM2 when compared to WT-KSHV (Fig. S4.2A). To determine if LANA overexpression leads to downregulation of MCM2, we transduced TIME cells with a LANA expressing lentivirus and found that LANA expression is not sufficient to downregulate MCM2 (Fig. S4.2B). Overall, these experiments reveal that WT-KSHV infection downregulates both CDC7 and MCM2, decreasing p-MCM2 which can be further depleted by CDC7 inhibitors.

4.3.4 Innate immune signaling downregulates MCM2 and sensitizes cells to CDC7 inhibition, but MCM2 downregulation during KSHV infection is independent of IFN-β

Because we were unable to specify a viral latent gene required for the downregulation of MCM2, we hypothesized that downregulation of MCM2 may be mediated by innate immune signaling. To test this, we transfected TIME cells with cGAMP or treated them with IFN-β and immunoblotted for MCM2 at different time points. Surprisingly, treatment with either cGAMP or IFN-β led to downregulation of MCM2, with depletion peaking at 48 hours post treatment (Fig. 4.4A, quantification shown in the right panel). To determine if IFN-β treatment confers sensitivity to CDC7 inhibition, we treated TIME cells with IFN-β and PHA767491. Interestingly, IFN-β in combination with PHA767491 led to an increase in cell death when compared to either condition alone (Fig. 4.4B). To determine if the downregulation of MCM2 during KSHV infection is dependent on IFN-β signaling, we created IFNAR1 and IFNAR2 stable knockout cell lines by transducing TIME cells with lentiviruses expressing CRISPR/Cas9 and gRNAs targeting these genes. We then tested the functionality of these mutants by pre-treating with IFN-β, then

infecting with KSHV-BAC16. The cells were then scanned for GFP positivity. While the scramble control cells displayed a marked reduction in GFP intensity when treated with IFN-β, IFNAR1 and IFNAR2 knockouts displayed similar levels of GFP intensity compared to the untreated controls, indicating that IFN-β treatment failed to rescue these cells from infection (Fig. S4.3, quantification shown in the lower panel). We next infected these cells with WT-KSHV and found that MCM2 expression was not rescued, indicating that the downregulation of MCM2 during KSHV infection is independent of IFN-β (Fig. 4.4C). Overall, these experiments show that while innate immune signaling confers sensitivity to CDC7 inhibition and downregulates MCM2, KSHV downregulation of MCM2 is independent of IFN signaling.

4.3.5 KSHV latent genes are not required nor are they sufficient to confer sensitivity to CDC7 inhibition.

We next sought to identify latent genes that may be required or sufficient for sensitivity to CDC7 inhibition. To test requirements, we infected TIME cells with different KSHV-BAC16 mutants (ΔvCycΔvFLIP, ΔKaposins and ΔmiR) and treated these cells with PHA767491. To test sufficiency, we treated the LANA-expressing cell line with PHA767491. Cell death was measured and infection with different mutant viruses failed to rescue cells from sensitivity to CDC7 inhibition (Fig. 4.5). Similarly, LANA overexpression failed to confer sensitivity to PHA767491. Overall, these experiments show that vCyc, vFLIP, Kaposins and the miR cluster are not required for sensitivity to CDC7 inhibition and LANA is not sufficient to confer sensitivity.

4.3.6 KSHV lytic gene expression is upregulated by PHA767491 but the proportion of ORF59+ cells is not increased.

Finally, we wanted to determine if cell death during CDC7 inhibition was caused by lytic reactivation. To test this, we infected cells with WT-KSHV and treated cells with PHA767491 and measured expression of the lytic genes K8.1 and ORF10 at the indicated time points. Interestingly, both K8.1 (Fig. 4.6A) and ORF10 (4.6B) mRNA transcripts were elevated in the

PHA767491-treated cells relative to the untreated cells at both 24 and 48 hours post treatment. To determine if this increase in lytic gene expressing correlated with an increase in ORF59+ cells, we infected cells with WT-KSHV and treated cells with PHA767491 and counted the number of ORF59+ cells by immunofluorescence microscopy 48 hours later. While the proportion of LANA+ cells was slightly increased in the untreated cells (Fig. 4.6B), the proportion of ORF59+ cells was similar between the treated and untreated conditions (Fig. 4.6C). Overall, these experiments show that while there is an increase in lytic gene expressing following CDC7 inhibition, there does not appear to be an increase in the proportion of ORF59+ cells.

4.4 Discussion

Here we identify the CDC7-MCM2 pathway as being required for survival of KSHV-infected cells. MCM2 depletion leads to a modest increase in cell death in KSHV-infected cells. However, this experiment needs to be optimized and studied further. Pharmacological inhibition of CDC7 is much more efficient at killing KSHV-infected cells. However, there appears to be a disconnect in the results from the screen/genetic experiments and the chemical inhibition experiments. MCM2 is depleted in the screen and validation experiments. However, chemical inhibition results in decreased phosphorylation of MCM2 in KSHV-infected cells only. MCM2 (and p-MCM2) is not depleted in the uninfected cells and levels of p-MCM2 only begin to decrease at 2 μM (but cell death effect can be observed at 0.5 and 1 μM). Therefore, it is possible that off-target effects of PHA767491 may also contribute to cell death.

That MCM2 would be identified in our CRISPR screen is surprising given the essential role of this protein during DNA replication. However, clues from cancer research may elucidate the mechanism behind this selective dependency. In normal cells, upon CDC7 and/or MCM2 depletion, the replication fork stalls, which activates ATM/ATR, which in turn, activate several different checkpoint proteins, including pRB, p53 and CHK1/CHK2, which all lead to cell cycle

arrest. In cancer cells, these checkpoint proteins are often inactivated, which means that ATM/ATR activate p38 and Caspase 3, leading to apoptosis. It is possible that KSHV is similarly deregulating these checkpoint proteins, which would sensitize cells to CDC7 inhibition in an ATM/ATR-p38-Caspase 3-dependent manner. Further studies are needed to tease out these possibilities.

The finding that both KSHV and IFN signaling downregulate MCM2 expression in distinct mechanisms is novel and worth exploring further in the future. Decreased expression of MCM2 has paradoxically been linked to cancer. Low levels of MCM2 leads to replication fork stalling, DNA damage and genomic instability. Since both KSHV and innate immune signaling decrease MCM2 expression, it is possible that both could promote DNA damage and genomic instability. Chronic viral infection can lead to persistent IFN production, which, over time, could lead to DNA damage due to sustained MCM2 repression. The same scenario may play out during KSHV infection. The repression of MCM2 expression appears to be long-term during KSHV infection and not just limited to initial infection as decreased MCM2 expression was observed up to 9 days post infection (data not shown). However, the downregulation of MCM2 is strongest during infection with WT-KSHV, whereas infection with KSHV-BAC16 results in a modest decrease in MCM2 levels. This disparity is not due to differences in infection rates, as cells infected with KSHV-BAC16 were selected to ensure all cells were infected. Further experiments are needed to determine this differential phenotype when using WT-KSHV versus KSHV-BAC16.

Overall, the finding that CDC7 inhibition leads to death of KSHV-infected cells is novel and identifies a promising new pathway that may be targeted by therapeutics. Further studies are needed to ensure that the increased cell death in latently infected cells upon drug treatment is indeed due to the decreased phosphorylation levels of MCM2 and not to an alternate pathway, off-target effects of the drug or caused by lytic reactivation of the virus.

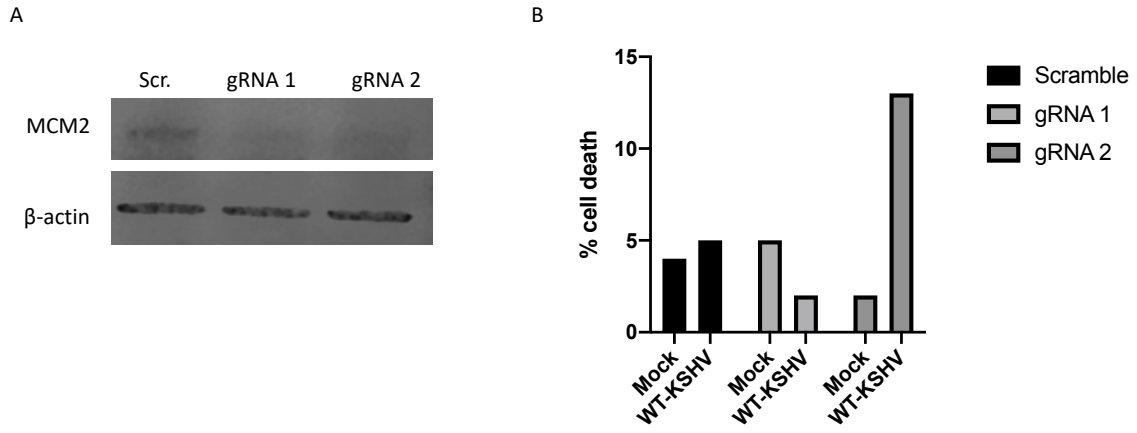

Figure 4.1: MCM2 may be required for survival of KSHV-infected cells. **(A)** TIME cells were transduced with a lentivirus expressing either a nontargeting "scramble" gRNA or 2 different gRNAs targeting MCM2. Whole cell lysate was immunoblotted for the indicated antibodies. **(B)** TIME cells were infected with WT-KSHV for 48 hours, then transduced with the corresponding lentiviruses. Cell death was measured at 48 hours post transduction by trypan blue. Data shown are from a single replicate.

