KSHV hijacks a non-canonical caspase activation pathway to block antiviral interferon signaling

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Abstract

Viruses must employ strategies to circumvent host immune defenses in order to establish successful infections. Herpesviruses are particularly adept at establishing long-term infections as they have long co-evolved with their hosts to become experts at avoiding immune detection. Kaposi's sarcoma-associates herpesvirus (KSHV) is an oncogenic herpesvirus that causes the AIDS-defining malignancy Kaposi's sarcoma (KS) and other lymphoproliferative disorders. KS is a leading cause of cancer in sub-Saharan Africa, and its available treatments have limited efficacy. Developing therapeutics that directly target KSHV could greatly improve prognosis as virus-directed strategies have proven effective for other viral-induced cancers. Lytic reactivation of KSHV is essential for virus spread and disease progression and KSHV relies heavily on immune suppression to reactivate and replicate in its hosts. Thus, understanding the mechanisms of KSHV immune evasion mechanisms may allow us to disrupt these strategies as potential treatments.

Our lab previously discovered that KSHV usurps caspase-8 activity to block the type I interferon (IFN) response and promote lytic reactivation and viral replication. However, the details of this caspase-mediated IFN suppression were unclear. Here I report that during KSHV lytic infection, caspase-8 is activated through the pathogen sensing TLR3-TRIF pathway, and resultant caspase activity impedes activity of the DNA sensor cGAS to block IFN induction. This is the first report of caspase-mediated negative cross-regulation between these two pathogen sensing pathways. Caspase activity seems to target a cGAS regulator, rather than cGAS itself, to block its activity. Our single-cell RNA sequencing analysis also reveals that the potent IFN response resulting from caspase inhibition occurs from >5% of cells expressing IFN-β. While caspase-8 activation canonically occurs through the FADD-dependent extrinsic apoptosis pathway, some

previous studies have similarly reported FADD-independent caspase-8 activation through TLR3. However, my data is the first to demonstrate TLR3-driven caspase-8 activation through natural virus infection rather than artificial stimulation of TLR3. Moreover, my data revealed that KSHV lytic reactivation strongly upregulates TLR3 and that this may be sufficient to activate TLR3 signaling in a ligand-independent manner to induce caspase-8 activation. This work reveals novel insights into how KSHV lytic infection alters TLR3 signaling and triggers caspase-mediated cross talk between two pathogen sensing pathways to block type I IFN signaling.

Dedication

To Debbie: the most compassionate, kindhearted, gentle and loving person that I have ever known in my life. I undoubtedly would not be where I am today without you. The impact that you had on my life is insurmountable. The light that you cast on everyone you encountered will forever live on in us. You always inspired the best in me, and I will carry that inspiration with me through the rest of my life. I am forever grateful to have been your “pooh” and that you loved me and Amanda as your own children. Our families will forever be intertwined, and you will always live on through the memories we share. As John recently put it: “What a web of loving and caring she spun!” Love you and miss you always.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
APAF1	Apoptotic protease activating factor 1
APCs	Antigen presenting cells
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BFP	Blue fluorescent protein
CARD	Caspase recruitment domain
Cas9	CRISPR-associated protein 9
cFLIP	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
cGAS	Cyclic GMP-AMP synthase
CHD1	Chromodomain Helicase DNA Binding Protein 1
CHX	Cyclohexamide
CIAP	Cellular inhibitor of apoptosis protein
CMV	Human cytomegalovirus
CRISPR/Cas9	Clustered regularly interspaced palindromic repeats
DAI aka ZBP1	DNA-dependent activator of IRFs
DAMP	Damage associated molecular pattern
DD	Death domain
DDX27	DEAD-Box Helicase 27
DED	Death effector domain
DENV	Dengue virus
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
dsRNA	dsRNA
DUSP11	Dual-specificity phosphatase 11
EBOV	Ebola virus
EBV	Epstein-Barr virus
EBER	Epstein-Barr virus–encoded small RNA
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FADD	Fas Associated Via Death Domain
FBS	Fetal bovine serum
GEM	Gel Beads-in-emulsion
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
G3BP1	Ras GTPase-activating protein-binding protein 1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IFN	Interferon
IFITM	Interferon-induced transmembrane protein
IFI16	Interferon gamma inducible protein 16

IFNAR	Interferon-α/β receptor
IKKε	Inhibitor of nuclear factor kappa-B kinase ε
IL-6	Interleukin-6
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon sensitive response element
IRF3	Interferon Regulatory Factor 3
IRF7	Interferon Regulatory Factor 7
JAK-STAT	Janus kinase/signal transducers and activators of transcription
KD	Knockdown
kDa	Kilodaltons
kicGAS	KSHV inhibitor of cGAS
KICS	KSHV-induced cytokine syndrome
KO	Knockout
KS	Kaposi's sarcoma
KSKV	Kaposi's sarcoma associated herpesvirus
LANA	Latency-associated nuclear antigen
LPS	Lipopolysaccharide
MAVS	Mitochondrial anti-viral signaling protein
MCD	Multicentric Castleman Disease
MDA5	Melanoma differentiation-associated gene 5
MHV68	Murine herpesvirus 68
miRNA	Micro RNA
MOMP	Mitochondrial outer membrane permeabilization
MSM	Men who have sex with men
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
ORF	Open reading frame
PAC	Puromycin-N-acetyl transferase
PAN	Polyadenylated nuclear RNA
PAMPS	Pathogen associated molecular patterns
PCA	Principal component analysis
PEL	Primary effusion lymphoma
PKR	Protein kinase RNA-activated
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptors
PTMScan	Post-translational modification scan
PVDF	Polyvinylidene difluoride
RFP	Red fluorescent protein
RIG-I	Retinoic acid inducible gene I
RIPA	Radioimmunoprecipitation assay
RIPK	Receptor-interacting serine/threonine-protein kinase
RLR	Retinoic acid-inducible gene I (RIG-I)-like receptor
RNAi	RNA interference
RSV	Respiratory syncytial virus
RTA	Replication and transcription activator
RT-qPCR	Real-time quantitative PCR
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scRNAseq	Single-cell RNA-sequencing
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

siRNA	Small interfering RNA
SOCS1	Suppressor of cytokine signaling 1
Sp1	Specificity protein 1
STING	Stimulator of interferon genes
SQSTM1	Sequestosome 1
TBK1	TANK-binding kinase 1
TBST	Tris-buffered saline with 0.1% Tween 20
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRAIL-R1/2	Tumor necrosis factor receptor superfamily member 10A/B
TRIF	TIR-domain-containing adapter-inducing interferon-β
UMAP	Uniform Manifold Approximation and Projection
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLICE-inhibitory protein
vIL-6	Viral Interleukin-6
vIRF	Viral IRF
VSV	vesicular stomatitis virus
VZV	varicella-zoster virus

Chapter 1. Introduction

1.1. Evolutionary virus-host arms race

Human biology is intimately intertwined with and influenced by the microorganisms that inhabit our bodies. The human microbiome consists of numerous bacteria, viruses, fungi, archaea, and protists that harbor symbiotic relationships with humans and are indispensable for many aspects human health. However, many microorganisms are pathogenic and cause a plethora of diseases in human hosts. Since pathogens have always presented a massive threat to human health and have historically been a leading cause of mortality, they exert immense selective pressure on the human genome and have played a key role in shaping it[1].

Viruses have a particularly intimate relationship with their hosts as they are obligate intracellular parasites that hijack the machinery of host cells to carry out their life cycle and propagate. As a result, viruses interact with a vast and diverse array of host proteins, often conserved proteins that drive essential cellular functions, and consequently drive adaptation of these proteins. One study conservatively estimates that viruses direct nearly one third of all adaptive amino acid changes within a subset of the human proteome that is conserved within mammals[2]. While viruses have broadly driven human evolution by affecting widespread protein adaptation, the proteins specifically involved in antiviral defense, rather than critical basic cellular functions, evolve particularly rapidly[2]. Despite the rapid evolution of these antiviral defense proteins, viruses themselves evolve at a far more astonishing rate than humans since they can rapidly produce numerous progeny viruses throughout their life cycle of infection. This is illustrated by the significant within-host virus diversity that develops from chronic infection by viruses like human immunodeficiency virus (HIV), influenza, and herpesviruses like Kaposi's sarcoma associated herpesvirus (KSHV)[3–5]. This constant

evolution of host antiviral defense strategies and viral mechanisms to thwart and counteract such host defenses is known as the evolutionary virus-host arms race. This is in line with the Red Queen hypothesis proposed by evolutionary biologist Leigh Van Valen in 1973 that co-evolving species, such as viruses and humans, must constantly adapt to maintain their relationship with each other and persist[6]. This hypothesis is derived from Lewis Carroll's novel *Through the Looking Glass* in which the Red Queen says to Alice that "here, you see, it takes all the running you can do to keep in the same place." This evolutionary arms race is a long-standing interest of many virologists and immunologists, and studying it has and will continue to have critical impacts on our ability to counteract current viral threats and prepare for inevitable emerging threats that have potentially devastating consequences for public health.

1.2. Host defenses to viral infections

1.2.1. Brief overview of the immune system and all barriers to infection

The human body has numerous barriers to infections, from our skin and mucous membranes to highly specialized immune cells. Apart from acting as a physical barrier to pathogens, our skin produces broadly acting antimicrobial peptides, such as defensins and psoriasin, to prevent pathogens from potentially breaching the skin barrier to invade the body. Bodily secretions such as tears, saliva, and mucous also broadly counteract pathogens via the antimicrobial peptides that they harbor. The microbiome also prevents foreign pathogens from establishing infection by competing for space and nutrients. These general physical and chemical barriers constantly limit our burden of infection by preventing the millions of pathogens that we encounter daily from establishing niches to thrive in our bodies[7]. However, many pathogens have evolved mechanisms to resist and overcome these barriers and invade the body where they encounter additional, more specialized levels of immunity.

The innate immune system consists of the aforementioned barriers as well as other defense mechanisms that are designed to non-specifically attack a breadth of pathogens. The innate immune system has been evolving since its origin approximately 1000 million years ago, in contrast to the adaptive immune system which began developing roughly 500 million years ago[8]. Pathogens that enter the body are met with tissue resident and circulating innate immune cells that can distinguish them as foreign invaders by detecting distinct moieties on them called pathogen associated molecular patterns (PAMPs). Recognition of PAMPs by pattern recognition receptors (PRRs) on immune cells causes them to become activated and enact processes that directly kill the pathogens, such as phagocytic cells engulfing and destroying pathogens and innate lymphoid cells like natural killer cells directing programmed death of infected cells. The latter is especially important for counteracting virus infections as viruses rely on the persistence of the cells they infect in order to hijack their machinery and replicate. The activation of immune cells also causes them to secrete chemical messenger molecules called cytokines and chemokines to communicate with a diverse range of cell types and coordinate an inflammatory response that recruits more immune cells to the site of infection for a more potent attack. Specific immune cells, predominantly dendritic cells, that can process antigens from pathogens and display them on specialized proteins on their cell surface are known as professional antigen presenting cells (APCs)[9]. These APCs bridge the gap between the innate and adaptive immune system as they, upon being activated by PRR stimulation and antigen uptake and undergoing maturation, migrate to lymphoid organs to present antigens to naïve T cells. Only T cells with a receptor that specifically recognizes the presented pathogen-derived antigen will become activated from this interaction, thus priming them to coordinate a highly specific attack on the invading pathogen[10]. While the onset of an adaptive immune response is far more delayed than innate immune responses, its calculated specificity for the

pathogen and lasting immunological memory generates a potent and powerful response to more effectively clear infections. Nonetheless, the highly tailored adaptive immune response would not be possible without the initial innate immune responses that immediately act to limit the spread of infection while alerting surrounding immune and non-immune cells of the infection.

1.2.2. Cell intrinsic and innate intracellular immune responses

While tissue resident and circulating innate immune cells are important for performing their specialized functions and activating the adaptive immune response, viruses first encounter cell-intrinsic and innate intracellular immune responses in the cells that they initially infect, which are not always immune cells themselves. When viruses breach barrier sites of the body, non-immune cells, especially epithelial cells, are the first group of cells that they encounter as these cells constitute the bulk of the invaded tissue. Intracellular defense mechanisms that occur in infected cells are key for controlling the initial infection by both directly restricting virus replication and consequent local spread, as well as by triggering an inflammatory response and recruiting innate immune cells to the site of infection[11]. While PRRs are most highly expressed in specialized immune cells that are exceptionally receptive to pathogens, many are also widely expressed among non-immune cell types in the body and lead to induction of intracellular innate immune responses upon sensing invading pathogens. Additionally, many cell types constitutively express certain viral defense mediators called restriction factors that impede various stages of the virus life cycle[12]. Thus, expression levels of these restriction factors is inversely correlated to a cell's permissiveness to virus infections[13]. In the laboratory, permissive cells with little to no expression of restriction factors are often used for measuring viral titers, while restrictive cells with high restriction factor expression intrinsically limit levels of viral replication and are useful for studying

host defense mechanisms. Due to their constitutive expression, restriction factors determine the susceptibility of hosts to emerging viruses that they lack prior exposure and immunological memory for. The first known restriction factors to inhibit viral entry into a cell are the human interferon-induced transmembrane protein (IFITM) family proteins[14]. They broadly impede this first step of the virus life cycle by structurally modifying cellular membranes to prevent the necessary membrane fusion reaction for enveloped virus entry, thus allowing them to restrict a diverse array of virus families including orthomyxoviruses, paramyxoviruses, rhabdoviruses, flaviviruses, filoviruses, poxviruses, bunyaviruses, alphaviruses, lentiviruses, and coronaviruses[14]. Constitutive expression of cell-intrinsic restriction factors presents a strong selective pressure for viruses to evolve strategies to evade and antagonize these factors. The rapid replicative nature of viruses causes them to evolve faster than their hosts, so many constitutive restriction factors are circumvented by modern day viral threats[15]. Thus, while IFITM proteins are highly constitutively expressed in many cell types, including the epithelial barrier cells that are often first encountered by an invading virus, many of them are also potently upregulated by innate intracellular immune responses that are triggered upon a host cell sensing a virus infection[16]. Thus, these responses offer an additional layer of first-line defense to control early stages of virus infection.

1.2.3. Type I interferon response

The cytokines produced as a first line of defense when cells sense viral infections are type I interferons (IFNs). Type I IFNs were discovered in 1957 as factors that could "interfere" with influenza A virus and have since been extensively studied for their multifaceted functions in antiviral immunity[17,18]. Barrier cells such as epithelial cells are crucial for sensing early virus infection and rapidly mounting local type I IFN responses. This induces expression of directly antiviral interferon-stimulated genes (ISGs) while

recruiting and augmenting the functions of specialized immune cells that combat intracellular infections. When viral PAMPs are recognized by host cell PRRs, they undergo conformational changes and sometimes oligomerization that prompts recruitment and association with their various adaptor proteins. This triggers downstream signaling to activate the kinases TBK1 and IKKε which phosphorylate the transcription factors IRF3 and IRF7, allowing them to dimerize and translocate to the nucleus to induce transcription of type I IFN cytokines (**Fig 1.1**)[19]. When secreted, these

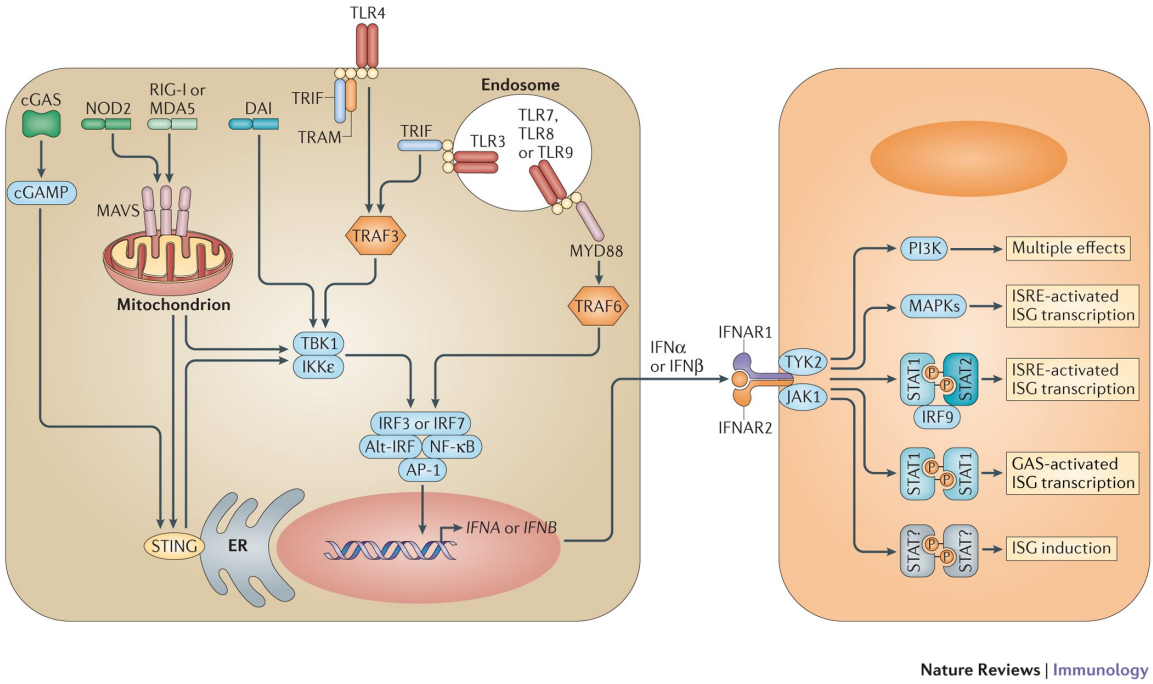

Figure 1.1: Pathways of type I interferon induction and receptor signalling. *Recognition of microbial products by a range of cell-surface and intracellular pattern recognition receptors, including Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I), can lead to induction of the genes encoding type I interferons (IFNs), which is mediated by several distinct signalling pathways. On the binding of type I IFNs to their receptor (IFNAR), multiple downstream signalling pathways can be induced, leading to a diverse range of biological effects. The canonical signal transducer and activator of transcription 1 (STAT1)–STAT2–IFN-regulatory factor 9 (IRF9) signalling complex (also known as the IFN-stimulated gene factor 3 (ISGF3) complex) binds to IFN-stimulated response elements (ISREs) in gene promoters, leading to induction of a large number of IFN-stimulated genes (ISGs). Type I IFNs can also signal through STAT1 homodimers, which are more commonly associated with the IFNγ-mediated signalling pathway. Other STAT heterodimers and homodimers may also be activated downstream, including STAT3, STAT4 and STAT5. Other signalling pathways that do not rely on Janus kinase (JAK) and/or STAT activity may also be activated, including*

mitogen-activated protein kinases (MAPKs) and the phosphoinositide 3-kinase (PI3K) pathway, thereby leading to diverse effects on the cell. Alt-IRF, IRFs other than IRF3 or IRF7; AP-1, activator protein 1; cGAMP, cyclic di-GMP-AMP; cGAS, cytosolic GAMP synthase; DAI, DNA-dependent activator of IRFs; ER, endoplasmic reticulum; GAS, γ-activated sequence; IKKε, IκB kinase-ε; MAVS, mitochondrial antiviral signalling protein; MDA5, melanoma differentiation-associated gene 5; MYD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor-κB; NOD2, NOD-containing protein 2; STING, stimulator of IFN genes; TBK1, TANK-binding kinase 1; TRAF, TNF receptor-associated factor; TRAM, TLR adaptor molecule (also known as TICAM2); TRIF, TIR domain-containing adaptor protein inducing IFNβ; TYK2, tyrosine kinase 2. Reprinted with permission from McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. Nat Rev Immunol. 2015 Feb;15(2):87-103.

cytokines bind to their cognate interferon-α/β receptor (IFNAR) that is ubiquitously expressed on nearly all body cells[19]. This occurs in an autocrine and paracrine fashion to direct a potent transcriptional program in the infected to combat the infection, and in uninfected bystander cells to prime them to resist infection and limit local virus spread. This antiviral program is enacted via activation of the JAK-STAT pathway downstream of IFNAR, and subsequent induction of hundreds of ISGs. Several ISGs have been characterized for their functions in restricting nearly all stages of the viral life cycle through various mechanisms. ISG15, an ISG that is often very rapidly and potently induced by type I IFNs, is a well-studied example that restricts many viruses at various replication steps. It is a small protein that can be conjugated to both viral and host proteins[20]. However, the role of many ISGs during infection with different viruses remains to be fully defined and is thus a very active area of research[21].