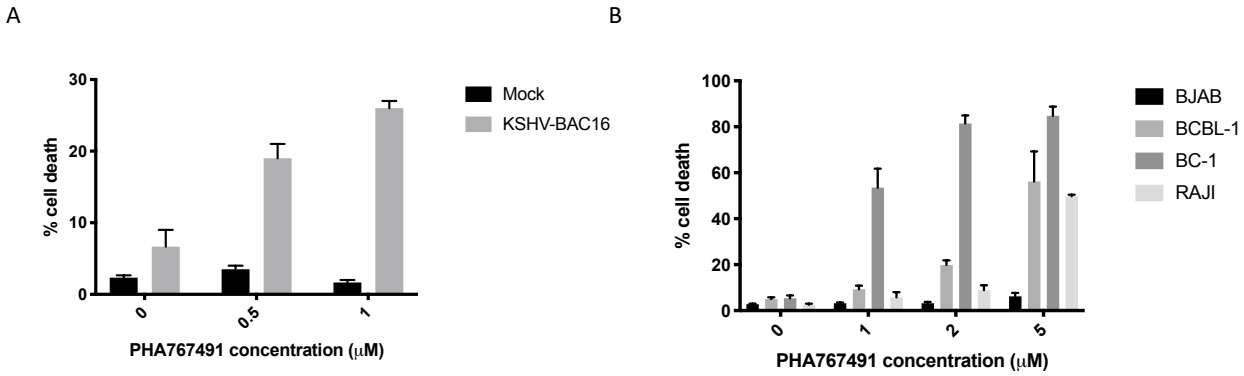

Figure 4.2: γ-herpesvirus-infected cells are sensitive to CDC7 inhibition. (A) TIME cells were infected with KSHV-BAC16 and treated with the indicated concentrations of PHA767491 at 24 hpi. Cell death was measured by trypan blue at 48 hours post treatment. **(B)** BJAB, BCBL-1, BC-1 and RAJI cells were treated with the indicated concentrations of PHA767491 and cell death was measured by trypan blue at 48 hours post treatment. Data are shown as mean ± SEM from at least 3 biological replicates.

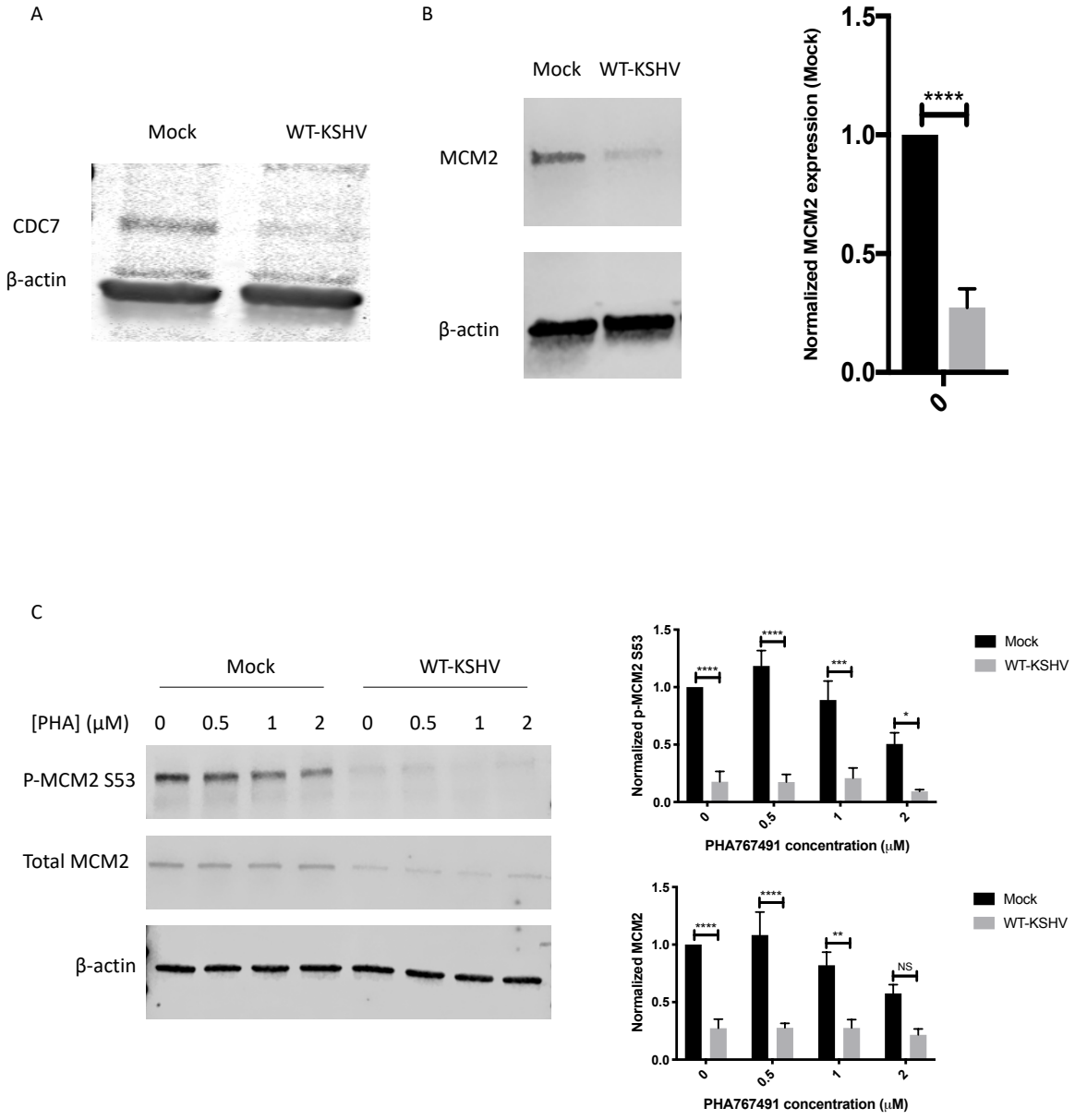

Figure 4.3: KSHV de novo infection downregulates CDC7 and MCM2, which is further reduced by PHA767491. TIME cells were mock or WT-KSHV-infected and whole cell lysate was immunoblotted at 48 hpi for β-actin and **(A)** CDC7 or **(B)** MCM2. The panel on the right is a quantification of (B). **(C)** TIME cells were mock or WT-KSHV-infected and treated with the

indicated concentrations of PHA767491 at 24 hpi. Whole cell lysates were immunoblotted for the indicated antibodies 24 hours post treatment. The blot shown is representative. The upper (p-MCM2) and lower (total MCM2) panels are quantifications of the left panel. Data are shown as mean ± SEM from at least 3 biological replicates. **** $p < 0.0001$