1.2.4. Innate Sensors

Unlike intrinsic restriction factors that are constitutively expressed, type I IFNs must be tightly regulated to avoid inappropriate and chronic inflammatory responses that can be detrimental to human health. The activation of PRRs, the innate sensor proteins, is key to this regulation. PRRs trigger a signaling cascade that induce type I IFN signaling only when activated by either PAMPS or damage associated molecular patterns

(DAMPs), which are released as a result of many types of infectious and non-infectious cellular stress[22]. Moreover, expression of PRRs is also often upregulated by type I IFN cytokines, which further contributes to the regulation and self-amplification of type I IFN responses in only the appropriate settings. Several PRRs are well characterized to sense virus infection by detecting viral nucleic acids. These include cyclic GMP-AMP synthase (cGAS), interferon gamma inducible protein 16 (IFI16), retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), certain toll-like receptors (TLRs), and certain NOD-like receptors (NLRs). These PRRs localize to different cellular compartments to sense various types of viruses at different stages of infection. Transmembrane PRRs sense viruses at the cell surface and endosome membranes (TLRs), while others signal from the cytoplasm (cGAS, IFI16, RIG-I, MDA5, NLRs) and sometimes nucleus (cGAS, IFI16). While PRRs are often known for each sensing a classical type of PAMP, some have been shown to respond to additional, non-canonical ligands. For example, TLR4 classically recognizes bacterial lipopolysaccharide (LPS) yet has been shown to respond to glycoproteins on dengue virus (DENV), Ebola virus (EBOV), respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[23]. TLR4 also plays a role in responses to certain herpesviruses like HSV-1 and KSHV through unknown mechanisms[24]. Several other PRRs have also been unexpectedly implicated in viral infections with an unclear route of activation. TLR3.

1.2.5. Toll-like receptors

The toll gene was first discovered in 1985 in Drosophila for its importance in embryonic development, and in 1996 it was found to have an essential role in host innate immunity against fungal infection[25,26]. Shortly after, the first mammalian Toll-like receptor, TLR4, was described as a Drosophila Toll homologue that drives expression of cytokines that activate adaptive immunity[27,28]. It was reported to do so by recognizing

and responding to a specific PAMP, lipopolysaccharide (LPS), found on gram-negative bacteria[28]. LPS became known as the classical PAMP recognized by TLR4, and excessive amounts of LPS can over-stimulate TLR4 and lead to life-threatening septic shock due to hyperinflammation caused by an over-production of cytokines, often referred to as a cytokine storm[23].

All TLRs are type I transmembrane proteins that localize to the cell surface or intracellular compartments including the endoplasmic reticulum, endosomes, and lysosomes[29]. Classically known cell-surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10. These generally respond to surface components of microbial pathogens[30]. Classically known intracellular TLRs include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13. These generally respond to nucleic acids derived from viruses and bacteria, as well as from a damaged host cell[30].

Several viruses including SARS-CoV-2, Ebola virus (EBOV), dengue virus (DENV), and respiratory syncytial virus (RSV) induce cytokine storms, often occurring in patients with higher levels of virus particles and/or proteins[31–38]. Interestingly, surface glycoproteins on these viruses have been found to non-canonically activate TLR4, which is key for triggering the hyperinflammatory cytokine responses[39–41]. In some cases, TLRs also mediate innate immunity during infection yet it remains unclear how they become activated, such as with TLR4 and KSHV[42]. Other TLRs have been shown to be stimulated by unexpected pathogens and molecular moieties that differ from their canonical ligands.

In addition, just as new research sheds light on non-canonical TLR ligands, recent studies have also shown non-canonical TLR localization. TLRs are paramount to innate and adaptive immunity. This is exemplified by several devastating conditions that result from genetic deficiencies in TLR signaling Thus, over 20 years of intensive research on this crucial group of proteins has led to TLRs becoming the best characterized PRRs.

However, much remains to be elucidated to polish our understanding of this complex and sophisticated system of innate immune signaling. The findings of this future research will hopefully allow us to more masterfully manipulate and fine tune innate immune responses with improved therapeutic strategies to benefit human health.

1.2.6. cGAS

In addition to transmembrane PRRs like TLRs, cytosolic PRRs are also crucial for responding to invading pathogens as well as markers of cellular damage. Since DNA in the cytoplasm is either the result of infection or disruption to the mitochondria or nucleus, several PRRs respond to cytosolic DNA. Indeed, since 2007, many cytosolic DNA receptors have been discovered that signal through stimulator of interferon genes (STING) to induce type I IFN expression. These include DNA-dependent activator of IRFs (DAI, aka ZBP1), RNA polymerase III, cyclic GMP-AMP synthase (cGAS), absent in melanoma 2 (AIM2), and interferon-gamma inducible 16 (IFI16)[43]. Of these, cGAS is the best characterized and most notable in its ability to directly activate STING. Since cGAS was discovered in 2013, it has become clear that it is essential for responding to self and foreign DNA, which has prompted intensive research on its structure, regulation, function, and implications in human disease[44]. DNA directly binds to cGAS and induces conformational changes that cause cGAS to dimerize. This exposes the catalytic pocket, which executes a two-step enzymatic reaction to synthesize cyclic GMP-AMP (cGAMP) from ATP and GTP[43]. cGAMP associates with STING dimers at the ER membrane, causing STING to alter its conformation, oligomerize, and become mobile. In turn, this allows STING to recruit TBK1, which phosphorylates itself, and the transcription factor IRF3. Phosphorylated IRF3 translocates to the nucleus, where it activates type I IFN transcription[45].

cGAS is widely considered a cytoplasmic protein, but recent work has also reported cGAS localization in the nucleus and at the plasma membrane[46]. The subcellular

localization of cGAS seems to be cell-type specific and influences cGAS functions, which have now been shown to extend beyond immune signaling[46,47]. Even in the context of immune signaling, the localization of cGAS dictates where it can recognize DNA to mount IFN responses in various disease states. Different viruses replicate in different cellular compartments, so it is not entirely clear how the genomic material of some DNA viruses would interact with cGAS to activate it. Many DNA viruses, such as herpesviruses, replicate in the nucleus where their viral DNA is thought to be contained separately from the cytoplasm, thus presenting a dilemma for how this viral DNA associates with cGAS localized to the cytoplasm. Herpes simplex virus-1 (HSV-1) was the first DNA virus found to activate cGAS-STING signaling[48], and transfection of a 120-mer synthetic dsDNA designed to represent the HSV-1 genome (HSV120) binds and activates cGAS[49]. Moreover, proteasomal degradation of the HSV-1 capsid in macrophages leads to release of viral DNA into the cytoplasm to activate the DNA sensor IFI16 and STING[50]. Thus, this may also mediate recognition of viral DNA by cGAS for HSV-1 as well as other herpesviruses. It is also possible that viral DNA is released into the cytoplasm during nuclear replication or that cGAS localizes to the nucleus during herpesvirus infection to recognize viral DNA. However, another known mechanism that contributes to cGAS activation during infection with HSV-1 and several other viruses is stress-induced leakage of mitochondrial DNA into the cytoplasm[51]. Indeed, cGAS activation by mitochondrial DNA also mediates immune sensing during infection with RNA viruses such as dengue virus, influenza A virus, picornaviruses, and noroviruses[52–55]. It is yet to be reported exactly how some viruses activate cGAS-STING signaling. For example, KSHV activates this pathway during primary infection of endothelial cells and when reactivated from latent infection, but the nucleic acid detected by cGAS remains unclear[56]. Nevertheless, it is clear that the cGAS-STING pathway is essential for controlling many virus infections. This is underscored by the various ways in

which herpesviruses are known to impede signaling through this pathway (**Figure 1.2**)[48].

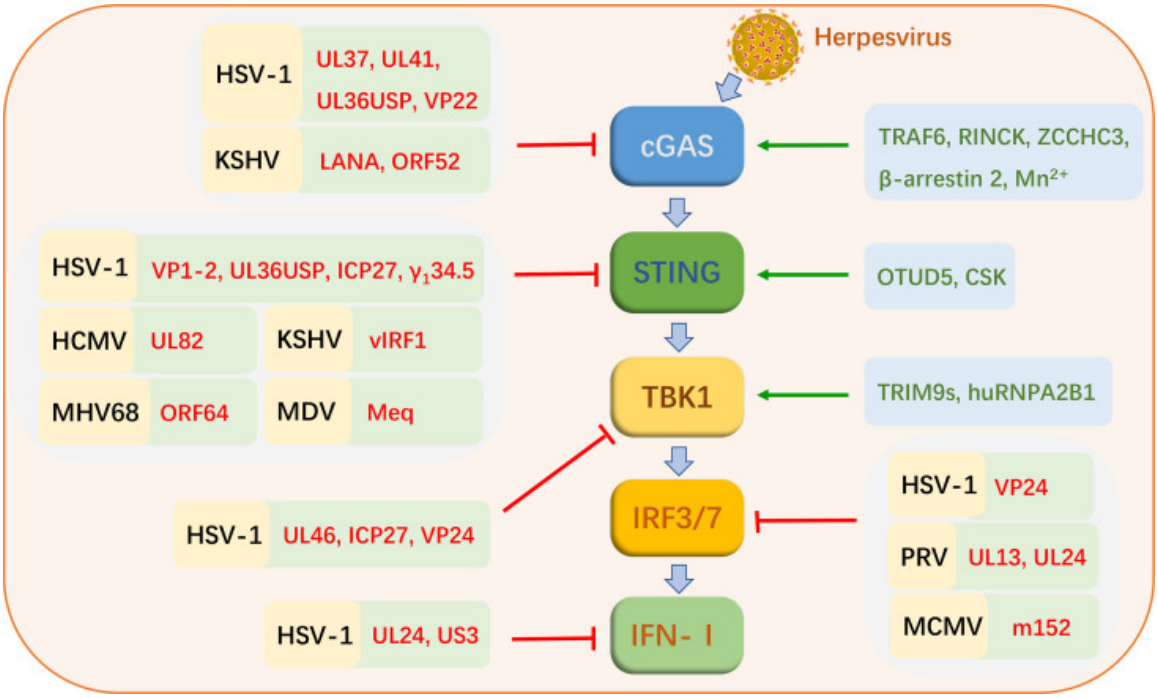

Figure 1.2: Strategies of cGAS-STING signaling pathway activation and virus evasion during herpesvirus infections.
Black letters represent herpesviruses. Red letters indicate the proteins of these viruses. Green letters indicate cellular proteins that favor cGAS-STING signaling pathway activation. Reprinted with permission from Deng L, Xu Z, Li F, Zhao J, Jian Z, Deng H, Lai S, Sun X, Geng Y and Zhu L (2022) Insights on the cGAS-STING Signaling Pathway During Herpesvirus Infections. Front. Immunol. 13:931885.

While proper activation of cGAS-STING signaling is key in many infectious and non-infectious contexts, it also must be tightly regulated to avoid the detrimental impacts of its over-activation in IFN-associated inflammatory diseases. Therefore, intensive research is underway to develop both activators and inhibitors of cGAS and STING to treat various diseases, including cancer and autoimmune diseases[45].

1.3. Herpesviruses

1.3.1. Overview and disease relevance

Herpesviruses are incredibly ubiquitous and successful pathogens that have co-evolved with their hosts for over 100 million years[57]. Thus, these large DNA viruses are

highly adapted to co-exist with and establish lifelong infections within their hosts. This is well depicted by the commonly known herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), as HSV-1 is thought to have infected human ancestors long before HSV-2 and thus is better adapted to persist in humans and more prevalent[58]. Herpesviruses likely infect every vertebrate species, but the herpesviruses that infect mammals, birds, and reptiles are classified as the *Herpesviridae* family[59]. This family is further divided into three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*, which differ in their host range, cellular tropism, and certain replicative mechanisms[58]. Nine herpesviruses within these subfamilies routinely infect humans. Human alphaherpesviruses, which have the broadest host range and a short replicative cycle, include herpes simplex virus-1 and -2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV)[60]. Human betaherpesviruses, which have a more restricted host range and a long replicative cycle, include human cytomegalovirus (CMV), and human herpesviruses 6A, 6B and 7 (HHV-6A, HHV6B, and HHV-7). Lastly, human gammaherpesviruses have the most restricted host range and include Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV, aka HHV-8). Common to all herpesviruses is their distinctive virion structure that includes an icosahedral capsid densely packed with genomic dsDNA, a "tegument" layer that contains key viral proteins for viral entry and egress, and an outer envelope containing glycoproteins[61]. As viral glycoproteins mediate cellular entry of enveloped viruses, they greatly influence the differences in host range and cellular tropism among herpesviruses.

Virtually all humans become infected with at least one herpesvirus in their lifetime[58]. The different human herpesviruses cause a range of disease phenotypes, which is largely dictated by the different body cell types and tissues that they establish infection in. Generally, herpesvirus infections do not result in severe morbidity and mortality in immunocompetent individuals[58]. One notable exception seems to be CMV infection of

certain organs, such as the gastrointestinal tract and central nervous system, causing life-threatening manifestations in apparently healthy individuals[62]. Nevertheless, most severe herpesvirus infections occur in patients with weakened immune systems like HIV and transplant patients, very young or aged individuals, and in cases where the virus infiltrates immune-privileged anatomical sites[58]. Viruses that have long co-evolved with hosts tend to establish relatively harmonious long-term infections, as viruses rely on intact and healthy hosts to propagate and persist within them. Thus, herpesvirus-associates symptoms in healthy individuals are often minor and help to facilitate viral spread, such as oral and genital sores (HSV-1 and HSV-2) and chickenpox blisters (VZV)[63]. The most significant factor contributing to the success of herpesviruses is their ability to persist in a dormant state of latency with periodic entry into their replicative lytic life cycle stage.

1.3.2. Latent and lytic stages of infection

All herpesviruses have both latent and lytic stages of infection that are essential for the virus to thrive in a host. Upon initial infection, herpesviruses typically undergo a short phase of productive lytic replication before establishing latency for long-term persistence[64]. During latent infection, the herpesvirus genome is carried within host cells in a dormant state without the active production of virions[63]. This involves circularization of the viral genome into an episome that remains tethered to the host cell chromatin and is replicated by cellular machinery to be maintained in dividing cells[63]. A small fraction of viral genes are expressed during latency to promote viral genome maintenance, cell survival, and immune evasion[63]. Various conditions can induce the virus to enter the lytic stage of infection from latency, a process termed lytic reactivation. Replication during lytic infection produces new virions to facilitate viral spread and reseeding of the latent

reservoir[65]. Lytic genes are expressed in three highly regulated temporal stages: immediate early, early, and late[58]. Early gene expression and consequent viral DNA replication is required for late gene expression, which produces structural virion components and facilitates viral egress[66]. Herpesvirus DNA replication and nucleocapsid formation occurs in the nucleus before nuclear budding and subsequent viral egress steps take place[67].

1.3.3. Gammaherpesviruses

A major characteristic of the human gammaherpesviruses EBV and KSHV is their ability to cause cancer, particularly in immunocompromised hosts. EBV was the first discovered gammaherpesvirus in 1964 and is associated with several malignancies including Burkitt's lymphoma, Hodgkin's disease, nasal T/natural killer non-Hodgkin's lymphomas, nasopharyngeal carcinoma, gastric carcinoma, leiomyosarcoma, and possibly breast cancer[68]. Additionally, up to half of adolescent's primary EBV infection in adolescents can cause an acute, non-malignant condition called infectious mononucleosis[69]. KSHV was first discovered in 1994 in AIDS-associated Kaposi's sarcoma (KS)[70]. Of all the oncogenic viruses, KSHV currently has the strongest connection to its associated tumors, KS and primary effusion lymphoma (PEL)[71]. Indeed, KSHV was detected in all cases of AIDS-associated as well as HIV-negative KS[68]. Gammaherpesviruses are lymphotropic and have the most restricted host range of all the *Herpesviridae* family[60]. This complicates *in vivo* studies of these viruses and necessitates the use of the murine gammaherpesvirus, murine herpesvirus 68 (MHV68), to model EBV and KSHV infection *in vivo*[72]. While notable discrepancies exist between MHV68 and the human herpesviruses, MHV68 is also lymphotropic and its infection displays similar disease phenotypes to EBV and KSHV[73].

1.3.4. KSHV

During the AIDS epidemic, the epidemiological patterns of high KS incidence in men who have sex with men (MSM) hinted that an infectious agent underlay the etiology of KS[74]. The discovery of KSHV in a KS lesion within an AIDS patient prompted intensive research into the tumorigenesis of KSHV and other viruses that were similarly associated with major AIDS-defining cancers[75]. Indeed, it was soon after recognized that most HIV-associated cancers are caused by oncogenic viruses including KSHV, (EBV), high-risk human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), and Merkel-cell polyomavirus[75]. The advent of antiretroviral therapy (ART) regimens markedly improved the prognosis of AIDS patients with access to this care and greatly reduced the incidence of the associated cancers[75]. However, the incidence of HIV is highest in low and middle income countries with poor access to care where there remains a large burden of these virus-associated cancers, particularly KS and cervical cancer[75]. Four major epidemiological forms of KS have long been recognized. The form of KS that was the first reported case in 1872 by Moritz Kaposi is categorized as classic KS or sporadic KS[76,77]. Classic KS is rarely aggressive and mainly occurs in HIV-negative elderly men of Mediterranean or Ashkenazi Jewish ancestry[77] . Endemic KS is a lymphadenopathic and typically aggressive form of KS that has been reported in Africa, predominantly in children, since 1947[77,78]. KS cases were generally rare before the AIDS epidemic of the 1980s, which drastically increased the incidence of KS. This form of KS was categorized as the AIDS-related or epidemic form of KS, which can be aggressive and occurred predominantly in MSM[77]. The fourth major KS form is iatrogenic KS, which often arises in immunosuppressed transplant patients, a population that is 200 times more likely to develop KS[77]. Iatrogenic KS typically presents as localized lesions, but it can also affect organs and its severity often correlates with the

degree of immunosuppression[77]. A recent, often non-aggressive, fifth form of KS has also begun to be recognized and occurs in HIV-negative MSM[79]. At present, the vast majority of KS cases occur in sub-Saharan Africa, often with poor prognosis. This region contains the highest seroprevalence of KSHV globally, which is >90% in some populations, largely due to childhood saliva transmission and the ongoing AIDS epidemic[77]. Conversely, KSHV seroprevalence is <10% in many developed regions including in the US, northern Europe and Asia, and is 20-30% in the Mediterranean[77].

While KS is the most prevalent KSHV-associated malignancy, other associated conditions include primary effusion lymphoma (PEL), a B-cell variant of Multicentric Castleman Disease (MCD), and the inflammatory KSHV-induced cytokine syndrome (KICS)[71]. The prognosis of these diseases is often very poor, as PEL and MCD patients typically only survive less than a year[71]. Like in KS, KSHV is found in all samples of PEL, which is an aggressive B-cell lymphoma found within body cavities[71]. KS and PEL are treated with conventional cancer treatments that have limited efficacy, and there are no available KSHV-targeted treatments. Thus, further research into KSHV biology and pathogenesis should aim to inform improved therapeutic avenues.