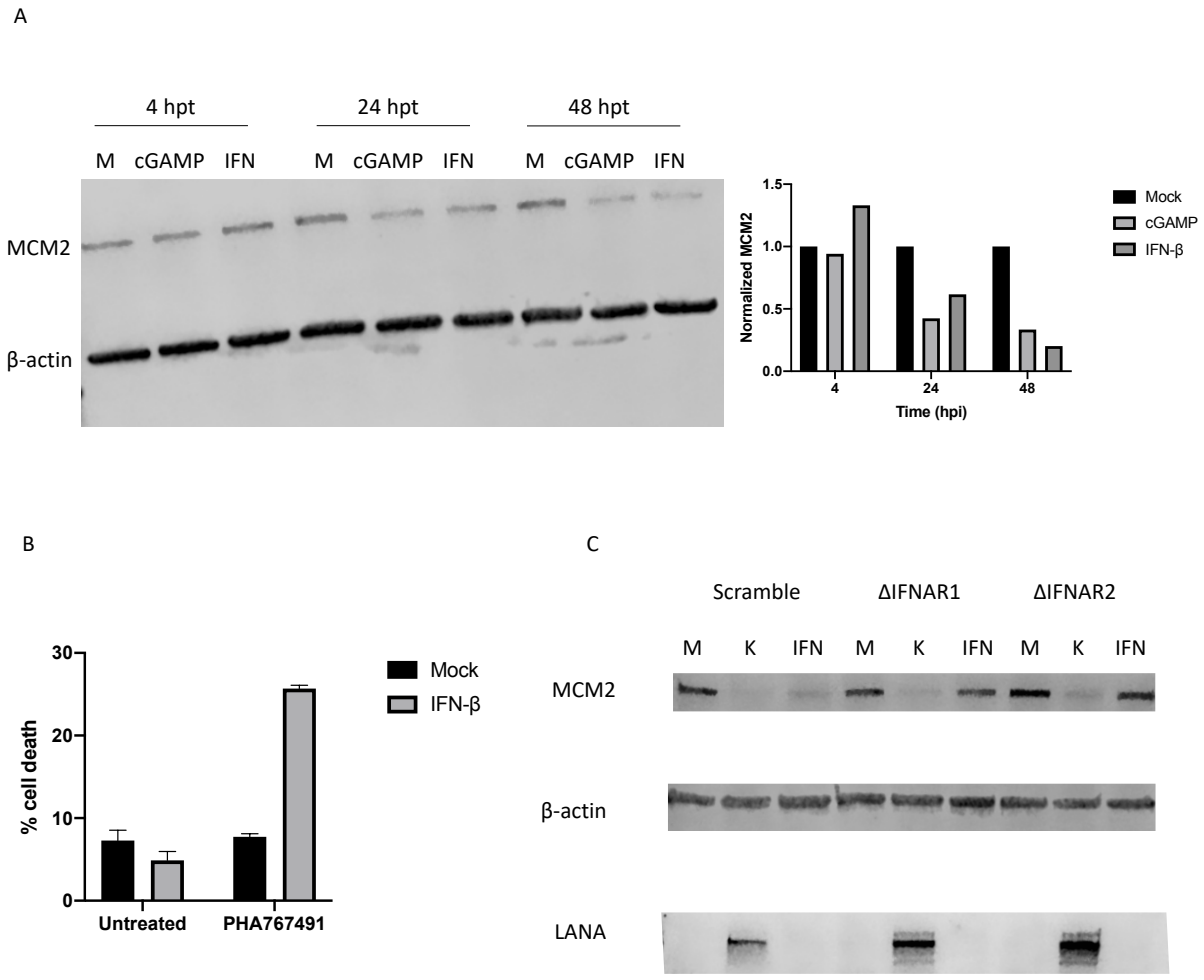

Figure 4.4: Innate immune signaling downregulates MCM2 and sensitizes cells to CDC7 inhibition, but MCM2 downregulation during KSHV infection is independent of IFN-β. (A) TIME cells were transfected with 5 μg/mL cGAMP or treated with 1000 IU/mL IFN-β or mock and whole cell lysate was immunoblotted at the indicated times for the indicated antibodies. The panel on the right is a quantification of the panel on the left. Data shown are from a single replicate. **(B)** TIME cells were treated with 1000 IU/mL IFN-β. Cells were treated with 1 μM PHA767491 24 hours later (but kept under IFN-β treatment). Cell death was measured by trypan blue 48 hours following PHA767491 treatment. Data are shown as mean ± SEM from 2 biological replicates. **(C)** TIME cells were transduced with lentiviruses expressing gRNAs targeting either IFNAR1, IFNAR2 or a nontargeting "scramble" control. The cells were then

infected with WT-KSHV and whole cell lysates were immunoblotted for the indicated antibodies at 48 hpi.

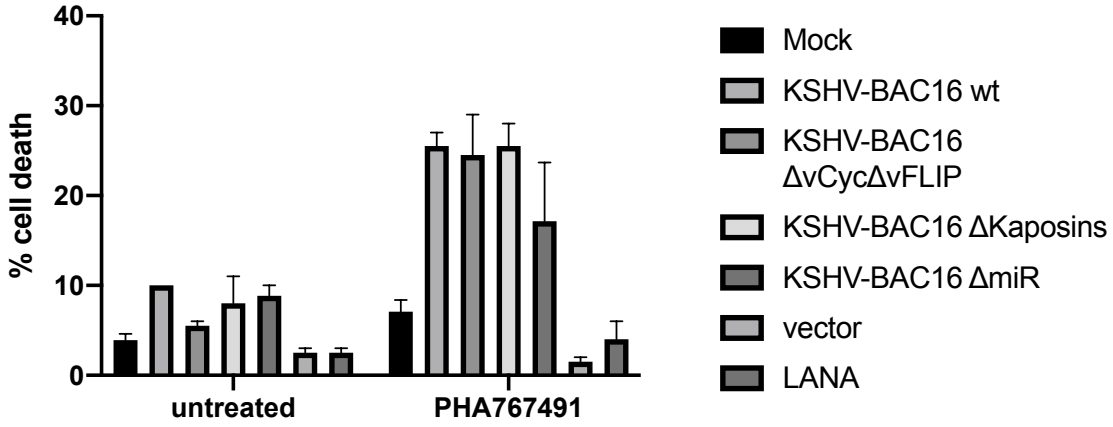

Figure 4.5: KSHV latent genes are not required nor are they sufficient to confer sensitivity to CDC7 inhibition. TIME cells were infected with either the indicated KSHV-BAC16 mutant or transduced with a vector control or LANA-expressing lentivirus. At 24 hpi, cells were treated with 1 μM PHA767491 and cell death was measured by trypan blue 48 hours post treatment. Data are shown as mean ± SEM from 2 biological replicates.

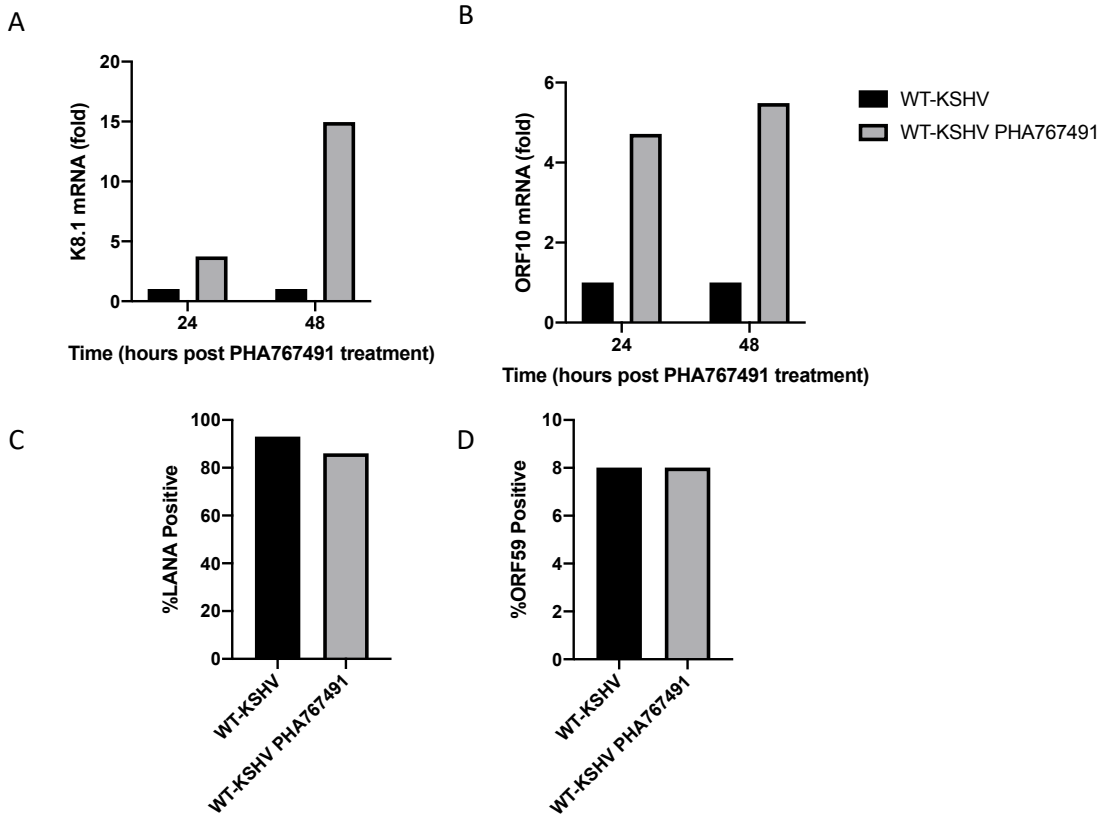

Figure 4.6: KSHV lytic gene expression is upregulated by PHA but the proportion of ORF59+ cells is not increased. TIME cells were infected with infected with WT-KSHV. At 24 hpi, cells were treated with 1 μM PHA767491. mRNA was harvested at 24 and 48 hours post treatment and **(A)** K8.1 or **(B)** ORF10 mRNA was quantified by RT-qPCR. The relative amount of mRNA for each gene was normalized to tubulin mRNA in each sample, and fold change was calculated as described previously. TIME cells were infected with infected with WT-KSHV. At 24 hpi, cells were treated with 1 μM PHA767491. At 48 hours post treatment, the proportion of **(C)** LANA+ and **(D)** ORF59+ cells were determined by immunofluorescence microscopy. Data shown are a single replicate.