The latent and lytic stages of KSHV infection are both crucial for its oncogenesis. KS and PEL tumors are mainly comprised of latently infected cells in which few viral genes are expressed[71]. A key latent KSHV protein is the multifaceted latency-associated nuclear antigen (LANA). A crucial function of LANA is tethering the viral episomal genome to host cell chromosomes, although it is also clear that it regulates host gene expression during latency[71]. Primary KSHV infection is established long before clinical manifestations, typically at least 10 years before the onset of KS in AIDS patients[80–83]. During this period of disease progression, lytic reactivation of KSHV from latent infection is essential for tumorigenesis[84]. Indeed, the replicative lytic cycle not only reseeds the latent population, but it also produces tumor-promoting and angiogenic signals such as

human and viral IL-6 (vIL-6) as well as VEGF[71]. In early stage KS, lytically infected cells are clearly detected in tumors, and the viral load of KSHV has been correlated with early KS progression in HIV-positive individuals[85–87]. Moreover, a drug that impedes KSHV lytic reactivation also promotes KS tumor regression in renal transplant patients[88]. Thus, the lack of virus-targeted treatments for KSHV-associated malignancies may be addressed by elucidating factors that affect lytic replication.

1.4. KSHV interference with intracellular innate immune signaling

As KSHV has co-evolved with its human hosts for millions of years, it has developed an arsenal of immune evasion strategies that allow it to avoid detection and successfully persist in long-term infections. Infection or lytic reactivation of KSHV can activate a variety of PRRs including several TLRs (TLR3, TLR4, and TLR9), RLRs, Nod-like receptors (NLRs), IFI16 and cGAS[77]. Thus, a diverse array of KSHV-encoded proteins have been reported to counteract various steps of these pathways (**Fig 1.3)**[89]. The DNA-sensing cGAS-STING pathway, which is recognized as being critical for controlling KSHV infections, is the most targeted by these proteins[89]. Cytoplasmic isoforms of LANA directly interact with cGAS, to suppress IFN induction and promote lytic reactivation[90]. Another protein that directly interacts with cGAS is ORF52, or KSHV inhibitor of cGAS (kicGAS), which inhibits cGAS enzymatic activity and resultant cGAMP production[91]. KSHV also encodes several homologs of cellular proteins that alter cellular pathways. Several KSHV viral IRFs (vIRF1, vIRF2, vIRF3) bind to the cellular transcription factors IRF3 and IRF7 downstream of cGAS-STING signaling, and other pathogen sensing pathways, to impede transcriptional activation of type I IFNs[92–94]. In addition, vIRF1 directly interacts with STING to prevent the recruitment of TBK1 and subsequent IFN induction[95]. Signaling through the RNA sensors RIG-I and TLR3 is also blocked by

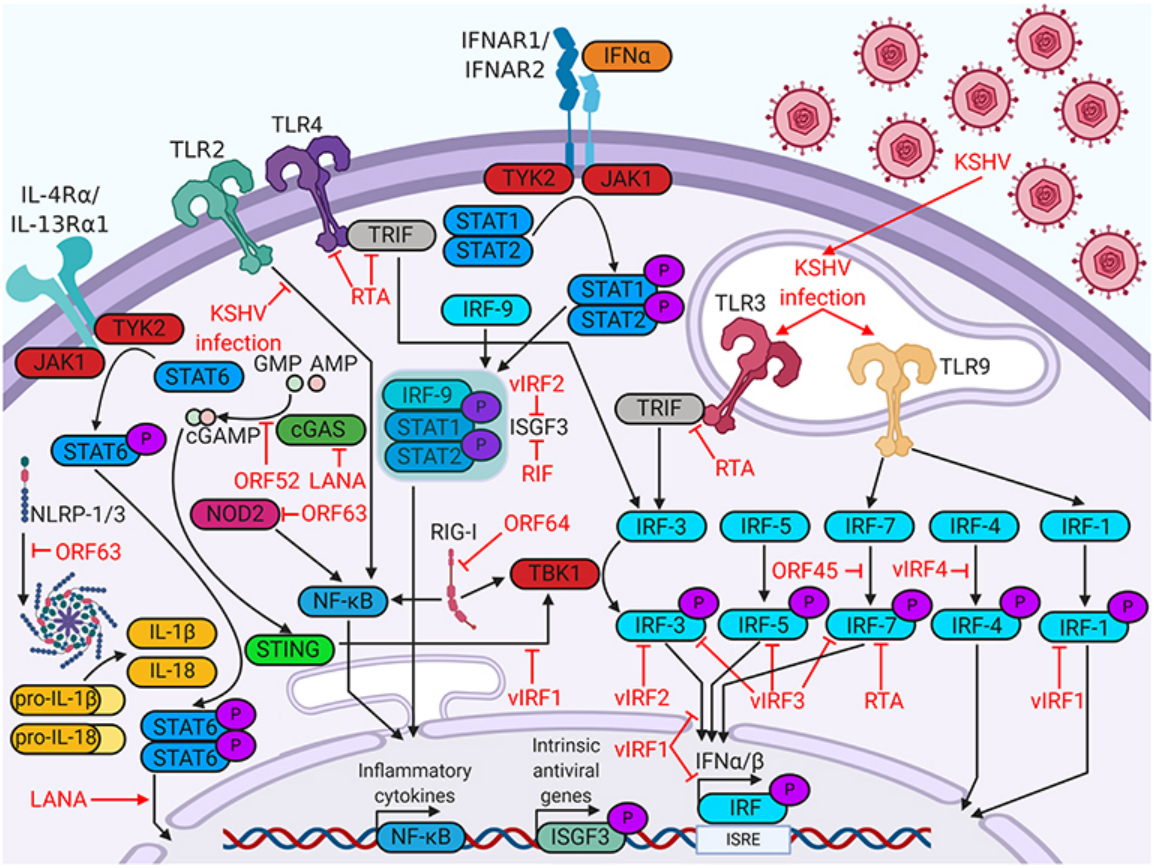

Figure 1.3: Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) proteins enable evasion of intrinsic immunity.
KSHV proteins antagonize viral sensing and interferon responses by targeting a wide variety of host immune factors. Reprinted with permission from Broussard G and Damania B (2020) KSHV: Immune Modulation and Immunotherapy. Front. Immunol. 10:3084.

KSHV proteins. KSHV ORF64 deubiquitinates RIG-I to impede its interaction with MAVS and thereby suppress IFN induction[96]. Also, over-expression of KSHV RTA in uninfected 293T cells promotes proteasomal degradation of the TLR3 adapter protein TRIF[97]. Clearly, many studies have described KSHV proteins that can impede type I IFN signaling. However, our lab previously reported a mechanism by which KSHV usurps the cellular protein caspase-8 to block IFN-β induction during lytic reactivation[98]. The work described in this thesis is aimed at further dissecting this non-viral-encoded immune evasion mechanism.

1.5. Caspases

Caspases are a highly conserved group of cysteine proteases that cleave their protein substrates at specific aspartic acid residues ("c" for cysteine, "asp" for aspartic acid)[99]. They all exist in a precursor form as inactive zymogens, called procaspases, before being cleaved to become active[99]. Once activated, each caspase has a unique repertoire of substrates that is dictated by the amino acid sequences preceding the aspartic acid cleavage site[99]. Two major groups of caspases are described based on their similar domain structures and functions: apoptotic caspases and inflammatory caspases (**Fig 1.4**)[100]. Exceptions include caspase-2 (human, functions in cell cycle),

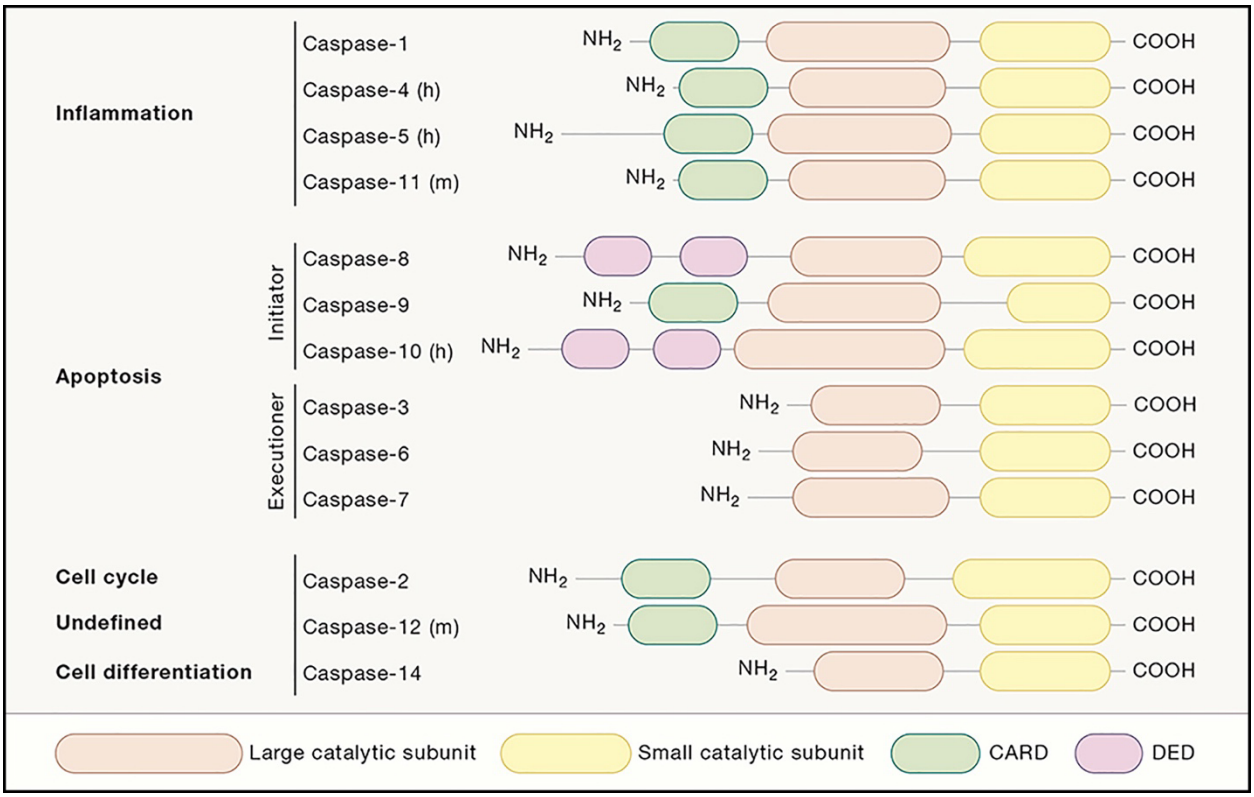

Figure 1.4: Functional Classification and Domain Architecture of Murine and Human Caspases.
The members of the caspase-family are classified as inflammatory or apoptotic based on the described functions and domain architecture. The caspases-1, -4, -5, and -11 are grouped as inflammatory caspases and share a CARD-domain at the N-terminal end. The apoptotic caspases can be further sub-categorized in initiator- and executioner caspases. The caspases-8, -9, and -10 have domain architecture akin to the

inflammatory caspases, however, their function is to initiate apoptosis through the activation of the executioner caspases-3, -6, and -7. In addition, caspase-2 shares domain structures with the inflammatory caspases; however, its function is described to be cell-cycle related. Structurally, murine caspase-12 clusters with the inflammatory caspases; however, until today its function remained undefined. Alternatively, for human caspase-12, it is generally accepted that 75% of the population is knockout, while the remaining 25% carry a proteolytically inactive pseudoprotease (h∗). The function of caspase-14 is linked to cell differentiation. m, murine and h, human. Reprinted with permission from Opdenbosch NV and Lamkanfi M (2019) Caspases in Cell Death, Inflammation, and Disease. Immunity. 50.

caspase-14 (human, functions in cell differentiation), and caspase-12 (typically not found in humans, undefined function in mice)[100]. The inflammatory caspases contain a caspase recruitment domain (CARD) that facilitates association with inflammasome complexes, which form downstream of pathogen sensing pathways and mediate inflammatory caspase cleavage and activation[101–104]. Once activated, inflammatory caspases drive maturation and secretion of inflammatory cytokines and promote the inflammatory form of cell death, pyroptosis[100]. However, caspases are most classically known as critical mediators of apoptosis, the most extensively studied form of cell death that is highly regulated and non-inflammatory[100,101].

1.5.1. Canonical apoptotic functions and activation of caspases

The apoptotic caspases are further subdivided into initiator (caspase-8, -9, and -10) and executioner or effector caspases (caspase-3, -6, and -7) (**Fig 1.4**)[100]. When apoptotic initiator caspases interact with adaptor proteins via homotypic interactions through their pro-domains, this allows them to dimerize and cleave themselves to become active[105,106]. Once activated, the initiator caspases cleave a range of substrates, including the effector caspases to activate them and prompt their cleavage of downstream apoptotic substrates[105,106]. Two different apoptotic pathways can trigger this cascade of caspase cleavage events: the extrinsic and intrinsic apoptosis pathways. The most common apoptosis mechanism in vertebrates is the intrinsic apoptosis pathway,

which becomes triggered by various states of cellular stress including DNA damage, ER stress, and growth factor deprivation[107]. These stressors induce mitochondrial outer membrane permeabilization (MOMP), which releases pro-apoptotic factors such as cytochrome c into the cytoplasm[107]. Cytochrome c is then able to bind and induce oligomerization of APAF1 to form the caspase-9-activating platform known as the apoptosome[107]. Once caspase-9 is recruited to this complex via its CARD-containing prodomain, it dimerizes and cleaves itself into its active form that can cleave the downstream apoptotic effector caspases[100,108].

Extrinsic apoptosis is initiated by death receptors, such as TNFR1, Fas, and TRAIL-R1/2, that become activated by binding to their extracellular cognate death ligands[107]. This ligation results in death receptor clustering and subsequent recruitment of the adaptor protein FADD via homotypic interactions between their death domains (DDs)[107]. The apoptotic initiators caspase-8 and caspase-10 contain a death effector domain (DED) that facilitates their recruitment to FADD, thus forming the death-inducing signaling complex (DISC)[109]. Association with this complex allows dimerization and autoproteolytic cleavage of caspase-8 to its active forms that can cleave its specific substrates, including the apoptotic effector caspases[109]. Caspase-8 activation occurs in two steps. The first cleavage step separates the large and small subunits to produce the DISC-bound p43/p41 fragment (the two numbers represent two isoforms of caspase-8 that differ by 15 amino acids) and p12, which gets further processed to p10[110,111]. The second cleavage step removes the prodomain (p26/p24) from the p43/41 fragment to yield the fully processed p18 fragment. The p10 and p18 fragments heterotetramerize to form the fully active caspase-8 enzyme, which is released from the DISC and can cleave a wide range of substrates, many of which facilitate apoptotic cell death[112]. The p43/p41 caspase-8 fragment is also active, but it is thought to have a more restricted substrate repertoire and does not drive apoptosis[113]. These caspase-8 cleavage steps are

regulated by the inactive caspase-8 homolog, cFLIP[114]. Two major cFLIP isoforms exist that form heterodimers with caspase-8 within the DISC to control the extent of its processing and activation. The short isoform, $cFLIP_S$, is a truncated form of caspase-8 containing only the tandem DEDs, and it binds to the DEDs of caspase-8 to completely block its processing and activation[115]. The long isoform, $cFLIP_L$, has a very similar structure to caspase-8 but it lacks the active site and proteolytic activity[115]. The role of $cFLIP_L$ in regulating caspase-8 activation is more complex as it is reported to both promote and inhibit apoptotic caspase-8 activation in different contexts. When caspase-8 heterodimerizes with $cFLIP_L$, its processing is limited to the partially active p43/41 form that remains DISC-associated[116]. However, this partially active heterodimer can still cleave adjacent heterodimers, as well as caspase-8 homodimers, to amplify caspase-8 activation[117]. Thus, a recent study revealed that the stoichiometric levels of caspase-8 and $cFLIP_L$ dictate the effect that $cFLIP_L$ will have on the kinetics and extent of caspase-8 processing and apoptosis[118]. In the absence of $cFLIP_L$, the extent of apoptosis is greatest since all DISC-associated caspase-8 will form homodimers that yield the fully processed, apoptotic p18 form[118]. Sub-stoichiometric levels of $cFLIP_L$ accelerate the rate of caspase-8 activation and apoptosis because the $cFLIP_L$-caspase-8 heterodimer is very efficient at promoting cleavage of the surrounding caspase-8 homodimers[118]. However, the ultimate levels of apoptosis are lower in this scenario since the heterodimers themselves will not yield apoptotic forms of caspase-8. When $cFLIP_L$ exists in a 1:1 ratio with caspase-8, it greatly inhibits apoptosis since this causes the levels of caspase-8 homodimers to be low[118]. Thus, cellular levels of $cFLIP_L$ and $cFLIP_S$ are key determinants of the extent of caspase-8 activation and overall cell fate. Indeed, the $cFLIP_L$-caspase-8 heterodimer also cleaves RIPK3 to prevent necroptosis, a programmed inflammatory form of cell death[119]. Thus, caspase-8 also has critical roles in

cell survival and its deletion in mice is embryonically lethal, except when RIPK3 is concomitantly ablated[119].

1.5.2. Caspases as emerging type I IFN regulators

Caspases are clearly well-defined for their key roles in controlling cell survival. However, they have also more recently emerged as important regulators of innate immune pathways, particularly type I IFN signaling. This was first discovered in a 2009 study that found negative regulation of IRF3 activation and murine skin inflammation by caspase-8[120]. In 2014, two different studies demonstrated that the apoptotic effector proteins caspase-3 and caspase-7 render caspase-9-mediated intrinsic apoptosis immunologically silent by preventing IFN induction[121,122]. In these studies, depletion of these apoptotic caspases caused the mitochondrial DNA that is released into the cytoplasm during intrinsic apoptosis to trigger IFN expression by activating the DNA-sensing cGAS-STING pathway[121,122]. The authors did not determine how the apoptotic caspases were silencing cGAS-STING-mediated IFN induction. However, more recent studies found evidence of direct caspase-mediated cleavage and inhibition of IFN induction through the cGAS-STING pathway. The inflammatory caspase-1 was found by the Jiang laboratory to cleave cGAS to impede IFN production during DNA virus infection[123]. A couple of years later, the Jiang laboratory also revealed that the apoptotic effector caspases-3 and -7 cleave cGAS, MAVS, and IRF3 to suppress IFN production during both DNA and RNA virus infection[124]. Moreover, our lab discovered in 2018 that caspase-8 blocks type I IFN induction during KSHV lytic infection[98]. However, it remained unclear how caspase-8 suppressed IFN signaling and how caspase-8 became activated during KSHV infection.

1.5.3. Non-canonical activation pathways of caspase-8

As described above, caspase-8 is canonically activated through the extrinsic apoptosis pathway, and most studies report a requirement for FADD in caspase-8 activation. However several studies have demonstrated activation of caspase-8 through TRIF-dependent TLR signaling, in both FADD-dependent and -independent manners. LPS treatment has been shown in several studies to activate caspase-8 through a TLR4- and TRIF-dependent mechanism[125–127]. Similarly, activation of TLR3-TRIF signaling via treatment with the synthetic dsRNA analog poly(I:C) has been shown to activate caspase-8 both directly and indirectly via upregulation of death ligands to trigger extrinsic apoptosis signaling[128–135]. The first evidence of direct caspase-8 activation and apoptosis by TLR3 and TRIF was found in 2006 via poly(I:C) treatment of human breast cancer cells[130]. Subsequent studies then unraveled the molecular basis for this TLR3-driven caspase-8 activation. One study in 2011 revealed that the "Ripoptosome" complex, which contains RIPK1, FADD, caspase-8, caspase-10, and cFLIP, associates with TRIF after poly(I:C) stimulation, and its formation is negatively regulated by cIAPs[129]. A year later, Estornes et al. demonstrated caspase-8 activation through a similar complex that is directly associated with TLR3 following poly(I:C) stimulation[128]. While this complex contained all the components of the Ripoptosome, its formation and subsequent activation of caspase-8 was FADD-independent, and it was also negatively regulated by cIAPs[128]. While all reports of TLR3-TRIF-dependent caspase-8 activation occur in the context of poly(I:C)-mediated TLR3 activation, these findings present the possibility that caspase-8 may be activated in physiological settings, such as viral infections, where TLR3 becomes activated.

1.6. Summary of main outstanding questions addressed in thesis

Herpesviruses are remarkably successful pathogens that have long co-evolved with their hosts to develop an extensive arsenal of immune-evasion strategies. This circumvention of immune responses is crucial for KSHV lytic replication and thus promotion of tumor development. While many KSHV-encoded proteins have been described for their roles in innate immune subversion, our lab was the first to discover that KSHV usurps the cellular protein caspase-8 to block type I IFN induction during lytic infection. Since this is a novel function of caspase-8 that is not accompanied by canonical caspase-8 mediated cell death, much remained unknown about the mechanistic details. To paint a more comprehensive picture of this viral immune evasion strategy, my thesis work has focused on deciphering the nature of caspase-8 activation during KSHV lytic infection as well as how it impeded type I IFN signaling.

Chapter 2. Caspase-8 is non-canonically activated through the TLR3-TRIF pathway during KSHV lytic infection

Lent RC, Tabtieng T, Lavin RC, Gaglia MM. To be submitted to *PLOS Pathogens*.

2.1. Introduction

Host innate immune responses are paramount for controlling viral infections. Many complex and intricately regulated systems have evolved to accomplish this, including the type I IFN response. Type I IFN cytokines potently counteract viral infections by inducing expression of directly antiviral interferon-stimulated genes (ISGs), as well as by triggering an inflammatory response via activation and recruitment of specialized immune cells to the site of infection[136]. When a virus infects a cell, its molecular moieties are detected by and trigger cellular pattern recognition receptors (PRRs) to activate the type I IFN response[137]. However, the potent pleiotropic immune effects of type I IFNs can be detrimental to the host when excessively activated. This is exemplified in many disease states that involve IFN dysregulation, including several autoimmune diseases, solid tumors, myocardial infarction, and chronic infections[138]. Therefore, sophisticated multilayered mechanisms of host regulation are in place to avoid this IFN-associated immunopathology. These regulatory pathways are sometimes hijacked by viruses as they are constantly evolving mechanisms to circumvent antiviral immune responses orchestrated by type I IFNs to evade destruction.

The oncogenic, AIDS-associated gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) relies heavily on immune suppression to successfully establish infection and replicate in a host. When lytically reactivating from latent infection, KSHV successfully impedes type I IFN signaling, resulting in little to no IFN induction in lytically infected cells. KSHV uses multiple mechanisms to achieve this almost complete block of IFN induction. First, KSHV deploys a number of viral proteins that impede IFN signaling. In addition, we recently established that KSHV usurps the activity of cellular caspase-8 to inhibit IFN induction[139]. In particular, caspase-8 blocks sensing of cytoplasmic DNA through the PRR cGAS and subsequent activation of the downstream effectors of this pathway, including the adaptor STING and the transcription factor IRF3[140].

Canonically, caspase-8 activation occurs through the extrinsic apoptosis pathway and results in cell death[109]. However, we have found that caspase-8 becomes activated during KSHV lytic infection without leading to cell death[139]. At present, we do not know the mechanism of this caspase-8 activation, especially as several KSHV proteins are thought to prevent apoptotic signaling[141–146]. In the extrinsic apoptosis pathway, ligation of death receptors recruits the adaptor protein FADD, which in turn promotes recruitment and activation of caspase-8 via autoproteolytic cleavage[109]. FADD is required for caspase-8 activation under most tested conditions. One known FADD-independent route of caspase-8 activation occurs through the PRRs Toll-like receptor (TLR) 3 and 4 and the adaptor TRIF. Activation of TLR3 and TLR4 via dsRNA and LPS, respectively, is more commonly linked to induction of type I IFN and inflammatory cytokines. LPS treatment has been shown to activate caspase-8 in a TLR4- and TRIF- dependent manner, which leads to inflammatory cytokine maturation and inhibition of necroptosis, an inflammatory form of cell death[125–127]. All reports of TLR3-driven caspase-8 activation have used artificial TLR3 stimulation with the dsRNA analog poly(I:C), which leads to apoptosis. Thus, it is unclear how caspase-8 becomes activated during KSHV lytic infection without resulting in cell death.

To better understand the pro-viral, immune-regulatory mechanism of caspase-8 activation that occurs during KSHV lytic infection, we used RNA interference (RNAi)- and CRISPR/Cas9-mediated depletion to determine which signaling components are essential for caspase-8 activation during KSHV lytic reactivation. This revealed that caspase-8 activation during KSHV lytic infection is independent of canonical FADD-dependent signaling that occurs through death receptors. We instead observe that caspase-8 activation occurs through TLR3-TRIF during KSHV infection, as caspase-8 activation is prevented when we deplete TLR3 and TRIF. Moreover, lytic reactivation of KSHV from latent infection enhances TLR3-TRIF signaling to activate caspase-8, likely

through the upregulation of TLR3. This upregulation may be sufficient, without additional stimuli, to induce caspase-8 activation, as caspase-8 is also activated by exogenous TLR3 overexpression in the absence of stimulus. Together, these findings raise an interesting model whereby RNA-independent TLR3 activation leads to antagonistic crosstalk between two pathogen sensing pathways during KSHV lytic infection.

2.2. Results

2.2.1. Caspase-8 activation during KSHV lytic infection does not occur through conventional death receptor signaling

Canonical caspase-8 activation through the extrinsic apoptosis pathway entails engagement of death receptors by their cognate ligands to facilitate clustering[109]. Receptor clustering induces recruitment of FADD and caspase-8 to form the death inducing signaling complex (DISC), and caspase-8 activation by autoproteolytic cleavage. Since FADD is normally strictly required for caspase-8 activation in this pathway, we first sought to determine whether FADD is also required during KSHV lytic infection using RNAi- and CRISPR/Cas9-mediated FADD depletion. For these and all other experiments in this paper, we used iSLK.219 and BC3 cells. Both of these cell lines are latently infected with KSHV. iSLK.219 cells also exogenously express a doxycycline-inducible copy of the KSHV master lytic regulator replication and transcription activator (RTA), allowing for induction of the KSHV lytic cycle from its latent state by doxycycline treatment[147]. BC3 cells were obtained from a primary effusion lymphoma (PEL) patient, and B-cells are the major latent reservoir for KSHV. KSHV can be lytically reactivated in BC3 cells via treatment with the chemical inducer 12-O-Tetradecanoylphorbol-13-acetate (TPA), which stimulates signal transduction cascades that activate the RTA gene[148]. Despite its almost ubiquitous requirement for caspase-8 activation in other systems, RNAi-mediated depletion in iSLK.219 cells and

CRISPR/Cas9 gene editing in BC3 cells revealed that FADD is dispensable for caspase-8 activation during KSHV lytic infection (**Fig 2.1A-B**). We measured caspase-8 by

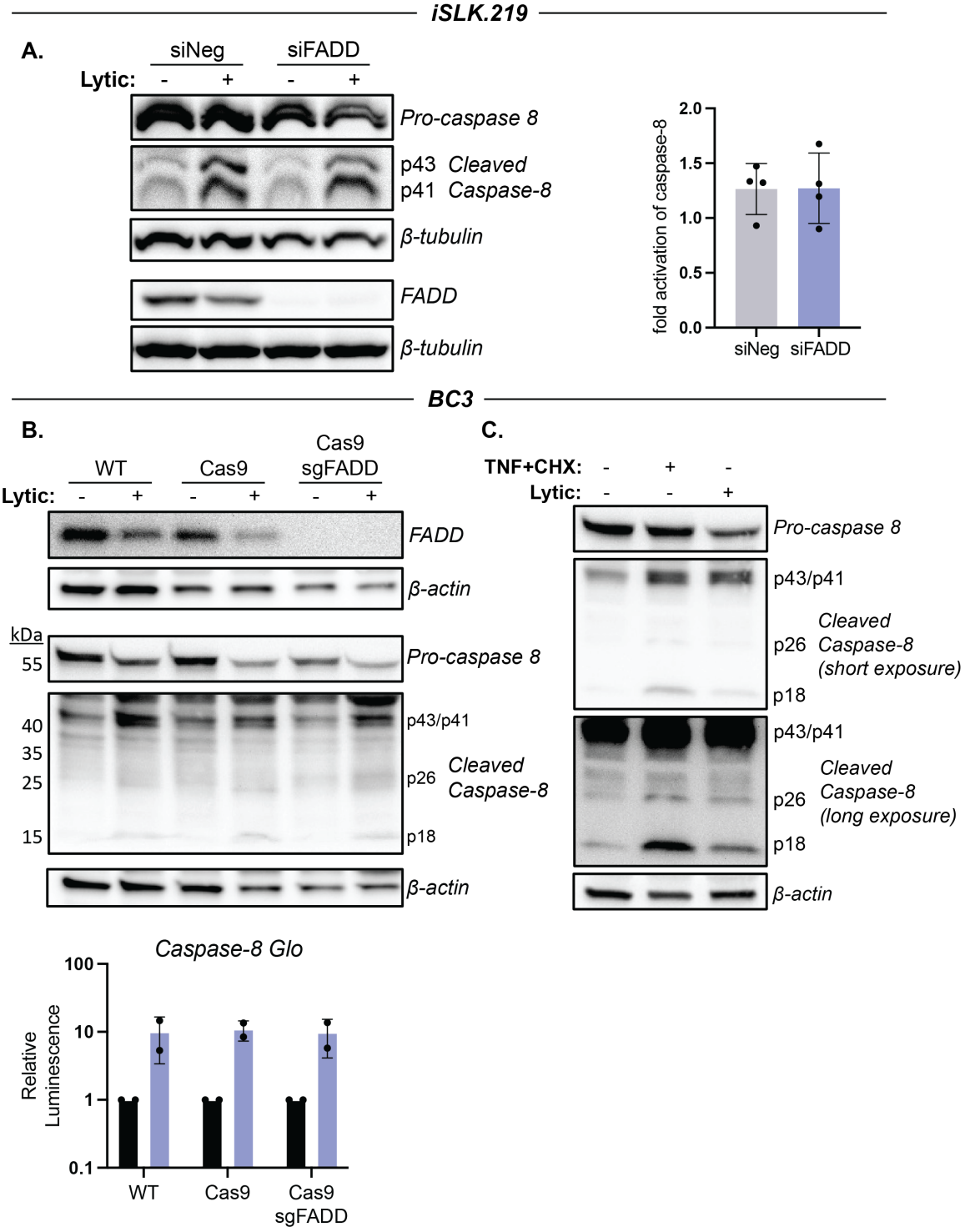

Figure 2.1: Caspase-8 activation during KSHV lytic infection does not occur through conventional death receptor signaling.

(A) iSLK.219 cells were treated with a negative control siRNA (siNeg) or an siRNA that targets FADD. Samples were collected at day 4 post lytic reactivation to assess knockdown efficiency and caspase-8 activation by western blot. (B) WT BC3 cells, Cas9-expressing BC3 calls, and Cas9/sgFADD-expressing BC3 cells were lytically reactivated for two days and samples were collected to check KO efficiency. Caspase-8 activity was measured by the luminescence based Caspase-8 Glo assay. (C) BC3 cells were either lytically reactivated for 2 days or treated with TNF and CHX for 6h. Samples were collected to assess caspase-8 activation by western blot.

detecting its cleavage products via western blot (**Fig 2.1A**) and by the luminescence based assay Caspase-8 Glo, which measures caspase-8 enzymatic activity (**Fig 2.1B**). As FADD is the major adaptor protein for death-receptor signaling, this result suggests that caspase-8 activation during KSHV infection occurs independently of death receptor signaling.

In previous studies where we had reported caspase-8 activation, we predominantly detected ~40 kDa fragments of caspase-8. This is generally considered an intermediate processed form, as procaspase-8 associated with the DISC undergoes two interdomain cleavage steps. First the large and small subunits are separated to yield the DISC-associated p43/p41 fragment of caspase-8 (the two numbers are due to the existence of two isoforms of caspase-8 that differ by 15 amino acids) and p12, which gets further processed to p10[110,111]. Then the prodomain (p26/p24) is removed from the p43/41 fragment to yield the fully processed p18 fragment. The p10 and p18 fragments heterotetramerize to form the fully active caspase-8 enzyme, which can cleave a wide range of substrates, many of which facilitate signaling cascades that culminate in apoptotic cell death[112]. However, the p43/p41 caspase-8 fragment is also active, although it is thought to have a more limited substrate repertoire and not to drive apoptosis. We thus compared the generation of both the p43/p41 and p18 processed forms upon lytic reactivation and canonical death receptor activation using tumor necrosis factor and cycloheximide treatment. Interestingly, we predominantly observe formation of only the p43/p41 fragment upon lytic reactivation, although the cells are

clearly capable of producing the p18 fragment upon death receptor stimulation (**Fig 2.1C-D**). This is in line with our previous finding that caspase activity during KSHV lytic infection does not lead to cell death[139]. Taken together, these findings all suggest that caspase-8 is activated through a distinct mechanism from canonical death-receptor mediated signaling that result mainly in partial processing of the caspase.

2.2.2. TRIF is important for caspase-8 activation during KSHV lytic infection

A few reports have identified FADD-independent mechanisms of caspase-8 activation, the predominant one being a pathway dependent on the signaling adaptor TRIF. Thus, we examined the role of TRIF in caspase-8 activation during lytic reactivation via RNAi-mediated depletion. Knockdown of TRIF results in decreased caspase-8 activation upon lytic reactivation of KSHV, indicating that this caspase-8 activation occurs through a TRIF-dependent mechanism (**Fig 2.2A**). As TRIF is a major signaling adaptor for certain TLRs, this suggests that caspase-8 activation is occurring through TLR signaling. Another major TLR signaling adaptor protein is MyD88[149]. To confirm that caspase-8 activation is specific to TRIF-dependent signaling and doesn't occur through a MyD88-dependent pathway, we knocked down expression of MyD88. This experiment showed that MyD88 is not needed for caspase-8 activation during KSHV lytic infection (**Fig 2.2B**).

2.2.3. Caspase-8 is activated through the TLR3-TRIF pathway during KSHV lytic infection

TRIF triggers signaling cascades downstream of TLR3 and TLR4. In particular, the TLR3-TRIF pathway has been reported to mediate caspase-8 activation and apoptosis in the context of poly(I:C) treatment and the TLR4-TRIF pathway in response to bacterial

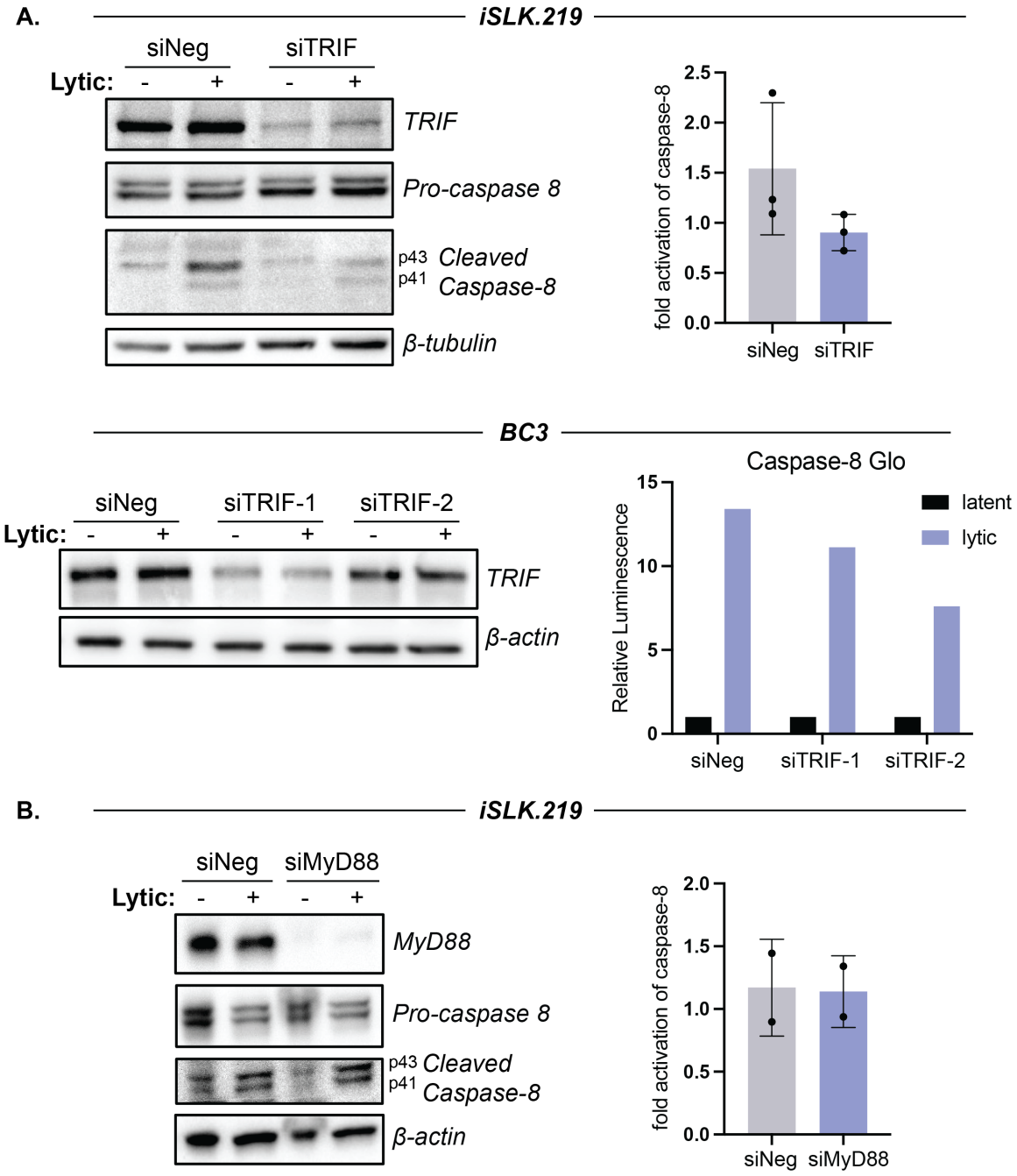

Figure 2.2: TRIF is important for caspase-8 activation during KSHV lytic infection. *(A and C) iSLK.219 cells were treated with negative control siRNAs (siNeg) or siRNAs that target TRIF and MyD88. Samples were collected at day 4 post lytic reactivation to assess knockdown efficiency and caspase-8 activation by western blot. (B) BC3 cells were treated with a negative control siRNA (siNeg) or siRNAs that target TRIF. Cells were lytically reactivated for 24h and protein samples were collected to check knockdown efficiency. Caspase-8 activity was measured by the luminescence based Caspase-8 Glo assay.*

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LPS. TLR3 is more ubiquitously expressed in a wide range of immune and non-immune cell types than many other TLRs, but its expression and activity varies amongst these cell types[150,151]. To test TLR3 activity in iSLK.219 and BC3 cells, we treated the cells with poly(I:C) and measured expression of IFN-β and IL-6 after 2-16 hours, a typical readout of TLR3 activity. TLR3 was active in both KSHV infected cell lines and could thus be involved in caspase-8 activation (**Fig 2.3A-B**). We also examined the expression and activity of TLR4 in these cells lines, because there is evidence that TLR4 may respond to KSHV through an unknown stimulus. However, we found that while TLR4 may be expressed in BC3 cells and iSLK.219 cells (**Fig 2.4A-B**), there was no IFN-β and IL-6 induction upon LPS treatment in BC3 cells (**Fig 2.4C**) and iSLK.219 cells (data not shown). This suggests that TLR4 is not active in these cells and thus likely not involved in caspase-8 activation (**Fig 2.4**). Given the expression and activity of TLR3 in iSLK.219 and BC3 cells, we assessed its role in caspase-8 activation during KSHV lytic infection via RNAi-mediated depletion. Like TRIF, TLR3 depletion reduced caspase-8 activation, indicating that caspase-8 activation during KSHV lytic infection occurs via the pathogen sensing TLR3-TRIF pathway (**Fig 2.3C-D**).