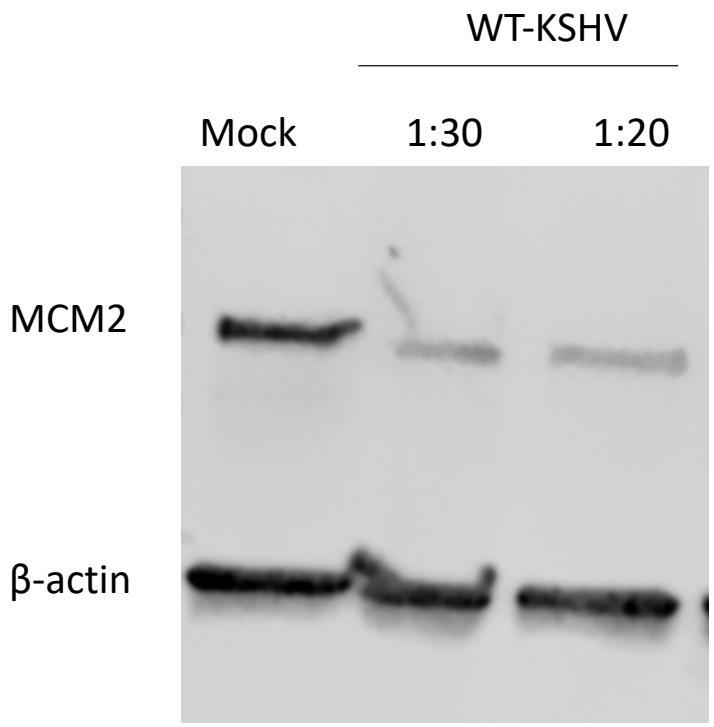

Figure S4.1: KSHV infection of primary BECs leads to downregulation of MCM2. Primary BECs were infected with WT-KSHV at two different dilutions and whole cell lysate was immunoblotted for the indicated antibodies 48 hpi.

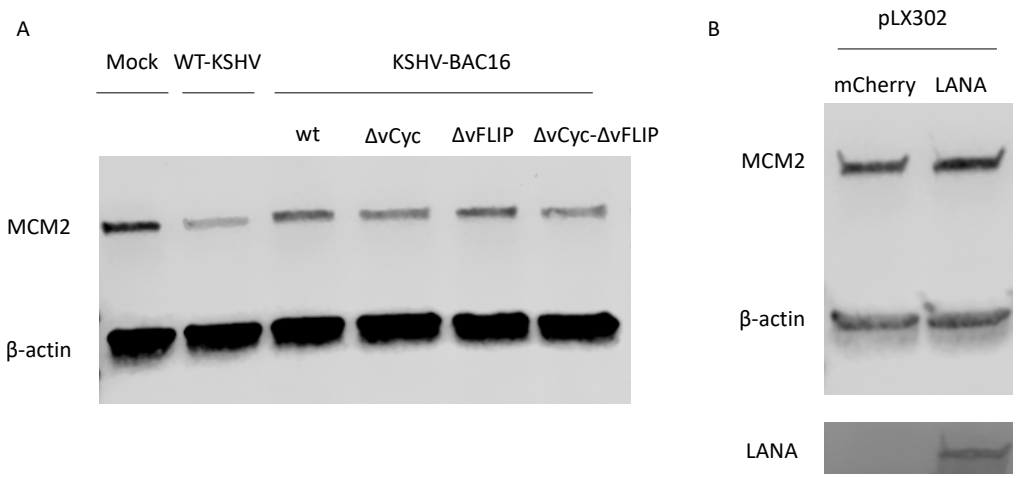

Figure S4.2: KSHV-BAC16 modestly downregulates MCM2 but LANA expression is not sufficient. (A) TIME cells were infected with mock, WT-KSHV or the indicated KSHV-BAC16 viruses (wt, ΔvCyc, ΔvFLIP, or ΔvCyc-ΔvFLIP). Cells infected with KSHV-BAC16 were selected with hygromycin B for 3 days. Whole cell lysates were immunoblotted for the indicated antibodies. **(B)** TIME cells were transduced with lentiviruses expressing either mCherry or LANA and whole cell lysates were immunoblotted for the indicated antibodies.

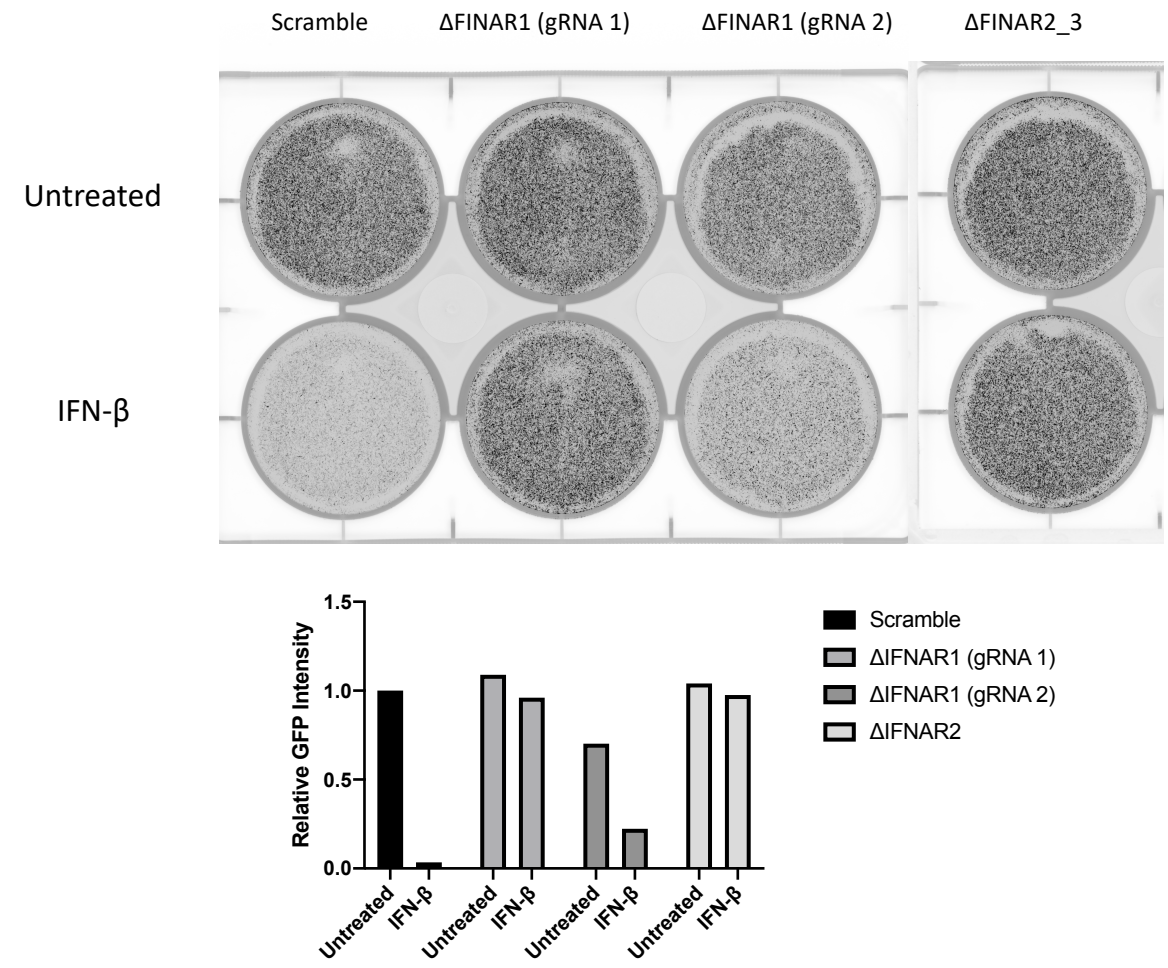

Figure S4.3: IFNAR knockout cells are unable to be rescued from KSHV infection by IFN-β. TIME cells were transduced with lentiviruses expressing CRISPR/Cas9 and gRNAs targeting IFNAR1 and IFNAR2 as well as a nontargeting scramble control. Cells were then treated with IFN-β for 24 hours and then infected with KSHV-BAC16. Cells were scanned for GFP intensity on the Typhoon fluorescent scanner. Raw plate data are shown in the top panel and the quantification is shown on the bottom panel. Data shown are a single replicate.