2.2.1. KSHV lytic reactivation alters the TLR3-TRIF pathway

Previous studies have shown that TLR3 is upregulated and activated during *de novo* infection of human monocytes and that KSHV-encoded proteins antagonize TLR3-mediated type I IFN induction[152,153]. Because upregulation of TLR3 is thought to potentiate its signaling in several contexts[154–162], we tested whether TLR3 was also upregulated during lytic reactivation. Our results showed that TLR3 mRNA and protein levels were higher upon KSHV lytic reactivation in both iSLK.219 and BC3 cells (**Fig 2.5A-B**). Moreover, we detected a 70 kDa processed form of TLR3 in both iSLK.219 and BC3 cells, and found that its abundance increased upon KSHV lytic reactivation in the

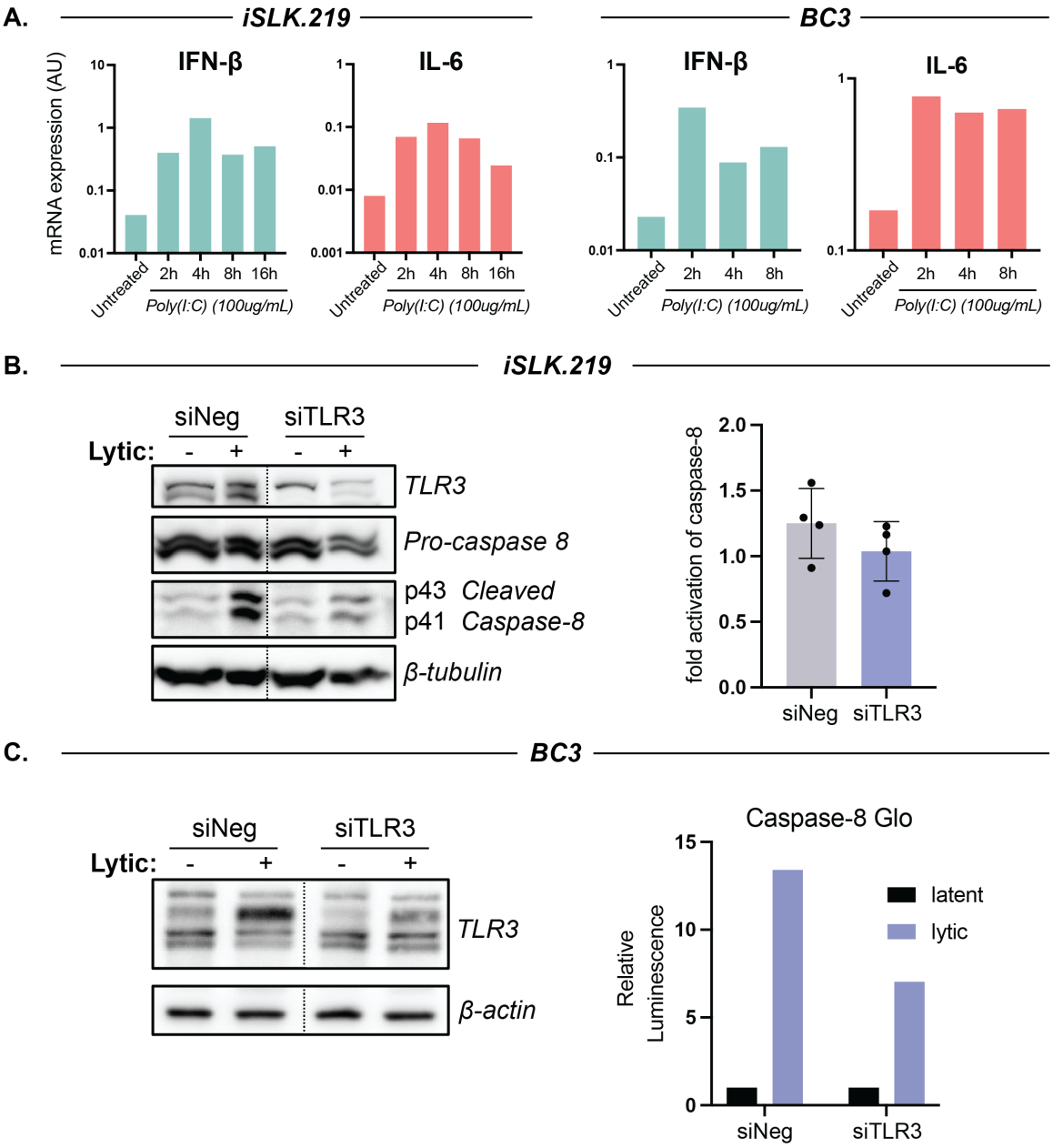

Figure 2.3: Caspase-8 is activated through the TLR3-TRIF pathway during KSHV lytic infection.

(A) iSLK.219 and BC3 cells were treated with 100 μg/mL poly(I:C) for the indicated time points. Total RNA was extracted and the levels of IFN-β and IL-6 mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA. (B and C) iSLK.219 and BC3 cells were treated with a negative control siRNA (siNeg) or an siRNA that targets TLR3. For iSLK.219 cells, two rounds of knockdown were performed. (A) Following second siRNA treatment, iSLK.219 cells were lytically reactivated for 4 days and protein samples were collected to assess knockdown efficiency and caspase-8 activation by western blot. (C) Two days after siRNA treatment, BC3 cells were lytically reactivated for 24h and protein samples were collected to assess knockdown efficiency. Caspase-8 activity was measured by the luminescence based Caspase-8 Glo assay.

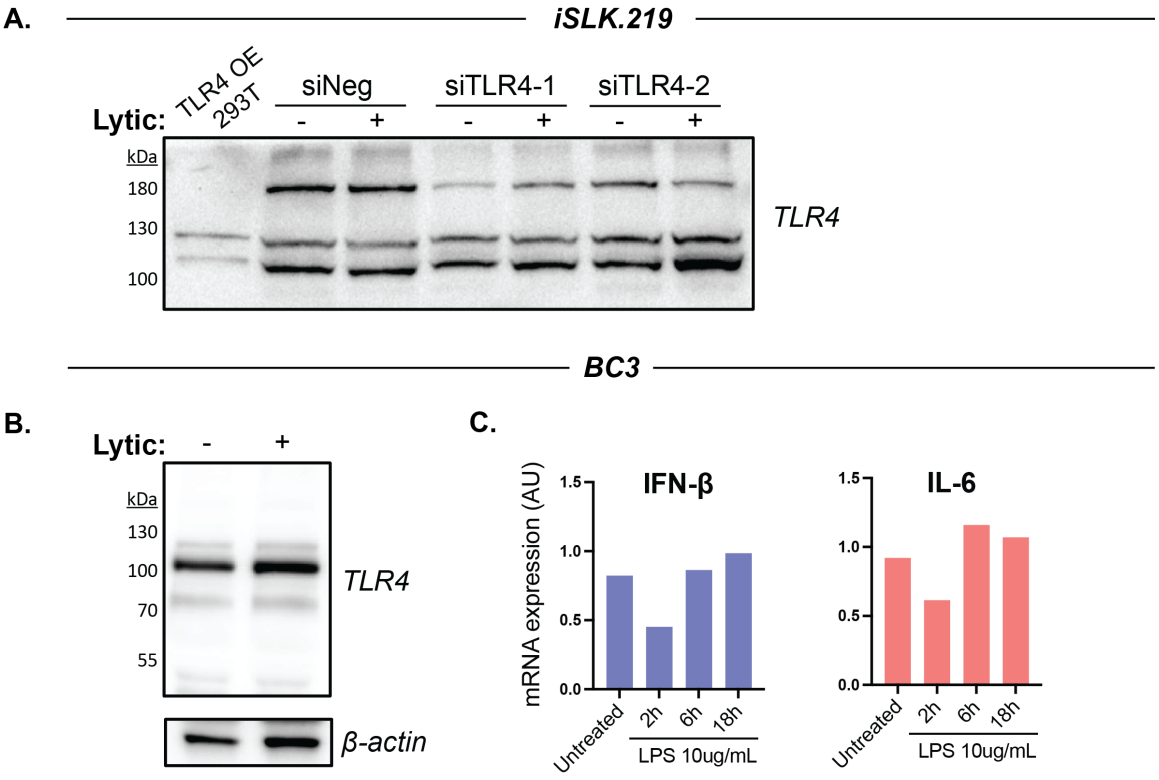

Figure 2.4: TLR4 may be expressed in KSHV-infected cells but it lacks clear activity.
(A) iSLK.219 cells were treated with a negative control siRNA (siNeg) or siRNAs that target TLR4. Samples were collected at day 4 post lytic reactivation to assess knockdown efficiency by western blot. As a positive control for TLR4 expression, lysates from HEK293T cells over-expressing TLR4 were analyzed on the same blot. (B) BC3 cells were lytically reactivated for 2 days and samples were collected to assess protein expression by western blot. (C). BC3 cells were treated with 10 μg/mL LPS for the indicated time points. Total RNA was extracted and the levels of IFN-β and IL-6 mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA.

iSLK.219 cells (**Fig 2.5A-B**). This form is produced by cathepsin cleavage upon maturation and acidification of endosomes, and this processing is required for TLR3 activity in some contexts[163,164]. Detection of the TLR3 processed form further points to TLR3 being active in these cells to mediate caspase-8 activation. It is unclear how TLR3 becomes upregulated during KSHV lytic infection. To test whether the dox-induced RTA expression in iSLK.219 cells is responsible for this TLR3 upregulation, the same treatment was performed in iSLK.RTA cells, which contain the doxycycline-inducible RTA transgene but are not infected with KSHV. TLR3 was not detected in these cells

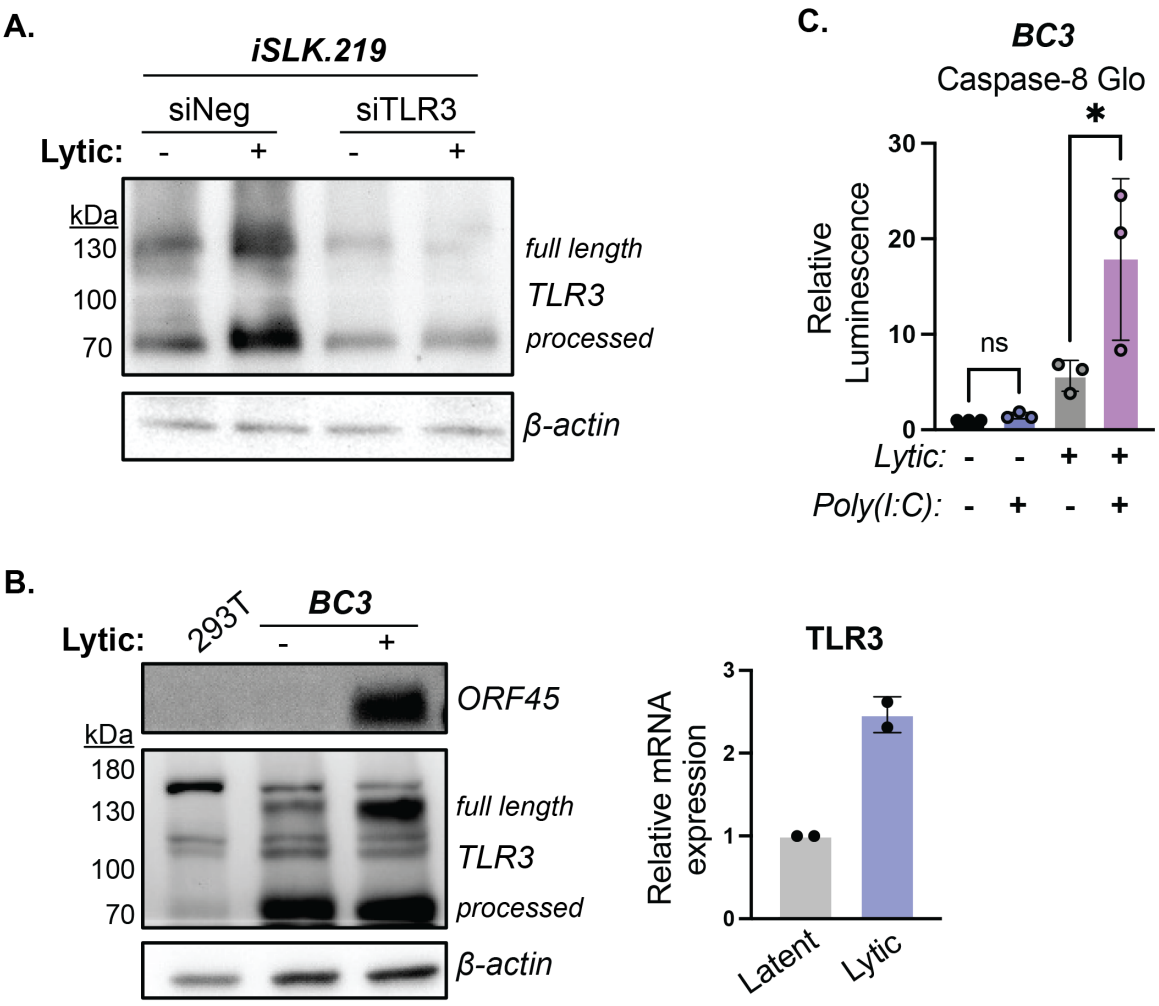

Figure 2.5: KSHV lytic reactivation alters the TLR3-TRIF pathway.
(A) iSLK.219 cells were treated with a negative control siRNA (siNeg) or an siRNA that targets TLR3. Samples were collected at day 4 post lytic reactivation to assess knockdown efficiency by western blot. (B) BC3 cells were lytically reactivated for 2 days and samples were collected to assess protein expression by western blot. As a negative control for TLR3 expression, lysates from HEK293T cells were analyzed on the same blot. The KSHV lytic protein ORF45 protein levels were measured as an indicator for lytic cycle induction. Total RNA was extracted and the levels of TLR3 mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA. (C) BC3 cells were lytically reactivated for 24h then treated with 100 μg/mL poly(I:C) for 6h. Caspase-8 activity was measured by the luminescence based Caspase-8 Glo assay.

both in the presence and absence of doxycycline treatment (**Fig 2.6**). Similarly, to test whether any effects of TPA treatment besides lytic cycle induction in BC3 cells causes TLR3 upregulation, this treatment was performed in BJAB cells, an uninfected Burkitt

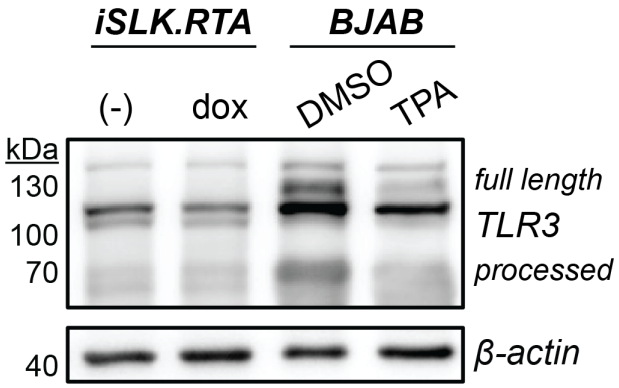

Figure 2.6: Doxycycline-induced RTA expression and TPA treatment do not upregulate TLR3.
iSLK.RTA cells were left untreated or were treated with doxycycline for 4 days. BJAB cells were treated with either DMSO (vehicle) or TPA for 48h. Samples were collected to assess TLR3 protein levels via western blot. The first two lanes (iSLK.RTA cells) are representative of two replicates. BJAB cells: n=1.

lymphoma B cell line. While TLR3 was detected in BJAB cells, TPA treatment seemed to actually downregulate its expression (**Fig 2.6**). Thus, other factors besides doxycycline-induced RTA expression and TPA treatment must be responsible for TLR3 upregulation. The upregulation and processing of TLR3 suggests that KSHV lytic reactivation may increase the responsiveness of TLR3 and potentiate caspase-8 activation through this signaling pathway. To examine this possibility, both latently infected and lytically infected BC3 cells were treated with poly(I:C) to stimulate TLR3 and caspase-8 activity was measured. Poly(I:C) treatment only induced significant caspase-8 activation in lytically infected cells but not latently infected cells (**Fig 2.5C**). Thus, cellular conditions that are induced upon KSHV lytic reactivation, likely the upregulation of TLR3, sensitizes cell to caspase-8 activation through TLR3-TRIF pathway.

2.2.2. Upregulation of TLR3 is sufficient to drive caspase-8 activation, as over-expression of TLR3 in latently infected cells induces caspase-8 activation in the absence of TLR3 stimuli

Significant alterations in both viral and cellular gene expression occur when KSHV is lytically reactivated from latent infection. Thus, while TLR3 upregulation during lytic infection is a likely culprit for sensitizing cells to caspase-8 activation, numerous factors can potentially influence this. As a reductionist approach to determine if TLR3 upregulation might yield this phenotype, we exogenously over-expressed TLR3 and measured levels of caspase-8 activation with and without poly(I:C) treatment in both iSLK.219 and BC3 cells. While TLR3 over-expression did sensitize cells to poly(I:C)-mediated caspase-8 activation, it was also surprisingly sufficient to induce caspase-8 activation even in the absence of an exogenous stimulus (**Fig 2.7A-B**). It is possible that TLR3 upregulation during KSHV lytic infection facilitates caspase-8 activation via sensitization to an unknown RNA species or other ligand. However, these data also raise the intriguing possibility that upregulation of TLR3 during KSHV lytic infection alone is sufficient to initiate signaling through the pathway and drive caspase-8 activation.

2.1. Discussion

Our experiments reveal a mechanism by which KSHV takes advantage of a pathogen sensing pathway, the TLR3-TRIF pathway, to activate caspase-8. In turn, caspase-8 activation blocks type I IFN induction through another pathogen sensing pathway, the cGAS-STING pathway. This demonstrates a novel mechanism of antagonistic crosstalk between these pathogen-sensing pathways. Instead of leading to cell death as activation of caspase-8 canonically does, KSHV lytic infection predominantly results in only partial activation of caspase-8 to its non-apoptotic form. This partial activation presumably prevents the antiviral effects of caspase-8-mediated

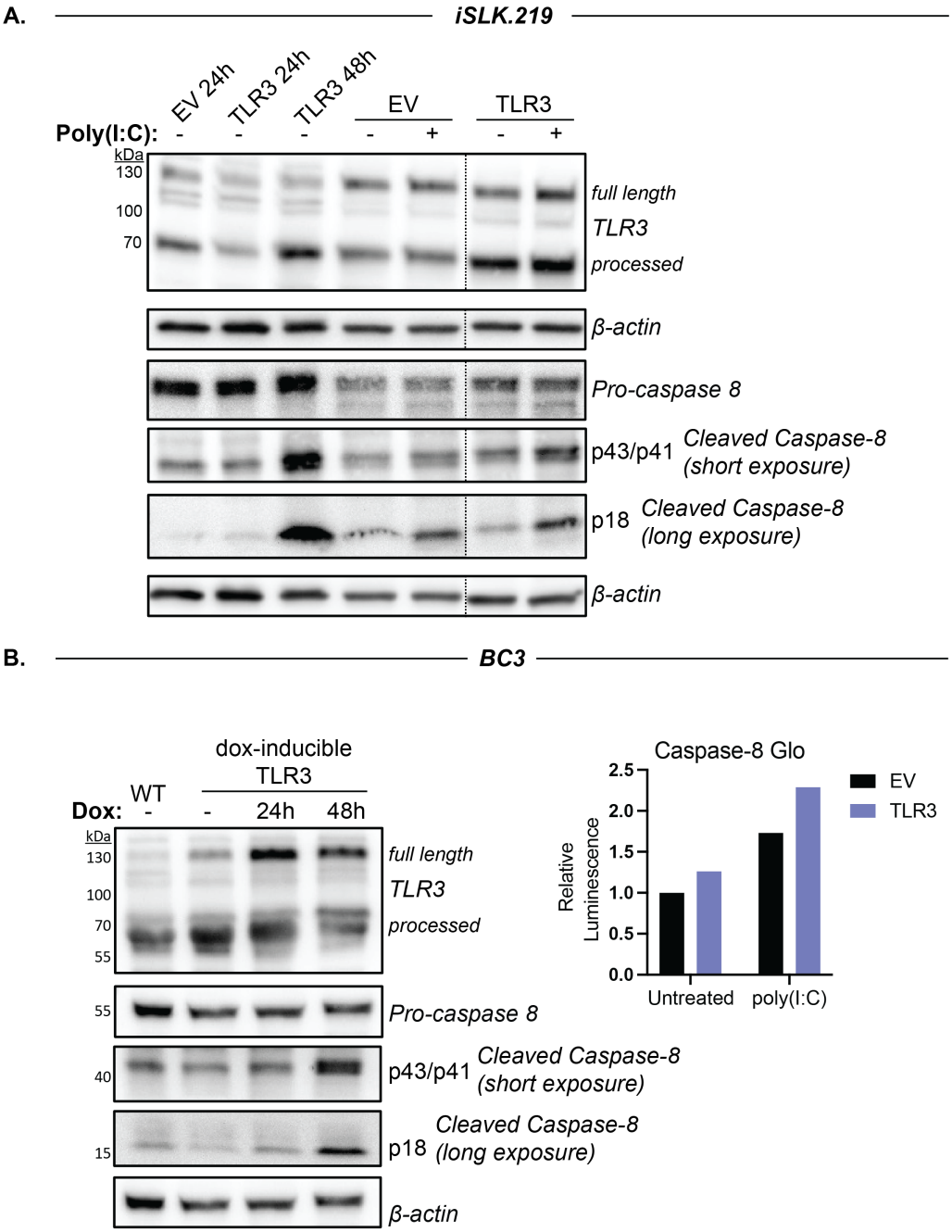

Figure 2.7: Upregulation of TLR3 is sufficient to drive caspase-8 activation. *(A) iSLK.219 cells were transfected with an empty vector or plasmid encoding TLR3. 24h later, indicated sampled were treated with 100 μg/mL poly(I:C) for 6h. Samples were collected at indicated time points to measure protein expression and caspase-8 activation by western blot. (B) BC3-pTRIPZ-flag-TLR3 cells were treated with doxycycline to induce TLR3 expression. Samples were collected at indicated time points to measure protein expression and caspase-8 activation by western blot. WT BC3 protein samples were also analyzed for comparison. 24h after doxyxcline treatment,*

indicated samples were treated with 100 μg/mL poly(I:C) for 6h and caspase-8 activity was measured by the luminescence based Caspase-8 Glo assay.

cell death while caspase-8 carries out its pro-viral IFN-regulatory function. Our findings also suggest that the activation of TLR3 during KSHV infection may occur solely from its upregulation without the requirement for a stimulus.

Due to the adverse effects of IFN-mediated immunopathology, many complex layers of regulation have evolved to prevent inappropriate and excessive type I IFN activation. For example, many components of IFN signaling pathways are ISGs themselves. This feed-forward system facilitates amplification of the type I IFN response by sensitizing these pathways to further signaling once they are initially activated[165]. This signal amplification poises cells to induce a potent antiviral state in only the necessary and appropriate contexts. Moreover, it can effectively prevent immunopathology. Indeed, early control of viral infections impedes the accumulation of excessive viral loads that overwhelm the immune system, leading to potentially fatal hyperinflammatory states, such as a cytokine storm.

Another aspect of regulation, as our study indicates, is cross-regulation of IFN-activating pathways. Examples of cross-regulation between different IFN-activating include STING- and MAVS-dependent induction of the suppressor of cytokine signaling 1 (SOCS1), which interacts with MyD88 and inhibits MyD88-dependent type I IFN signaling in murine plasmacytoid dendritic cells[166]. And the role of TRIF for STING-dependent type I IFN signaling in mouse embryonic fibroblasts[167]. The current study reveals a novel mechanism of cross regulation between the RNA sensing TLR3-TRIF pathway and the DNA sensing cGAS-STING pathway mediated by caspase activity. Dysregulation and hyperactivation of cGAS-STING signaling has negative implications for both pathogen-associated immunopathology and for many sterile chronic inflammatory diseases including some cardiovascular diseases, neurological disorders,

autoimmune diseases, and cancer[45]. Thus, the negative regulatory mechanism described in this study may have evolved to prevent detrimental outcomes that result from hyperactive cGAS-STING signaling . Nevertheless, KSHV is cleverly able to take advantage of this crosstalk by triggering it during lytic infection to evade type I IFN induction and consequently replicate more efficiently[139].

TLR3 has significant clinical implications for infections with several DNA viruses as some TLR3 polymorphisms are associated with more severe disease in infections with HSV-1 and HCMV, as well as with the EBV-associated nasopharyngeal carcinoma[168–171]. In contrast, some TLR3 polymorphisms protect against severe disease in infections with KSHV and HSV-2[172,173]. Moreover, in addition to KSHV, other herpesviruses such as HSV1, HSV2, and EBV activate TLR3-TRIF signaling during infection. We find that TLR3 is upregulated in lytically infected iSLK.219 and BC3 cells, which is in line with a previous study that reported TLR3 upregulation upon *de novo* infection of KSHV in THP-1 monocytes and primary human monocytes[152]. As lytic reactivation modifies the transcriptional landscape of the host cell, numerous factors could account for the increase in TLR3 transcription. In several other virus infections, TLR3 is upregulated through IFN-dependent mechanisms. One example is during measles virus infection where TLR3 upregulation occurs via an interferon sensitive response element (ISRE) in the TLR3 promoter region[174]. However, it is likely that TLR3 upregulation upon KSHV lytic reactivation is IFN-independent because TLR3 upregulation occurs upon lytic reactivation without the need for caspase inhibition (**Fig 2.5**). Indeed, our lab only detects IFN expression during lytic infection when we concomitantly treat with a caspase inhibitor[98]. Moreover, infection with several other viruses has been shown to upregulate TLR3 expression through unknown mechanisms. One possibility we considered is that the treatments used to induce the lytic cycle, doxycycline-induced RTA expression for iSLK.219 cells and TPA treatment for BC3 cells, could be upregulating TLR3. However,

we found that these conditions did not upregulate TLR3 in uninfected cell lines (**Fig 2.6**). This suggests that other factors during KSHV lytic infection induce TLR3 upregulation.

The TLR3-TRIF pathway is well known for sensing foreign dsRNA species within endosomes. Thus, understanding how DNA viruses trigger this pathway is an active area of research[175]. The best potential mechanism has been described in EBV, which produces the small non-coding EBER RNA. EBER forms a double-stranded stem-loop structure[176] and is released from EBV infected cells, thus inducing TLR3 signaling. HSV-1, like several other DNA viruses, forms dsRNA intermediates that are hypothesized to activate signaling of RNA sensors like TLR3. However, it is not clear how these transcriptional byproducts would end up in endosomes to associate with TLR3. Several sources of cellular dsRNA species can also trigger RNA sensors like TLR3, RLRs, and PKR in both infectious and sterile contexts[177]. Indeed, a recent study revealed that KSHV lytic reactivation in BC3 cells impedes DUSP11-dependent host RNA processing[178]. The resulting misprocessed dsRNAs trigger RIG-I-dependent interferon signaling and thereby restrict KSHV lytic reactivation[179]. While this study suggested that cellular RNAs transcribed by RNA polymerase III activate RIG-I signaling in KSHV infection, as they do in other infectious and sterile contexts[177], another study found that RIG-I is activated in an RNA polymerase III-independent manner in iSLK.219 cells[180]. Thus, multiple sources of immunostimulatory RNAs are produced during KSHV infection, yet it is unclear if and how they engage with TLR3 to activate this pathway.

Interestingly, it is also possible that no ligand is required for the TLR3 activation in KSHV-infected cells. We found that over-expression of TLR3 in latently infected cells in the absence of an RNA stimulus is sufficient to induce caspase-8 activation. TLR3 activation occurs via RNA ligand-induced clustering, which facilitates TRIF recruitment and downstream signaling[181]. Antibody-mediated crosslinking of TLR3 in the absence of an RNA ligand can also enable this clustering and stimulate TLR3 signaling[181]. It is

possible that mere overexpression of TLR3 increases the density of the receptor in endosomal or plasma membranes. The increased density could facilitate clustering and stimulate downstream signaling events. However, it is also possible that yet unknown RNA species generated during KSHV infection are released from infected cells to activate TLR3 at the cell surface or via endocytosis, such as the case for the EBV EBER[176].

All previous studies that demonstrate TLR3-driven, apoptotic caspase-8 activation are performed in the context of artificial TLR3 stimulation poly(I:C)[128–135]. Thus, our data are the first to our knowledge to show TLR3-driven, non-apoptotic caspase-8 activation during natural virus infection. We do not know what prevents induction of casaspe-8-mediated cell death during KSHV lytic infection. It is possible that poly(I:C) treatment in previous studies activated TLR3 to a threshold level that induced apoptotic caspase-8 activation. If this is true, perhaps TLR3 is more weakly activated during KSHV lytic infection, either through a ligand-dependent or independent manner, and thus does not reach this threshold. There are also many cellular and/or viral factors that could regulate the extent of caspase-8 processing and/or prevent downstream cell death. cFLIP is a major cellular regulator of caspase-8 activation, and both the long and short cFLIP isoforms of ($cFLIP_L$ and $cFLIP_S$) directly bind caspase-8 to control the extent of its processing and apoptotic activity[113,182]. Additionally, cellular inhibitor of apoptosis proteins (cIAPs) can prevent both death receptor and TLR3-triggered apoptosis, although the mechanism is unclear[128,132,183]. Future studies should examine the potential role of these cellular proteins in regulating caspase-8 activation and cell death during KSHV infection. Moreover, KSHV encodes miRNAs and proteins that could regulate caspase-8 activation and inhibit apoptosis. The KSHV homolog of cFLIP, vFLIP, can regulate caspase-8 activation, although previous studies point to this being dependent on the upregulation of cFLIP through NFκB signaling. Another example is KSHV K7

which inhibits downstream apoptotic signaling by binding caspase-3 to block its activity[146]. These viral factors should be investigated along with the potential cellular regulators of caspase-8 activation and apoptosis.

Caspases have only recently emerged as regulators of type I IFN signaling, and much research is needed to fully understand the roles of these multifaceted proteins in this context. While we demonstrated caspase-8-mediated crosstalk between the TLR3-TRIF pathway and the cGAS-STING pathway during KSHV infection, it is likely that this also occurs in other virus infections where both of these pathways become activated. Indeed, many viruses are known to stimulate multiple pathogen sensing pathways simultaneously. Moreover, TLR3-driven caspase-8 via poly(I:C) stimulation had previously been shown in a variety of cell types. Thus, future studies should examine whether this crosstalk occurs in other infectious contexts, as well as sterile contexts where cellular stress may activate both TLR3 and cGAS. Elucidating these mechanisms in various contexts may inform the development of therapeutic strategies to either impede or enable this IFN suppression to promote viral clearance or limit excessive IFN in inflammatory diseases.

2.2. Acknowledgments

We thank members of the Gaglia laboratory for suggestions and insights on the project. We thank the Tufts Laser Cytometry core staff for technical assistance. We thank Drs. Gack, Trono, Sabatini, and Manel for sharing reagents. This work was supported by American Cancer Society grant 131320-RSG-17-189-01-MPC and NIH R01 R01CA268976 to MMG. TT was supported by the Tufts Collaborative Cancer Biology Award. RCL was supported by NIH training grant T32 GM007310.

2.3. Declaration of interests

The authors declare no competing interests.

2.4. Methods

2.4.1. Contact for reagent sharing

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Marta M. Gaglia (Marta.Gaglia@tufts.edu).

2.4.2. Cell lines, constructs, reagents, and treatments

All cells were cultured at 37 °C in 5% CO_2. iSLK.219, iSLK.RTA, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS) (HyClone). BC3 and BC3-pTRIPZ-TLR3-flag cells were grown in RPMI supplemented with 20% FBS, 2 mM GlutaMAX supplement, and 50 μM β-mercaptoethanol (Gibco/Thermo Fisher). BJAB cells were grown in RPMI supplemented with 10% FBS and 2 mM GlutaMAX supplement. iSLK.219 cells were reactivated with 1 μg/ml of doxycycline (Thermo Fisher) for 4 days. Reactivation was confirmed visually for all iSLK.219 samples by examining the appearance of RFP-positive cells (RFP is driven by the lytic PAN promoter). BC3 cells were reactivated with 20 ng/ml of TPA for 24h or 48 h. BC3- pTRIPZ-flag-TLR3 cells were generated by transducing BC3 cells with lentiviruses containing pTRIPZ-flag-TLR3. Lentiviral packaging was carried out using the packaging plasmids psPAX2 and pMD2 (Addgene #12260, #12259). For pTRIPZ-flag-TLR3, the 3'UTR of pTRIPZ-shNS (a gift from Sandra Demaria ; Addgene plasmid # 127696 ; http://n2t.net/addgene:127696 ; RRID:Addgene_127696) was replaced with with SV40 3' UTR and TurboRFP was replaced with flag-hTLR3. Where indicated, cells were treated with 100 μg/mL poly(I:C) (MedChem Express or Selleck Chemicals), 20 μM TNF (Invivogen), and 10 μM

cyclohexamide (CHX) . The concentrations of all drug treatments were chosen based on common concentrations used in previously published studies, and the concentration of poly(I:C) was optimized to induce IFN-β in iSLK.219 and BC3 cells.

2.4.3. siRNA knockdown

iSLK.219 cells were transfected while in suspension (reverse transfection) with 5 nM siRNAs (purchased from Thermo Fisher) against FADD (HSS112893), TRIF (siRNA#1, HSS152365; siRNA#2, HSS152364), TLR3 (siRNA#1, s236; siRNA#2, s235), MyD88 (siRNA #1, s9136; siRNA #2, s9137), or a nontargeting siRNA (4390846) at a density of 16,700 cells/cm^2 using the Lipofectamine RNAiMAX transfection reagent (Life Technologies/Thermo Fisher) per the manufacturer's protocol. Six hours later, the medium was replaced. The reverse transfection process was repeated two days later for a second round of knockdown. Upon replacing the media after the second transfection, the lytic cycle was induced. All siRNAs were obtained from Life Technologies/Thermo Fisher. For BC3 cells, 2 x 10^6 cells were gently centrifuged at 200xg for 10 min. Pellets were resuspended in 100 µl SF Cell Line Nucleofector solution, and siRNAs were added to a final concentration of 200 nM. Cells then transferred to the Lonza Nucleocuvette Vessel and nucleofected using program CM-137 of the Amaxa 4D Nucleofector system (Lonza) composed of the core unit and the X unit. Cells were then incubated for 5 min at room temp, and 500 µl prewarmed media was added to cells in the Nucleocuvette Vessel. The single use pipettes included in the SF Cell Line 4D-Nucleofector X Kit L (Catalog #: V4XC-2012) were then used to transfer cells to a 6 well plate in which the wells contained 1.5 mL of pre-warmed media. Two days later, the lytic cycle was induced.

2.4.4. CRISPR-Cas9 gene editing

BC3 cells stably expressing Cas9 (BC3-Cas9 cells) were a kind gift from Eva Gottwein's laboratory. BC3-Cas9-sgFADD cells were generated by transducing BC3-Cas9 cells with lentiviruses containing pU6-sgFADD-Zeo. Cells underwent antibiotic selection for 2 weeks with zeomycin and protein samples were collected to assess KO efficiency.

2.4.5. TLR3 over-expression

iSLK.219 cells were plated in 6 well plates to be 60-80% confluent the following day. 24h later, cells were transfected with hTLR3-pcDNA3 (a gift from Saumen Sarkar (Addgene plasmid # 32712 ; http://n2t.net/addgene:32712 ; RRID:Addgene_32712)) using JetPRIME transfection reagent (Polyplus transfection, VWR). 24 hours post-transfection, some samples were treated with 100 µg/mL poly(I:C) for 6h. Samples were collected at indicated timepoints to assess TLR3 expression levels and caspase-8 activation by western blot.

2.4.6. Realtime quantitative polymerase chain reaction (RT-qPCR)

For RNA analysis, samples were collected at the indicated time points and cell pellets were lysed in RNA lysis buffer (Zymo Research). Total RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research) by following the manufacturer's protocol. For mRNA measurements, cDNA was prepared using an iScript cDNA synthesis kit (Bio-Rad) per the manufacturer's protocol. In all cases, 18S rRNA levels were used as an internal standard to calculate relative mRNA levels. Real-time quantitative PCR (RT-qPCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in a CFX Connect real-time PCR detection system (Bio-Rad). No-template and no-RT controls

were included in each replicate. CFX Manager software was used to analyze the data. Primers are listed in Table 2.1.

2.4.7. Protein analysis

iSLK.219 cells were seeded at a density of 167,000 cells per well in a 6-well plate and treated with doxycycline (1 µg/ml). Lysates were collected at the indicated time points. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) supplemented with cOmplete protease cocktail inhibitor (Roche). Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The following Cell Signaling Technologies antibodies were used on PVDF membranes at 1:1,000 dilution in 5% BSA in Tris-buffered saline with 0.1% Tween 20 (TBST): anti-TRIF (no. 4596), anti-TLR3 (no. 6961), anti-MyD88 (no. 4283), anti-caspase-8 (no. 9746), anti-cleaved caspase-8 (no. 9496), anti-β-tubulin (9F3) (no. 2128). Anti-β-actin (ab8229; 1:500, Abcam) and anti-TLR4 (sc-293072; 1:500, SCBT), and anti-FADD (BD610399; 1:1000, BD biosciences), and anti-KSHV ORF45 (MA5-14769; 1:1000, ThermoFisher) were also diluted in 5% BSA in TBST and used with PVDF membranes blocked in 5% BSA in TBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (both 1:5,000 in blocking buffer) were purchased from Southern Biotechnology. HRP-conjugated donkey anti-goat IgG (1:5,000 in 0.5% milk in PBST) was purchased from Santa Cruz Biotechnology. All membranes were exposed using Pierce ECL Western blotting substrate (Thermo Fisher) and imaged with a Syngene G:Box Chemi XT4 gel doc system.

2.4.8. Statistical analysis

All statistical analysis was performed using GraphPad Prism version 8.4.2 or later (GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical significance was determined by analysis of variance (ANOVA) followed by a *post hoc* multiple comparison test (Dunnett's, Sidak's or Tukey's) when multiple comparisons were required. All data are plotted as mean ± standard deviation.

Table 2.1: Primers used for qPCR.