Chapter 5. Conclusions and Future Directions

5.1 Summary

Here we show that KSHV effectively renders primary endothelial cells STING-null. After characterizing a batch of primary LECs (LEC4) that are defective for STING signaling, we show that there is no difference in susceptibility when comparing LEC4 to LEC8 (STING-intact). We then show that genetic ablation of STING fails to increase susceptibility to initial infection and does not restrict the spread of KSHV following lytic reactivation. These results are consistent with the finding that KSHV either does not detectably activate STING signaling during latent and lytic infections or the virus simultaneously activates and represses this pathway. Adv, on the other hand, is restricted from spreading through the culture by STING.

In Chapter 4, we show that the CDC7-MCM2 pathway may be required for survival of latently infected cells. Ablation of MCM2 shows a modest increase in cell death during KSHV infection whereas chemical inhibition of CDC7 results in a much greater degree of cell death is KSHV-infected cells. Further experiments failed to specify which latent gene may be responsible for this sensitivity to CDC7 inhibition, raising the possibility that several latent genes may be required for conferring sensitivity to CDC7 inhibition. Finally, we show that WT-KSHV and IFN signaling downregulate MCM2 but appear to be independent of each other because KSHV infection of IFNAR knockout cells fails to rescue MCM2 downregulation.

5.2 STING is dispensable during KSHV infection of primary endothelial cells

This study identified a primary sample of neonatal LECs that are intrinsically defective in innate immune activation by the cGAS-STING pathway. This defect is not universal in LECs, as other primary LECs (both adult and neonatal) were responsive to cGAS-STING agonists. The block in the pathway occurs prior to the phosphorylation of TBK1, STING and IRF3. In addition, pre-treatment with cGAMP revealed that STING is unable to activate any antiviral pathways in LEC4 since cGAMP pre-treatment did not decrease susceptibility to KSHV but pre-treatment

with IFN-β strongly inhibited KSHV infection. There was no mutation in the genomic sequence of STING so further work will be required to identify the defect in this pathway. Our study narrowed down the LEC4-specific defect in innate immune activation by cytosolic DNA to an inability to activate the phosphorylation of key proteins in the pathway. Transfection of RNA mimics induced a robust interferon response indicating the defect is specific to the DNA sensing pathway and not downstream induction of IFN. We chose to focus on STING due to the potential for cGAMP as a therapeutic for cancer. We hypothesize that the failure of STING, TBK1 and IRF3 to undergo phosphorylation could be due to a lack of expression of a positive regulator or overexpression of a negative regulator, or a mutation in an important cofactor. There are several steps leading up to the phosphorylation of TBK1, which initiate phosphorylation of STING and IRF3, where the block could be occurring. These include ubiquitination, translocation and palmitoylation of STING (139). Future studies will focus on the events that lead up to these processes. The finding that some primary neonatal LECs are defective in DNA sensing through STING is highly intriguing. Characterizing a large number of primary isolates would be required to determine how common this defect is in the population. It is likely that there is heterogeneity in the population in the degree to which cells respond to cytosolic DNA. Our own studies hinted at this, given that the magnitude of the response varied from isolate to isolate (compare Fig. S3.2 C to D, and E to F), suggesting that there may be a spectrum of responses across the population and the degree to which cells express IFN may vary from person to person. If this is the case, one could reasonably assume that individuals with cells that express lower levels of IFN during DNA virus infection would be more susceptible to infection by DNA viruses compared to individuals with cells capable of producing higher amounts of IFN during DNA virus infection.

Our studies with infection of LECs defective in STING signaling, led us to further characterize the role of STING during KSHV infection in primary endothelial cells. Interestingly, we found no difference in the susceptibility of infection when comparing the STING-null LEC4 to

the STING-intact LEC8. Consistent with this, when compared to the parent endothelial cells, KSHV infection rates, lytic reactivation and spread of KSHV through cultured endothelial cells was unchanged in endothelial cells where STING was knocked out. Therefore, STING does not play a significant role in KSHV biology as it is dispensable during both the establishment of latency as well as during lytic reactivation and spread of virus through cultured endothelial cells. However, this is not true for the role of STING during other DNA virus infections. The effect of STING on Adv infection was more nuanced, with STING being required for the induction of IFN-β during Adv infection and for restricting Adv spread but not initial infection or replication. This result is consistent with a previous report, although the finding that STING restricts Adv spread has not been reported previously (111).

Since the discovery of cGAS and STING, numerous studies have highlighted the importance of this pathway during both DNA and RNA virus infections (58, 79, 121, 124). While this pathway is undoubtedly critical for innate immune surveillance, there are likely scenarios where the pathway is either (1) activated too late to influence the course of infection, (2) not activated due to the lifecycle of the virus or (3) antagonized by viral proteins to prevent activation or limit downstream signaling. Our results suggest that during KSHV infection, all three scenarios could play a role in decreasing the importance of STING signaling for KSHV biology. This is supported by the minimal innate immune activation observed during de novo infection in BECs, which peaks at 48 hpi. The lifecycle of KSHV likely limits the amount of DNA exposed to the cytosol, as viral DNA is shielded following entry of the virion by endocytosis by the capsid until it is injected into the nucleus. Latency, the default program of KSHV in cultured cells, further limits the amount of viral DNA present in the infect cells. For these reasons we likely see limited innate immune activation following KSHV infection and no difference in susceptibility between STING-deficient and control cells. That being said, KSHV encodes numerous proteins that antagonize innate immune activation, including three that target cGAS or STING directly (14, 77, 78). These proteins likely limit production of IFN-β during lytic

reactivation, which may explain why there was no difference in the ability of KSHV to reactivate and spread in STING-deficient cells compared to the control. Our study demonstrates that it is likely that these proteins do not just modulate the innate immune response but render STING irrelevant for KSHV infection of endothelial cells

During Adv infection, it appears that STING is more important for controlling spread of the virus to bystander cells than it is for restricting initial infection or replication. This is supported by the observation that during both high and low MOI infections, the proportion of infected cells after a few days is the same in the STING deficient cells compared to the control (data not shown). However, at low MOI, a difference is observed after several days, with the virus spreading more readily in the STING-deficient cells than in the control cells. At high MOI, the virus is already infecting a high percentage of cells, so its replication is unabated in the presence of STING. This experiment suggests that while STING plays no significant role in restricting the virus once it enters the cell, it is important for paracrine signaling of IFN-β to protect bystander cells from infection. Our pre-treatment experiments also support this observation as cells are protected from infection by KSHV if pre-treated with either cGAMP or IFN-β.

This study examines the role of endogenous cGAS-STING activity during de novo infection and lytic reactivation of KSHV in primary endothelial cells. While many other studies have made important contributions to our understanding of KSHV-innate immune interactions and have pointed to a role for STING in restricting lytic replication (14, 98), our study is the first to explore the role of STING in primary endothelial cells. KS spindle cells appear to be most closely aligned with endothelial cells and therefore, the use of primary endothelial cells is important for studying KSHV interactions with its target cells, thus potentially providing in vivo relevance.

5.3 KSHV requires CDC7 for host cell survival

Our lab identified MCM2 as a potential host factor that is essential for the survival of latently infected cells. Subsequent experiments to validate the finding from the screen proved to be difficult to carry out due to the importance of MCM2 for cellular proliferation. Indeed, when MCM2 was knocked out, we found that cell growth halted, which is consistent with the essential role of MCM2 for replicating DNA during mitosis. Since MCM2 knockout cells ceased proliferating, it was difficult to conduct experiments with these cells. As a result, we tried an alternative approach where we infected cells first with KSHV, then transduced with the lentivirus to knock out MCM2. This resulted in a modest increase in cell death with one of the gRNAs.