PRIMER	**SOURCE**
18S rRNA Forward: GTAACCCGTTGAACCCCATT	Abernathy et al., 2015
18S rRNA Reverse: CCATCCAATCGGTAGTAGCG	Abernathy et al., 2015
IFN-β Forward: CAGCAATTTTCAGTGTCAGAAGC	Jacobs et al., 2013
IFN-β Reverse: TCATCCTGTCCTTGAGGCAGT	Jacobs et al., 2013
IL-6 Forward: GGTACATCCTCGACGGCATCT	Keller et al., 2003
IL-6 Reverse: GTGCCTCTTTGCTGCTTTCAC	Keller et al., 2003
TLR3 Forward: CCTGGTTTGTTAATTGGATTAACGA	Rasheed et al., 2020
TLR3 Reverse: TGAGGTGGAGTGTTGCAAAGG	Rasheed et al., 2020

2.5. Author contribution

One replicate of a TRIF KD in iSLK.219 cells was performed by Tate Tabtieng (Figure 2.2A). One replicate of a FADD KD in iSLK.219 cells was performed by Richard Lavin (Figure 2.1A). The rest of the experimental work was carried out by Rachel Lent.

Chapter 3. Caspase-mediated regulation and cellular heterogeneity of the cGAS/STING pathway in Kaposi's sarcoma-associated herpesvirus infection

Tabtieng T, Lent RC, Kaku M, Monago-Sanchez A, Gaglia MM. 2022. *mBio* 13:6. e02446-22.

3.1. Introduction

Type I interferon (IFN) cytokines are potently anti-viral and play an essential role in controlling infections and starting systemic immune responses. Type I IFNs are generally turned on during viral infection after sensing of viral nucleic acids by cellular pattern recognition receptors such as the cytosolic DNA sensor cGAS and RNA sensor RIG-I[137]. These sensors signal through adaptor proteins (STING and MAVS, respectively) and the kinases TBK1 and IKKε to activate the transcription factor IRF3 and trigger induction of type I IFN cytokines[137]. Type I IFNs in turn stimulate expression of interferon stimulated genes (ISGs) in an autocrine and paracrine manner to limit viral replication and infection, as well as alert the immune system of the infection[184]. As ISGs create a strong antiviral response, all viruses have evolved one or more strategies to block IFN induction[185]. The AIDS-associated oncogenic gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV), which is the etiological agent for one of the leading causes of cancer death in sub-Saharan Africa, Kaposi's sarcoma, is a prime example of this. KSHV induces minimal type I IFN responses when reactivating from latency[90,139], demonstrating it can strongly block this response.

While many viral encoded factors directly target the type I IFN response, viruses like KSHV have also evolved strategies to usurp cellular pathways to counteract immune responses. We recently established that KSHV exploits the activity of the apoptotic caspases to suppress the type I IFN response and promote viral replication[139]. Indeed, while KSHV lytic reactivation normally does not induce a strong type I IFN response, lytically infected cells express and secrete high levels of IFN-β if caspases are inhibited[139]. Although apoptotic caspases are canonically thought to be antiviral by facilitating cell death of infected cells, multiple recent studies have demonstrated caspase-dependent inhibition of innate immune signaling during apoptosis and viral infection[121,122,139,186]. However, it remained unclear from our previous studies how

caspases enact this IFN regulatory function during KSHV infection. In particular, while caspase-3 and -7 can directly cleave cGAS, MAVS, and IRF3 to block type I IFN signaling[186], our previous findings suggest that in the case of KSHV infections, caspase-8 is the major caspase suppressing the type I IFN response[139]. Moreover, caspase-8 appears to act independently of caspase-3/7 activation and of cell death to regulate IFNs in KSHV-infected cells[139]. As different caspases have different proteolytic targets, these results suggest the mechanism we found in KSHV infected cells may also be different than what has previously been reported.

To decipher how caspases disrupt IFN signaling during KSHV infection, we used RNAi-mediated depletion and pharmacological inhibition to identify which IFN signaling components are targeted by caspase activity. This revealed that caspase activity specifically inhibits cGAS to impede IFN signaling during KSHV lytic infection. We also performed single-cell RNA-sequencing (scRNAseq) to examine the heterogeneity of immune responses and viral gene expression during KSHV lytic infection. This uncovered considerable heterogeneity of the KSHV lytic cycle. Moreover, it surprisingly revealed that a small percentage of infected cells produce IFN and suggested that differences in cellular gene expression may account for this heterogeneity.

3.2. Results

3.2.1. Caspase activity blocks cGAS/STING signaling during KSHV lytic infection

We previously showed that the type I IFN response that occurs upon caspase inhibition in KSHV-infected cells is dependent on the kinase TBK1[139]. TBK1 can be activated by several pathogen sensors, including sensors of viral nucleic acids[137], and both the DNA and the RNA sensing pathways can be activated during KSHV infection[56,90,96,179,180,187–189]. To discern which pathogen sensing pathway is inhibited by caspase activity during KSHV infection, we knocked down key proteins for DNA and

RNA sensing: the DNA sensors cGAS and IFI16 and their signaling adaptor STING, and the RNA sensor RIG-I and its signaling adaptor MAVS (**Fig. 3.1A-C, Fig. 3.2**). For these experiments, we used iSLK.219 cells, which are latently infected with recombinant rKSHV.219. rKSHV.219 expresses GFP constitutively and RFP under the control of a KSHV lytic promoter[147]. These cells also express an exogenous copy of the KSHV master lytic regulator RTA under a doxycycline inducible promoter, so that reactivation of the viral lytic replication cycle can be induced by doxycycline treatment[147]. IFN-β induction upon caspase inhibition was severely reduced by STING and cGAS depletion, suggesting that caspase activity targets STING-dependent type I IFN signaling during KSHV replication (**Fig. 3.1A**). Moreover, upon STING and cGAS knock-down, caspase inhibitors no longer caused a reduction in KSHV replication, which we previously showed is a result of type I IFN induction[139] (**Fig. 3.1B**). In contrast, IFI16, RIG-I, and MAVS knockdowns had minimal effects on IFN-b induction and viral gene expression in the presence of caspase inhibitors (**Fig. 3.1A, B**). These results suggest that cGAS/STING-mediated DNA sensing is disabled by caspase activity. To corroborate this conclusion, we also tested pharmacological inhibition of cGAS enzymatic activity with RU.521. When bound to DNA, cGAS synthesizes the cyclic dinucleotide 2'3'-cGAMP, which in turn binds and activates STING[190]. Consistent with our knock-down results, cGAS inhibition reduced induction of IFN-β and of the interferon-stimulated gene ISG15 upon caspase inhibition (**Fig. 3.1D**). Collectively, these findings suggest that caspase activity prevents activation or signal transduction of the cGAS/STING DNA sensing pathway during KSHV lytic replication.

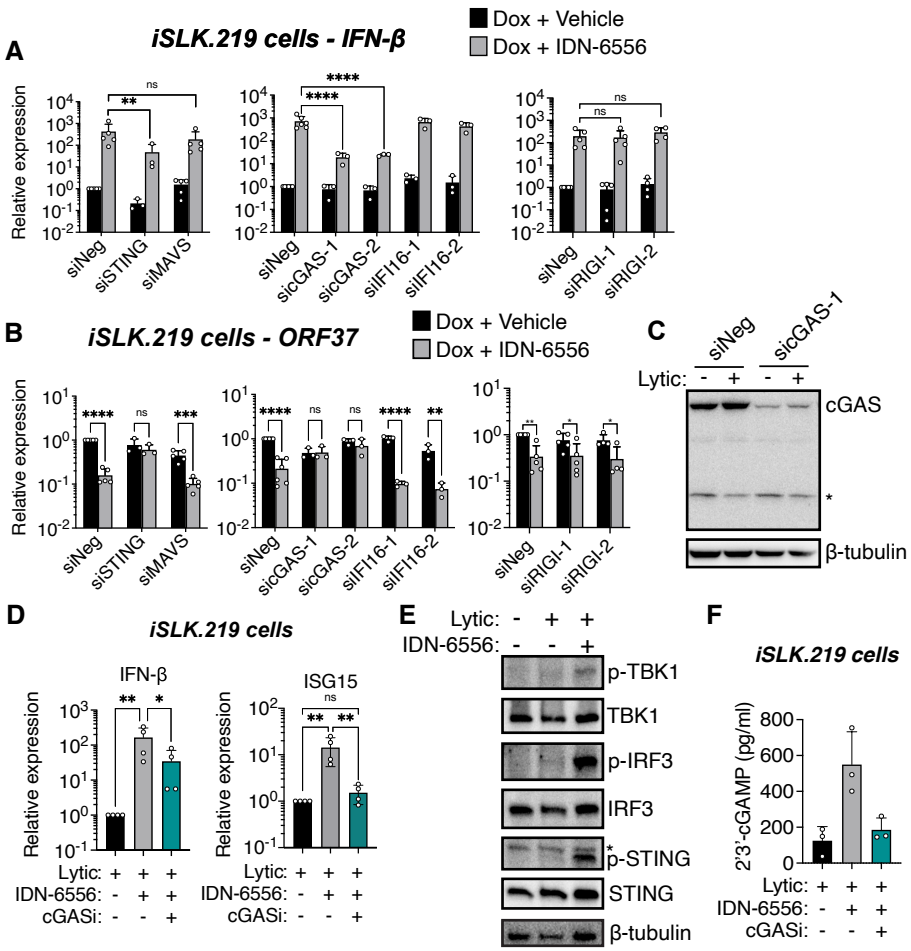

Figure 3.1: Caspase activity prevents cGAS activation during KSHV lytic replication to block IFN-β induction.

*(A-C) iSLK.219 cells were transfected with a negative control siRNA or siRNAs targeting the indicated proteins. For cGAS and RIG-I, the transfection was carried out twice, two days prior to and on the day of lytic cycle induction; for STING, MAVS and IFI16, one transfection was carried out two days prior to induction. The cells were then lytically reactivated with doxycycline (1 μg/ml) and treated with either DMSO (vehicle) and IDN-6556 (10 μM) as indicated. (A-B) Total RNA was extracted at day 5 post reactivation and the levels of IFN-β (A) and ORF37 (B) mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA (n ≥ 3). (C) Cell lysates were harvested at day 4 post reactivation and subjected to western blot for cGAS and β-tubulin as a loading control. Asterisk indicates non-specific band. Blots are representative of 3 replicates. (D-F) iSLK.219 cells were lytically reactivated with doxycycline (1 μg/ml) and treated with either DMSO (vehicle) or IDN-6556 (10 μM) and the cGAS inhibitor RU.521 (cGASi, 24.1 μM), where indicated. (D) Total RNA was extracted from iSLK.219 cells 3 days after reactivation. Levels of IFN-β mRNA were measured by RT-qPCR and normalized to 18S rRNA (n = 3). (E) Cell lysates were harvested at day 4 post reactivation and subjected to western blotting for p-TBK-1, TBK-1, p-IRF-3, IRF-3, p-STING, STING, and β-tubulin (as a loading control) as indicated. Asterisk indicates non-specific band. Blots are representative of 3 replicates. (F) Levels of 2'3'-cGAMP in lysate collected from iSLK.219 cells at day 4 post reactivation were measured by ELISA (n = 4). ns, *, **, ***, ****: p value > 0.05, < 0.05, < 0.01, < 0.001, < 0.0001, Tukey's multiple comparisons test after two-way ANOVA..*

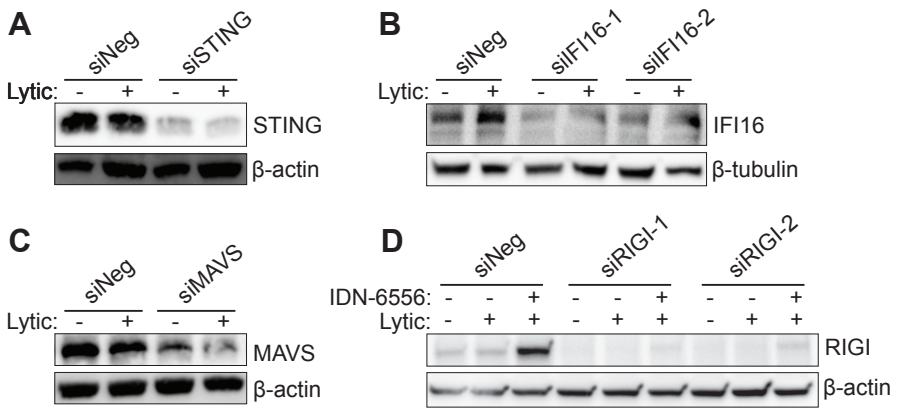

Figure 3.2: Knockdown of proteins in the type I IFN induction pathway.
iSLK.219 cells were transfected with a negative control siRNA and siRNAs targeting STING (A), IFI16 (B), MAVS (C), or RIG-I (D). Cell lysates were harvested at day 4 post reactivation with doxycycline and treatment with IDN-6556 (where indicated) and subjected to western blotting for the target proteins and β-actin or β-tubulin as loading controls. Blots are representative of 3 replicates.

3.2.2. Caspases inhibit cGAS activity without reducing cGAS protein levels

To determine how caspases interfere with cGAS/STING signaling, we tested whether there was differential activation of various effectors in the cGAS/STING pathway in the presence *vs*. absence of caspase activity. We observed increased phosphorylation of the downstream proteins STING, TBK1, and IRF3, which indicates that they are activated upon caspase inhibition (**Fig. 3.1E**). These results suggest that activation of the entire pathway is blocked by caspases. Indeed, we also found that 2'3'-cGAMP levels were increased upon caspase inhibition and were reduced by treatment with a cGAS inhibitor (**Fig. 3.1F**). Taken together with our results from the knockdown experiments, these findings identify the target of caspases as a component or regulator of the cGAS/STING pathway that acts to promote activation or activity of cGAS.

We considered the possibility that the target was cGAS itself. One report suggests that caspase-3 and -7 can cleave cGAS during apoptosis and other viral infections[186]. However, we have found that the activity of these caspases is

dispensable for IFN regulation during KSHV lytic infection, and that caspase-8 is likely the key regulator[139]. Also, we do not detect an overall reduction in cGAS levels nor any cGAS cleavage fragments during lytic reactivation (**Fig. 3.1C**). Ectopic co-expression of caspase-8 and cGAS also did not reveal caspase cleavage fragments, even though caspase-8 overexpression also leads to activation of caspase-3 and -7 (data not shown). It is thus likely that during KSHV lytic infection, caspases do not cleave cGAS directly, but instead target a regulatory factor that modulates its activity

3.2.3. Caspase activity also regulates cGAS in KSHV-infected primary effusion lymphoma B cells

To determine if caspases also suppress the cGAS/STING pathway in a cell type that is physiologically relevant for KSHV infection and tumorigenesis, we examined the activity of caspases in BC3 cells, a KSHV-infected B cell line derived from a primary effusion lymphoma (PEL) patient[191]. Similarly to iSLK.219 and BCBL1 cells[139], caspase-8 was also activated upon lytic reactivation of BC3 cells (**Fig. 3.3A**). Moreover, caspase inhibition increased expression of both IFN-β and ISGs (ISG15 and IFIT1, **Fig. 3.3B**), as well as IRF3 phosphorylation (**Fig. 3.3C**). Consistent with what we observed in iSLK.219 cells, caspase inhibition potentiated 2'3'-cGAMP production in lytically reactivated BC3 cells (**Fig. 3.3D**). Moreover, inhibition of cGAS with RU.521 reduced the induction of IFN-β (**Fig. 3.3E**). These results strengthen our conclusion that caspases have a major role in inhibiting cGAS activity or activation to and blocking IFN induction during KSHV lytic infection in multiple cell types, including cell types that are relevant to KSHV infection in humans and KSHV-associated diseases.

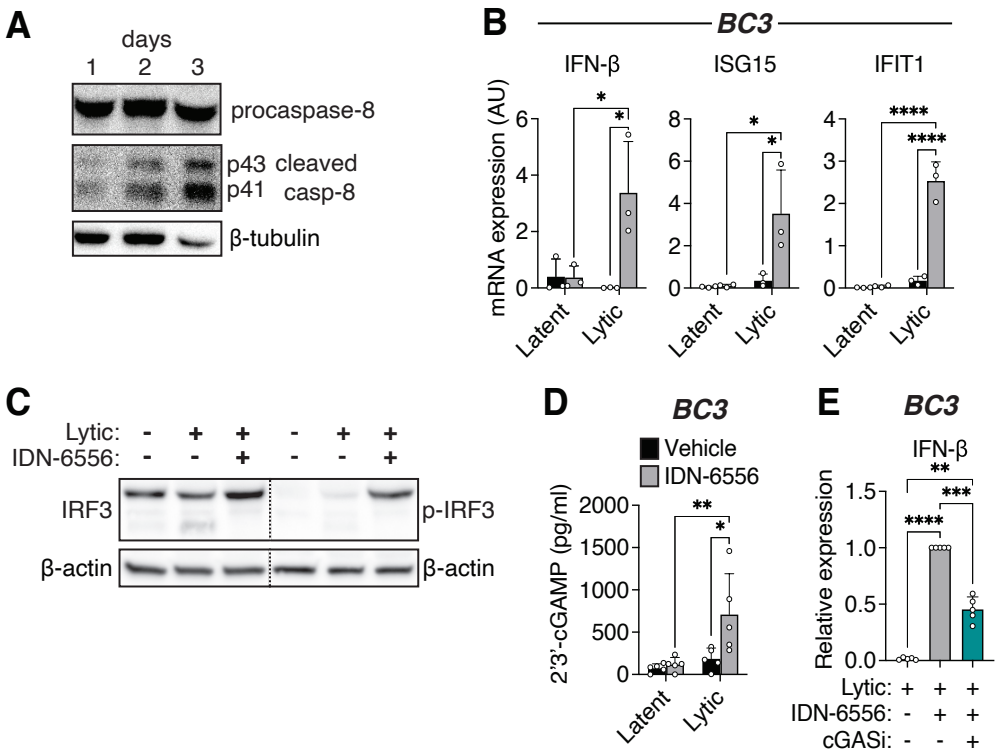

Figure 3.3: Caspases also inhibit cGAS activity in KSHV infected B cells.
(A) iSLK.219 cells were treated with doxycycline (1 μg/ml) to reactivate the lytic cycle, as well as IDN-6556 and anti-IFN antibodies where indicated. mRNAs levels of IFN-β, the ISGs ISG15 and IFIT1, and the KSHV gene ORF37 were measured by RT-qPCR four days after doxycycline addition. (B-C) Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. (B) Heatmaps of expression of KSHV genes in each cell in the three lytic samples, sorted by cluster. The legend below the heatmaps defines what expression level the colors represent (arbitrary units). (G) UMAP diagram of cell cycle stage classification of cells in the 4 samples.