Our model explaining the essentiality of CDC7 and MCM2 during KSHV infection relies on work done on cancer cells lines. We propose the following: similar to cancer cells, KSHV deregulates key checkpoint proteins, including pRB, p53 and Chk1/Chk2 (Fig. 5.1). Normally, these proteins would become activated through ATM/ATR following DNA damage caused by CDC7 and/or MCM2 depletion, resulting in cell cycle arrest. However, deregulation of these proteins would shuttle the cell down a p38-Caspase 3-dependent pathway, leading to apoptosis. There is some evidence suggesting that KSHV inhibits p53 and pRB, but further work is required to determine if inhibition of these proteins is required for sensitivity to CDC7 inhibition (140, 141).

The finding that both KSHV-infected endothelial cells as well as KSHV-infected B cells are sensitive to CDC7 inhibition suggests that the sensitivity is a consequence of latency and cellular reprogramming and not an artifact of response to initial infection. However, use of mutant viruses and LANA overexpression failed to specify a particular latent gene as being required or sufficient for sensitivity to CDC7 inhibition. This could be due to redundancy in the effects of latent genes. The fact that RAJI cells, a KSHV-/EBV+ cell line, are sensitive to CDC7 inhibition hints at an evolutionarily conserved pathway that alters the cell cycle and may be common among γ-herpesviruses. Additionally, there appears to be an additive effect in BC-1

cells (KSHV+/EBV+), as these cells are more sensitive to CDC7 inhibition than BCBL-1 or RAJI cells. It is tempting to envision that KSHV and EBV encode orthologues that target the cell cycle and that their combined expression increases sensitivity of these cells to CDC7 inhibition.

KSHV downregulation of MCM2 and CDC7 was an unexpected finding. To our knowledge, this is the first report describing a viral infection leading to the downregulation of MCM2. The consequence of this downregulation is unclear, and it does not appear to be required for sensitivity to CDC7 inhibition given that MCM2 is not downregulated in cells infected KSHV-BAC16, but these cells still die upon treatment with PHA767491. However, given the importance of MCM2 for normal cellular processes, including DNA replication, proliferation and transcription, it is hard to believe that downregulation of such an important protein would occur without a phenotype. Indeed, decrease expression of the MCM complex, including MCM2, has been linked to inflammation, genomic stability and cancer due to replication fork stalling and DNA damage (142–146). Therefore, exploring the functional consequence of MCM2 depletion following KSHV infection should be prioritized.

We also found that innate immune signaling phenocopied KSHV infection in that MCM2 expression was decreased following treatment with cGAMP or IFN-β. In addition, IFN-β was sufficient to render cells sensitive to CDC7 inhibition. However, further experiments showed that the downregulation of MCM2 during KSHV was independent of IFN-β signaling as infection of IFNAR knockout cells failed to rescue cells from MCM2 depletion. However, we did not determine if sensitivity to CDC7 inhibition during KSHV infection is reliant on IFN-β. However, given the high dose of IFN-β (1000 IU/mL) required for cell death during CDC7 inhibition, it is unlikely that the sensitivity to CDC7 inhibition is dependent on IFN-β. In addition, our experiments ruled out IFN signaling as being required for MCM2 downregulation during KSHV infection, but not upstream innate immune signaling. It remains a possibility that IRF3 or NF-κB-

dependent pathways upstream of IFN may be required for the downregulation of MCM2 during KSHV infection. There are two interesting phenotypes that warrant further experimentation.

The first is that IFN-β downregulates MCM2 expression. This has not been described before and given that MCM depletion leads to genomic instability and cancer, it is possible to imagine a scenario where chronic viral infection (perhaps hepatitis B or C virus) leads to persistent production of IFN, which, in turn, represses MCM2 expression. MCM2 repression over time would then lead to genomic instability and possibly cancer. It does, however, logically make sense for cells to limit the pool of available resources that may be used for replication during viral infection given that the MCM complex is a helicase that is used for both DNA replication and transcription. Therefore, downregulation of MCM2 (and likely the entire MCM complex) may act as part of the antiviral state to limit the suitability of the host cell to viral replication. However, sustained downregulation of MCM2 may lead to DNA damage, genomic instability and cancer. This newly described model where IFN signaling represses MCM2 to promote genomic instability is an exciting new area to explore.

The second phenotype that requires further investigation is the observation that KSHV infection of IFNAR knockout cells results in increased LANA expression. This finding was unexpected given that KSHV results in minimal IFN-β expression. It is also unlikely that IFNAR knockout cells are more susceptible to infection given that STING knockout cells are no more susceptible than wild-type cells. It is possible that the increase in LANA expression is due to an increase in lytic reactivation in IFNAR mutant cells. Given that innate immune signaling suppresses lytic reactivation, there may be increased lytic reactivation in the IFNAR-null cells (147). We therefore propose that IFN signaling represses LANA expression.

It is possible that CDC7 inhibition leads to an increase in lytic reactivation. Indeed, we observed an increase in expression of lytic genes when infected cells were treated with PHA767491. However, this increase was modest, and we did not observe an increase in the

proportion of ORF59+ cells. More experiments are required to determine if the cell death is caused by lytic replication.

5.4 Future Directions

5.4.1 KSHV and STING

Future experiments should focus on determining the block in STING signaling in LEC4. Because this defect appears to be specific to this batch of primary cells, the defect is likely caused by a SNP. We have ruled out a SNP in the *Tmem173* exon through RNA sequencing. However, we cannot rule out a SNP in the noncoding parts of the *Tmem173* gene or other cofactors and essential accessory proteins. Therefore, to determine the location of the block, whole genome sequencing should be done on LEC4 (with LEC6 as a comparison). Once potential candidate genes have been identified, we can screen for which novel mutations might be contributing to the defect by using site-directed mutagenesis and expressing these plasmids in 293T cotransfected with a STING plasmid and an IRF3 reporter plasmid.

STING is likely dispensable during KSHV infection due to the efficient antagonism by the virus of this pathway. Our results in Fig. S3.4 hint at antagonism by KSHV due to the modestly reduces levels of p-STING and p-IRF3 when cells are infected with KSHV, then transfected with cGAMP. However, cGAMP transfection may overwhelm any antagonism by KSHV, which could mask subtle effects. In order to directly test KSHV antagonism of the pathway, we can infect BECs with KSHV, then superinfect with Adv and measure IFN-β induction. If KSHV is efficiently blocking innate immune activation, then it would be expected that IFN-β expression would be far lower in KSHV and Adv-infected cells compared with cells infected with Adv alone. A potential caveat to this experiment is that KSHV infection of BECs may alter susceptibility to superinfection by Adv, which may affect the results.

In order to determine if antagonism of the cGAS-STING pathway enhances initial infection and spread, we can use mutant viruses for ORF52 and vIRF1. In these experiments,

we would expect that mutant viruses for these genes would be less efficient at spreading through the culture following lytic reactivation and would exhibit lower infection rates following initial infection when compared to wild-type virus. To determine if reduced susceptibility to infection and reduced ability to spread following lytic reactivation is due to diminished antagonism of the cGAS-STING pathway (and not due to other unrelated functions of these genes), we can infect STING KO cells with either ORF2 or vIRF1 mutants and measure infection rates and spread through the culture. The expectation would be that susceptibility to initial infection and ability to spread during lytic reactivation would be restored to wild-type virus levels in STING KO cells. That way, we can rule out the possibility of any observed phenotypes being due to other functions of these genes that are unrelated to innate immune antagonism. A complementary approach to these experiments would be to overexpress these viral genes in primary endothelial cells and then infect these cells with KSHV. If these genes efficiently block activation of the pathway, then we would expect that cells overexpressing ORF52, vIRF1 or LANA would be more susceptible to initial infection and that the virus would spread more easily in these cells when compared to cells expressing a vector control.

The fact that the KSHV genome contains at least three genes that directly target cGAS or STING suggest strong evolutionary pressure by this pathway. However, another contributing factor as to why STING is dispensable is the lifecycle of the virus. Following entry, the viral genome is shuttled from cytosol to the nucleus by the capsid. Assuming cGAS is cytosolic (there is some controversy about the subcellular localization), this does not leave many opportunities for sensing of the viral DNA by the host. Other DNA sensors, including IFI16, may sense KSHV DNA in the nucleus. However, KSHV encodes an antagonist that functions by degrading IFI16 (148). In addition, because the default pathway is latency, the viral DNA is only replicated by host machinery, viral DNA is not produced in large quantities in most cells. This further limits the amount of available PAMP that can be sensed by cGAS. Other DNA viruses, particularly those that default to lytic replication, including HSV-1 (in nonneuronal cell types) and

Adv, may be more vulnerable to inhibition by the cGAS-STING pathway. However, these viruses replicate quickly and to high titers, so it is possible that the ability to replicate quickly and to produce a large number of progeny virions helps to overcome inhibition by the cGAS-STING pathway.

5.4.2 KSHV and CDC7

It is important to repeat the screen validation experiments. For that experiment, only a single replicate was obtained. There are several approaches that can be taken to obtain more robust data. The first approach is to repeat the experiment exactly (infect with KSHV first, then transduce with the lentivirus to create MCM2 knockout cells). One limitation of this approach is that by transducing after infecting, a homogenous population of MCM2 knockouts is not obtained since the cells are not selected after transduction. To get around this, it is possible to transduce first, select, then infect. However, given that MCM2 knockout cells cease proliferating, it is necessary to transduce a large number of cells to ensure that there are enough cells to use for experiments.

The second way to validate the screen data relies on use of deactivated (d)Cas9 targeting MCM2 under a doxycycline-inducible promoter (149). dCas9 utilizes a mutant or nuclease-deficient Cas9, which is turned on by doxycycline. Upon activation and under the direction of the gRNA, dCas9 binds to but does not cut the corresponding regions of the genome. However, the binding of dCas9 blocks transcription of the gene. By using dCas9, cells will continue to proliferate following transduction. The cells can then be treated with doxycycline, resulting in loss of MCM2 expression, and then infected with KSHV. This approach circumvents the issue of halted proliferation and allows for enough cells to be obtained by allowing the cells to proliferate following transduction and selection and treating cells with doxycycline before infecting with KSHV. Another possibility is to transduce cells with a lentivirus expressing MCM2 gRNA and doxycycline-inducible Cas9 to prevent editing until later.

The final approach to validating the screen data involves targeting CDC7. MCM2, which is phosphorylated by CDC7, was identified in the screen as one of the top hits whereas CDC7 was not. However, use of the CDC7 inhibitor PHA767491 was far more efficient than the genetic ablation experiments. Therefore, it may be more effective to validate the results from the screen by knocking out CDC7 rather than MCM2. Similar to MCM2, CDC7 knockout cells are viable, but proliferation is severely reduced. Therefore, it may be necessary to employ the aforementioned techniques in order to obtain enough cells to work with.

Given that MCM2 forms a heterohexamer with other MCM proteins, it is possible that other MCM proteins may also be essential for cell survival during KSHV infection. Indeed, MCM4, MCM5 and MCM7 were all identified to varying degrees by the screen as potentially being essential for KSHV latency. While none of these “hits” were as strongly identified as MCM2, it is still worth investigating the potential for each to contribute to survival of KSHV-infected cells. In addition, the fate of the other MCM proteins during KSHV infection and innate immune signaling is unknown. It is possible that the expression of the other MCM proteins may phenocopy MCM2, and that KSHV infection and innate immune signaling both result in decreased expression of the entire MCM2-7 complex. Perhaps a more interesting possibility is that KSHV infection selectively downregulates MCM2 expression while levels of MCM3-7 remain unchanged. Future studies should investigate these possibilities.

Chemical inhibitors often have off-target effects. The CDC7 inhibitor PHA767491 has strong cross-reactivity to CDK9 (106). One of the functions of CDK9 is to phosphorylate RNA polymerase II to promote transcription. Inhibition of CDK9 leads to degradation of labile transcripts, including several different anti-apoptotic genes. To tease apart whether CDC7 or CDK9 activity is required for survival of latently infected cells, the use of CD7-specific and CDK9-specific inhibitors is required (150–152). However, given that CDK9 was not identified in the screen as being essential for the survival of latently infected cells, these experiments may only result in ruling out the requirement of CDK9 during latency.

In our model, we suggest that KSHV may deregulate checkpoint proteins, which shuttles cells along an ATM/ATR-p38-Caspase 3 pathway upon CDC7 inhibition (Fig. 5.1). To test whether this cell death is dependent on ATM/ATR-p38-Caspase 3, we can use different inhibitors of each of these proteins to see if this rescues KSHV-infected cells from cell death upon CDC7 inhibition. For example, we can treat infected cells with PHA767491 and either caffeine (ATM/ATR inhibitor), SB202190 (p38 inhibitor) or Z-DEVD-FMK (Caspase 3 inhibitor) to determine if blocking this pathway rescues cells from cell death. Alternatively, we can treat infected cells with PHA767491 and retinoic acid, which activates pRB, results in cell cycle arrest rather than cell death.

To further explore the functional consequences of MCM2 depletion, we propose infecting cells with KSHV and examining rates of DNA synthesis, proliferation and transcription. In addition, it may be possible to rescue MCM2 depletion during KSHV infection by overexpressing MCM2. If MCM2 depletion is part of the cellular response to limit resources during infection, rescuing MCM2 expression during viral infection may actually promote viral replication. To determine if sustained MCM2 repression by IFN signaling promotes genomic instability, cells should be treated with IFN-β and examined for DNA damage by flow cytometry.

To determine if the cell death observed during CDC7 inhibition is due to lytic replication, the experiments examining lytic gene expression must be repeated. In addition, infected cells can be treated with PHA767491 and either ganciclovir or phosphonoacetic acid. If cell death is caused by lytic replication, treatment with replication inhibitors would rescue cells from death.

Finally, to further explore the repression of LANA expression by IFN-β, the experiments where IFNAR knockout cells are infected with WT-KSHV must be repeated. Cells can also be treated with IFN-β following KSHV infection to determine if this results in decreased expression of LANA.

5.5 Conclusions

Studying host factors that either restrict or are required for viral infection is essential for understanding the lifecycle of the virus and for the development of antiviral factors that can be used as therapeutics. Here we show that an important innate immune protein, STING, is rendered dispensable during KSHV infection. Our study bridges the gap in the field, connecting previous reports hinting at the importance of STING with those identifying antagonists of the pathway. We show that KSHV minimally activates innate immune signaling, and that genetic ablation of STING fails to increase susceptibility to initial infection and does not increase the ability of the virus to spread following lytic reactivation. These experiments are complemented by those with Adv, which show that Adv potently activates innate immune signaling in a STING-dependent manner. Finally, STING is required for restricting Adv spread through the culture, but not replication or initial infection. These experiments further contribute to our understanding of the role STING plays in restricting viral infection and how viruses overcome and evade this pathway. In our other study, we show that MCM2 and CDC7 may be essential factors for the survival of KSHV-infected cells. CDC7 inhibition potently induces cell death in KSHV-infected cells, and KSHV infection downregulates MCM2 expression. Finally, we show that innate immune signaling phenocopies KSHV in that IFN-β sensitizes cells to CDC7 inhibition and downregulates MCM2 expression. Together, these studies improve our understanding of host factors important during KSHV infection and identify a potential therapeutic target for treatment during KSHV infection and KS.

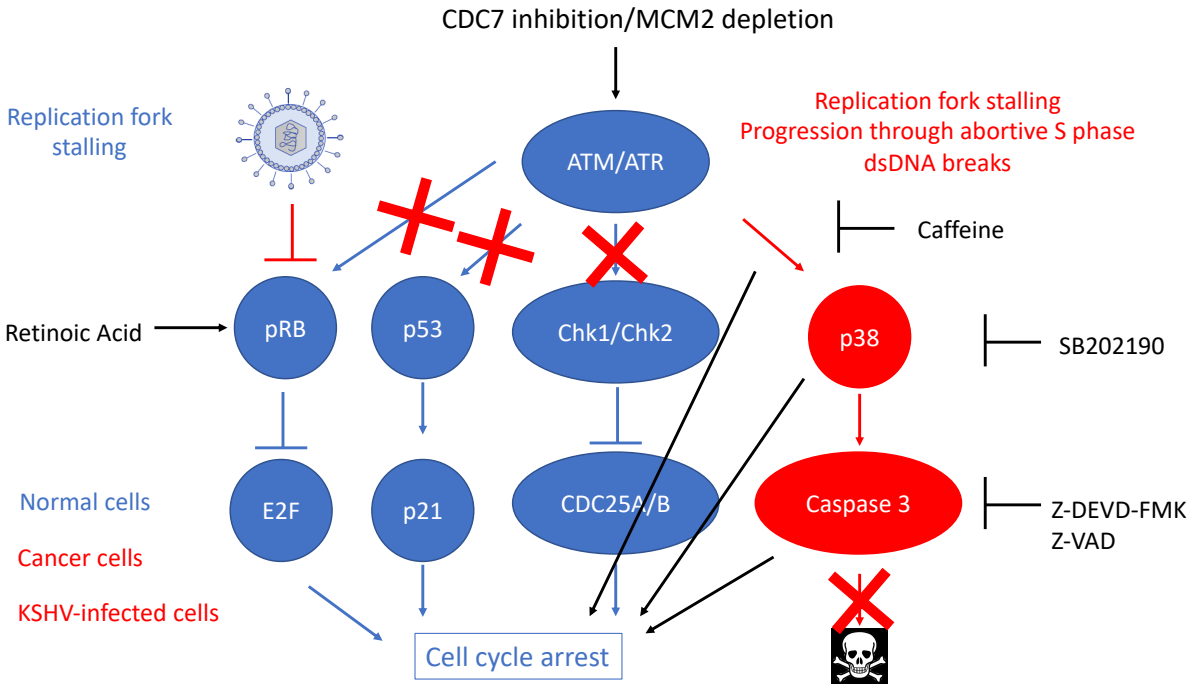

Figure 5.1: Model depicting the requirement of CDC7 and MCM2 during KSHV latent infection.

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CV

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Summary

Industrious and ambitious scientists with 8 years of experience in both basic and translational research with an interest in leveraging my life sciences expertise to help develop therapeutics to improve human health

- Strong interpersonal skills and ability to work well in a team setting
- Exceptional organizational skills and ability to manage multiple projects simultaneously
- Meticulous attention to detail and strong critical thinking skills
- Proven experience managing research groups and projects

Education

- University of Washington, Seattle, Ph.D. Microbiology, 09/2015-01/2020
- University of Wisconsin, Madison, B.S. Microbiology, 01/2013-05/2015

Research and Professional Experience

PPD, Middleton, WI, Senior Scientist 01/2020-Present

- **Method validation and stability testing of cell and gene therapy pharmaceutical products**

University of Washington, Seattle, Graduate Research Assistant
06/2016-01/2020
Principal investigator: Michael Lagunoff

- **Characterization of the role of the innate immune protein STING during DNA virus infection of primary endothelial cells**
    - Used CRISPR/Cas9 to create STING-deficient cells and infected with KSHV and Adenovirus. We found that STING is dispensable during KSHV infection but required for restricting Adenovirus spread.
- **CDC7 is required during KSHV latency**
    - Utilized both genetic and pharmacologic methods to test the requirement of CDC7 during KSHV latency. We found that CDC7 inhibition leads to selective killing of KSHV-infected cells.

University of Washington, Seattle, Graduate Research Assistant
03/2016-06/2016
Principal investigator: Nina Salama

- **Detection of fluoroquinolone-resistant *Helicobacter pylori* in stool**
    - We developed and optimized an assay to detect single-nucleotide polymorphisms conferring resistance to fluoroquinolones in *Helicobacter pylori* from patient stool.

University of Washington, Seattle, Graduate Research Assistant
09/2015-12/2015
Principal investigator: Deb Fuller

- **Assessment of polyfunctionality as a correlate of CD8-mediated *in vitro* control of SIV**
    - Tested the role of polyfunctional CD8 T cells in controlling of SIV in vitro.

University of Wisconsin, Madison, Undergraduate Research Assistant
01/2014-05/2015
Principal investigator: Heidi Goodrich-Blair

- **Pathogen-Immune interaction between *Xenorhabdus nematophila* bacteria vectored by *Steinernema carpocapsae* nematodes and the immune system of the insect model *Manduca sexta***
    - Characterized *X. nematophila* mutants for virulent and immunosuppressive phenotypes. We found that mutants for a gene encoding a putative toxin were attenuated during insect infections.

University of Wisconsin, Madison, Undergraduate Research Assistant
08/2013-12/2013
Principal Investigator: Timothy Kamp

- **Lineage reprogramming of mouse fibroblasts to proliferative and multipotent induced cardiac progenitor cells by defined factors**
    - We tested the ability of different transcription factors to reprogram mouse fibroblasts into cardiac precursor cells.

Public Health Madison and Dane County, Summer intern
05/2013-08/2013
Supervisor: John Hausbeck

- **West Nile virus mosquito vector surveillance and control**
    - Assisted in the surveillance and eradication of mosquito species capable of spreading West Nile virus in the Madison metro area.

University of Wisconsin, Madison, Undergraduate Research Assistant
06/2012-01/2013
Principal Investigator: Matthew Walsh

- **Analysis of socioeconomic factors as correlates of oral health in Wisconsin adults**
    - We analyzed socioeconomic factors and found a positive correlation with oral health.

Selected Publications and Presentations

Vogt, D., Zaver, S., Ranjan, A., Lagunoff, M. STING is dispensable during KSHV infection of primary endothelial cells. *Virology.* 2020, 540, 150-159.

DiMaio, T., **Vogt, D**., Lagunoff, M. KSHV Requires vCyc to Overcome Replicative Senescence in Primary Human Lymphatic Endothelial Cells. *In revision*.

Vogt, D., Zaver, S., Ranjan, A., Lagunoff, M. STING is dispensable during KSHV infection of primary endothelial cells.

- *Oral presentation.* International Herpesvirus Workshop. Knoxville, TN. July 2019.

- *Poster presentation.* International Herpesvirus Workshop. Knoxville, TN. July 2019.

Vogt, D., Zaver, S., Ranjan, A., Woodward, J., Lagunoff, M. KSHV does not induce interferon in primary endothelial cells.

- *Poster presentation*. Pathogen-associated Malignancy Integrated Research Center Retreat at Fred Hutch Cancer Research Center. Seattle, WA. April 2019.
- *Poster presentation*. Viral Pathogenesis Retreat at Fred Hutch Cancer Research Center. Seattle, WA. June 2018.
- *Oral presentation*. Department of Microbiology Research in Progress meeting at University of Washington. Seattle, WA. February 2018.
- *Poster presentation*. Department of Microbiology Annual Retreat at University of Washington. Leavenworth, WA. September 2017.

Skills

- Extensive experience in mammalian cell culture, viral expression vectors, transfection, drug treatment and cell proliferation and death assays
- Demonstrated experience in standard molecular biology techniques, such as cloning, PCR-based assays, gene expression (qRT-PCR), etc.
- Highly proficient in immunoassays, such as western blotting, ELISA, immunofluorescence, flow cytometry, and immunoprecipitation
- Strong background in analysis of data and scientific literature, writing, and oral presentations
- Experience working in a cGMP lab, including documentation and following SOPs

Honors and Awards

- Viral Pathogenesis Training Grant, NIH, University of Washington, Seattle, 2017
- Microbiology High Achievement Award, University of Wisconsin, Madison, 2015
- National Science Foundation Graduate Research Fellowship Honorable Mention, 2015
- CALS Dean's List, University of Wisconsin, Madison, 2014-2015
- CALS Undergraduate Research Scholar Award, University of Wisconsin, Madison, 2014
- Philipp and Vera Gerhardt Undergraduate Excellence Award, Bacteriology Department, University of Wisconsin, Madison, 2014
- Dean's List, University of Wisconsin, Oshkosh, 2011-2012

Leadership, Broader Impacts and Service

- Mentor to several graduate students, postbaccalaureates and undergraduates 06/2017-01/2020
    - Responsible for managing projects, supervising experiments, aiding in data analysis and presentation
- Graduate Advisor to the University of Washington Microphiles Club 03/2019-06/2019
    - Responsible for organizing events, mentoring, facilitating discussion and providing a resource for undergraduates interesting in microbiology
- President of the Department of Microbiology Graduate Students 08/2018-08/2019
    - Responsible for organizing events, coordinating the student-invited seminar speaker
- Program leader at Madison School and Community Recreation 06/2015-07/2015

    - Responsible for supervising and leading activities for children ages 5-7 enrolled in the summer program with a particular emphasis on science-based events
- Volunteer soccer coach at Mt. Horeb Recreation Department
  07/2010-08/2012
    - Responsible supervising and leading soccer drills and promoting teamwork and sportsmanship

Teaching experience

- Lecturer for introductory microbiology course
  10/2020
    - Department of Microbiology, University of Washington. Seattle, WA
- Lecturer for molecular biology techniques lab
  02/2019
    - Department of Microbiology, University of Washington. Seattle, WA
- Lab instructor molecular biology techniques lab
  01/2017-03/2017
    - Department of Microbiology, University of Washington. Seattle, WA
- Lab instructor for introductory microbiology lab
  01/2017-03/2017
    - Department of Microbiology, University of Washington. Seattle, WA

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