3.2.4. Single-cell RNA sequencing (scRNA-Seq) highlights heterogeneity of KSHV reactivation and confirms the pro-viral role of caspase activity

The results so far provide a bulk analysis of the IFN pathway during KSHV infection. However, not all iSLK.219 cells in the population re-enter the lytic cycle upon doxycycline addition[147], suggesting there could be significant cell-to-cell variability in responses to infection. To understand both lytic reactivation and IFN signaling on a single cell basis, we carried out scRNA-Seq on uninfected cells, latently infected cells, lytically infected cells, and lytically infected cells treated with IDN-6556 (**Fig. 3.4A, Table 3.1**). Uninfected cells are SLK cells containing the inducible KSHV RTA transgene, but

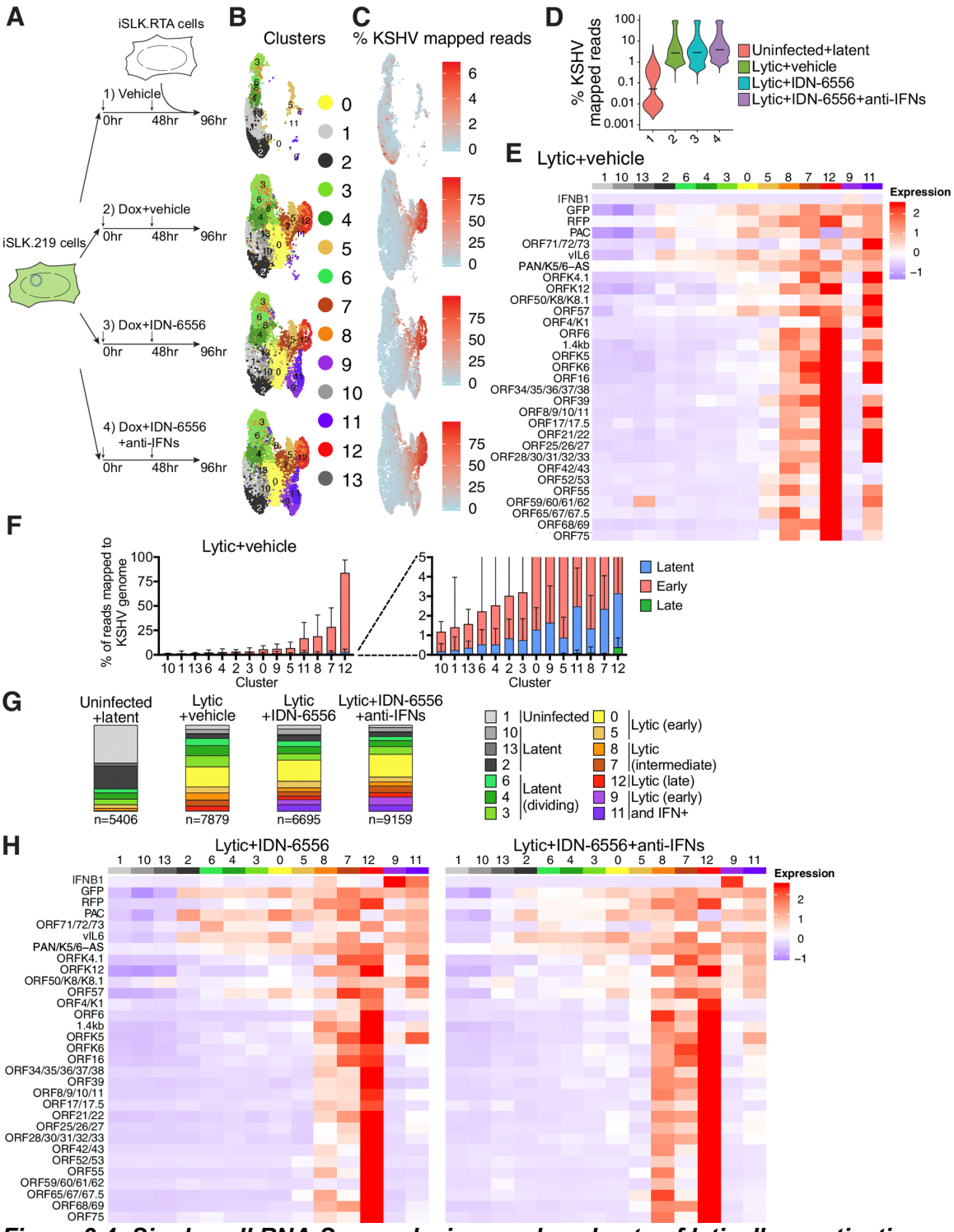

Figure 3.4: Single-cell RNA-Seq analysis reveals subsets of lytically reactivating cells.

(A) Diagram of the single-cell RNA-Seq (scRNA-Seq) experiment using 4 samples. Sample 1 was a mixture of uninfected iSLK.RTA and latently infected iSLK.219 cells treated with DMSO (vehicle). Samples 2-4 were iSLK.219 treated with doxycycline (Dox) to reactivate lytic replication ("Lytic") and treated with DMSO (vehicle), the caspase inhibitor IDN-6556, or IDN-6556 and a cocktail of anti-IFN antibodies to block paracrine

IFN signaling. All samples were collected 4 days after the start of treatments. (B-H) Analysis of data from the scRNA-Seq experiment presented in (A). (B) Uniform manifold analysis and projection (UMAP) diagram of the clusters unbiasedly defined by Seurat in the 4 samples. (C) UMAP diagram showing what percentage of reads from each cell comes from the KSHV genome. Legends on the right define what percentage the colors represent. (D) Violin plot of the distribution of cells in each sample based on percentage of KSHV reads. Sample 1 has a bimodal distribution because it is a mix of uninfected and latent cells. (E) Heatmap of average expression of KSHV genes in each cluster in the lytic + vehicle sample. The legends on the right define what expression level the colors represent (arbitrary units). Corresponding heatmap with gene expression in each individual cell can be found in Fig. 3.5B. (F) Plot of percentage reads per cell that map to each of three types of KSHV genes (latent, early and late) in each cluster in the lytic + vehicle sample. Clusters are sorted based on the total average percentage of KSHV reads. (G) Proportion of cells that map to each cluster in the four samples. (H) Heatmap of average expression of KSHV genes in each cluster in the lytic + IDN-6556 and lytic + IDN-6556 + anti-IFN samples. The legends on the right define what expression level the colors represent (arbitrary units). Corresponding heatmaps with gene expression in each individual cell can be found in Fig. 3.5B.

Table 3.1: Characteristics of scRNA-Seq datasets.

	Uninfected + latent	Lytic + vehicle	Lytic + IDN-6556	Lytic + IDN-6556 + anti-IFNS
Total reads	142,704,565	138,925,899	154,624,957	160,218,965
Total cells	5,777	8,310	7,029	9,638
Median reads per cell	22,881	14,739.5	19,083	14,085
Total reads used in analysis	130,047,844	136,742,207	148,241,619	156,671,882
Cells used in analysis	5,406	7,879	6,695	9,159

no rKSHV.219 (iSLK.RTA)[147]. We also included lytically infected cells treated with IDN-6556 and anti-IFN antibodies to block autocrine and paracrine signals (**Fig. 3.4A, Fig. 3.5, Table 3.1**). Analysis using the Seurat package[192,193] unbiasedly identified 13 clusters with different gene expression across the 4 samples (**Fig. 3.4B**). Analysis of the mapped reads showed that, as expected, there was a higher percentage of KSHV reads in cells that were lytically reactivated (**Fig. 3.4C, D**). Certain clusters were clearly enriched in KSHV transcripts (**Fig. 3.4C, E; Fig. 3.5B**) and these corresponded to clusters that only

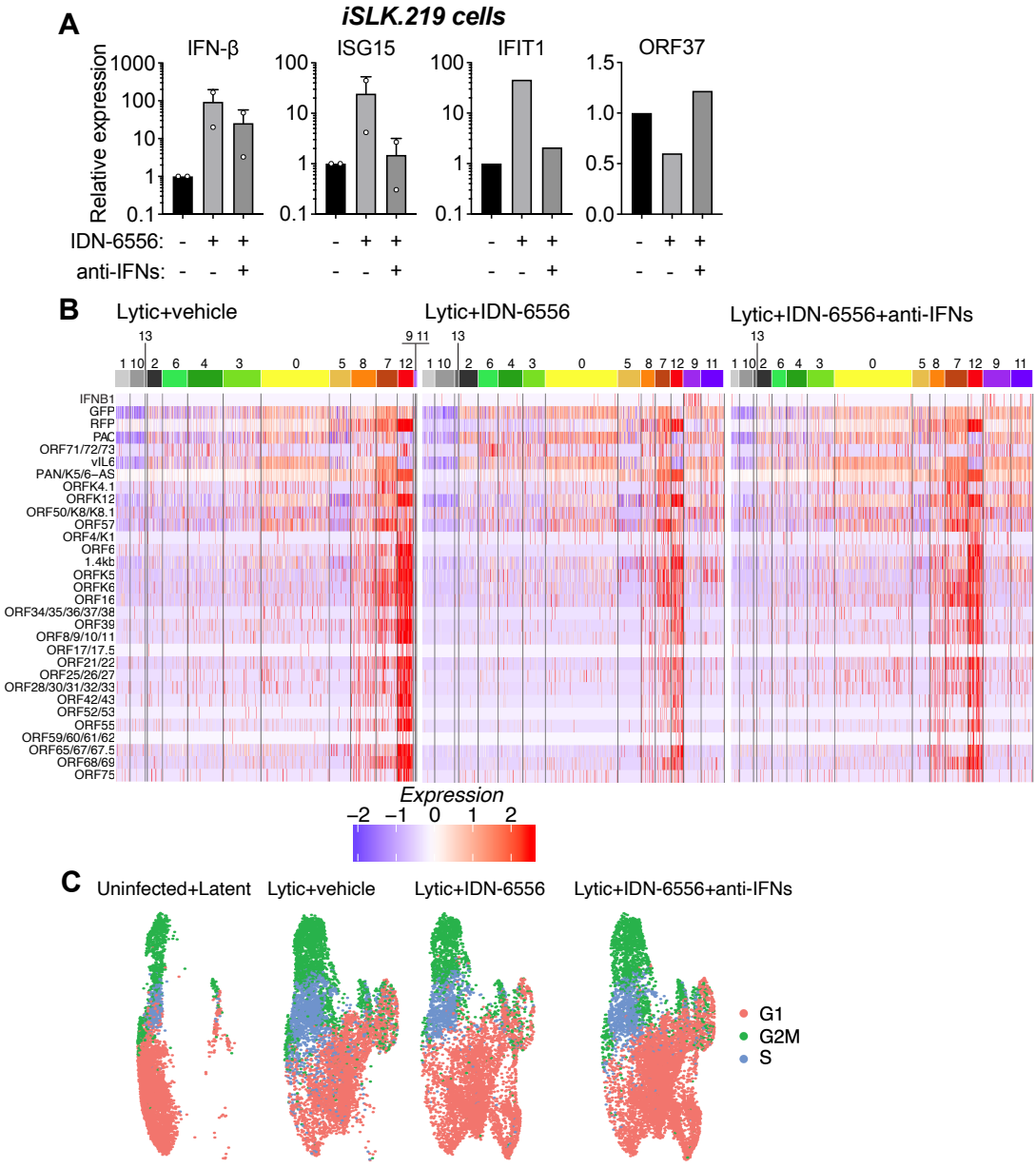

Figure 3.5: scRNAseq controls and additional analyses.
A) iSLK.219 cells were treated with doxycycline (1 μg/ml) to reactivate the lytic cycle, as well as IDN-6556 and anti-IFN antibodies where indicated. mRNAs levels of IFN-β, the ISGs ISG15 and IFIT1, and the KSHV gene ORF37 were measured by RT-qPCR four days after doxycycline addition. (B-C) Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. (B) Heatmaps of expression of KSHV genes in each cell in the three lytic samples, sorted by cluster. The legend below the heatmaps defines what expression level the colors represent (arbitrary units). (G) UMAP diagram of cell cycle stage classification of cells in the 4 samples.

appeared in the lytic samples (**Fig. 3.4B, C**). Analysis of the cell cycle status indicated that most of the cells were in G1, but some of the clusters were enriched in cells in S or

G2/M phase, and this tended to correlate with lower viral gene expression, presumably indicative of lower reactivation (**Fig. 3.5C**).

We further analyzed the type of genes that were seen in the different clusters in the lytic+vehicle sample. We saw that several clusters had cells with on average 5-30% of reads mapping to KSHV genes, while more than 75% of the reads mapped to KSHV genes in cells from cluster 12 (**Fig. 3.4E-F, Fig 3.5B**). Because our scRNA-Seq is directed at the 3' end of RNAs and many mRNAs in KSHV have the same 3' end[194,195], we were not able to conclusively distinguish expression of all the viral genes. Therefore, in the heatmaps we list together all the ORFs that each 3' end corresponds to. Nonetheless, analysis of viral genes and of the rKSHV.219-encoded GFP, RFP and puromycin-N-acetyl transferase (PAC) reporters agrees with the general picture obtained from percentage reads. Most clusters show some lytic gene expression but only a few demonstrate substantial expression of lytic genes (**Fig. 3.4E, Fig. 3.5B**). Interestingly, we note that some of the clusters that expressed lytic genes did not express high levels of RFP (cluster 0 for example, **Fig. 3.4E**). Because KSHV lytic genes are expressed in several waves, termed early lytic, delayed early and late[196–198], we roughly categorized the clusters based on the pattern of lytic gene expression, as well as the cell cycle stage and the expression of IFNs. We classified clusters as "uninfected", "latent", "latent (dividing)", "lytic (early)", "lytic (intermediate)", "lytic (late)", "lytic (early) and IFN+" (**Fig. 3.4E, 3.4G-H, Fig. 3.8A-C**). Most of the viral transcripts detected increased in levels monotonically in cells that had progressed further through the lytic cycle, except for ORF57, K4.1, and vIL-6, which appeared to decrease in levels in the "late" lytic cell cluster (cluster 12, **Figure 3.4E**).

Figure 3.4G and **Figure 3.6B** show how the different clusters expanded and contracted in each sample. Interestingly, addition of the caspase inhibitors and the consequent type I IFN induction did not reduce the overall percentage of cells

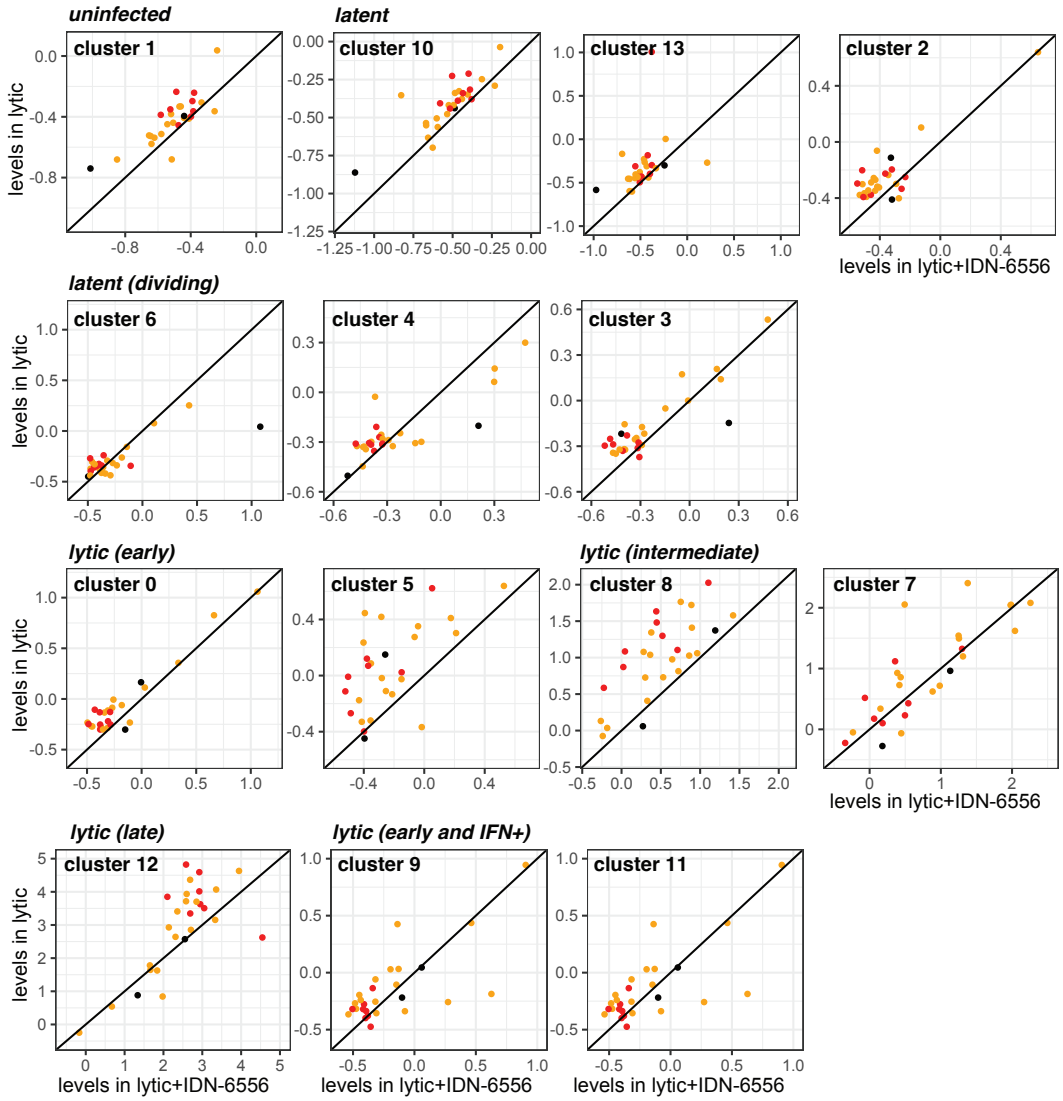

Figure 3.6: Changes in viral gene expression between lytic iSLK.219 cells and lytic iSLK.219 cells treated with caspase inhibitors.
Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. Scatter plots show the average expression of viral genes in each of the clusters (ordered based on our classification) in iSLK.219 cells treated with doxycycline (lytic, y-axis) and iSLK.219 cells treated with doxycycline and caspase inhibitors (lytic + IDN-6556, x-axis). Black dots represent latent genes, yellow dots early genes and red dots late genes. The diagonal line represents equal levels

expressing lytic genes (**Fig. 3.4G, Table 3.2**), but it reduced the number of cells that we classified as "lytic (intermediate)" and "lytic (late)" (particularly clusters 7, 8, and 12) (**Fig. 3.4G**). Moreover, caspase inhibitor treatment reduced the levels of most viral genes in many clusters, most notably the "lytic" clusters 5, 8, and 12 (**Fig. 3.4E, H, Fig. 3.5B, Fig.**

Table 3.2: Number and percentage of cells in each cluster.

Cluster	Classification	Dataset							
		Uninfected and latent		Lytic + vehicle		Lytic + IDN-6556		Lytic + IDN-6556 + anti-IFNs	
1	uninfected	2365	43.7%	378	4.8%	278	4.2%	251	2.7%
10	latent	16	0.3%	387	4.9%	439	6.6%	420	4.6%
13	latent	185	3.4%	40	0.5%	77	1.2%	79	0.9%
2	latent	1435	26.5%	390	4.9%	429	6.4%	457	5.0%
6	latent (dividing)	255	4.7%	663	8.4%	436	6.5%	437	4.8%
4	latent (dividing)	387	7.2%	942	12.0%	555	8.3%	624	6.8%
3	latent (dividing)	350	6.5%	1004	12.7%	499	7.5%	831	9.1%
0	lytic (early)	3	0.1%	1829	23.2%	1645	24.6%	2416	26.4%
5	lytic (early)	231	4.3%	559	7.1%	517	7.7%	523	5.7%
8	lytic (intermediate)	156	2.9%	667	8.5%	306	4.6%	453	4.9%
7	lytic (intermediate)	2	0.0%	555	7.0%	341	5.1%	693	7.6%
12	lytic (late)	0	0.0%	412	5.2%	274	4.1%	456	5.0%
9	lytic (early) and IFN+	7	0.1%	35	0.4%	385	5.8%	849	9.3%
11	lytic (early) and IFN+	14	0.3%	18	0.2%	514	7.7%	670	7.3%
	total	5406		7879		6695		9159	

3.4). The effect of caspase inhibitors on the size of these clusters and the reduction in lytic gene expression was largely rescued by blocking type I IFN signaling with anti-IFN antibodies (**Fig. 3.4G, H, Fig. 3.5B, Fig. 3.7**). These results confirm that caspase activity is required for reactivation and/or progression through the lytic cycle and that inhibition of the lytic cycle upon caspase inhibition is due to the paracrine and autocrine effects of type I IFN secretion. These data thus recapitulate our bulk level findings on the role of caspases during KSHV replication[139]. The scRNA-Seq results also confirm the wide variability in stage of reactivation of iSLK.219 cells and reveal that the RFP signal in iSLK.219 cells may underestimate the cells that are expressing lytic genes.

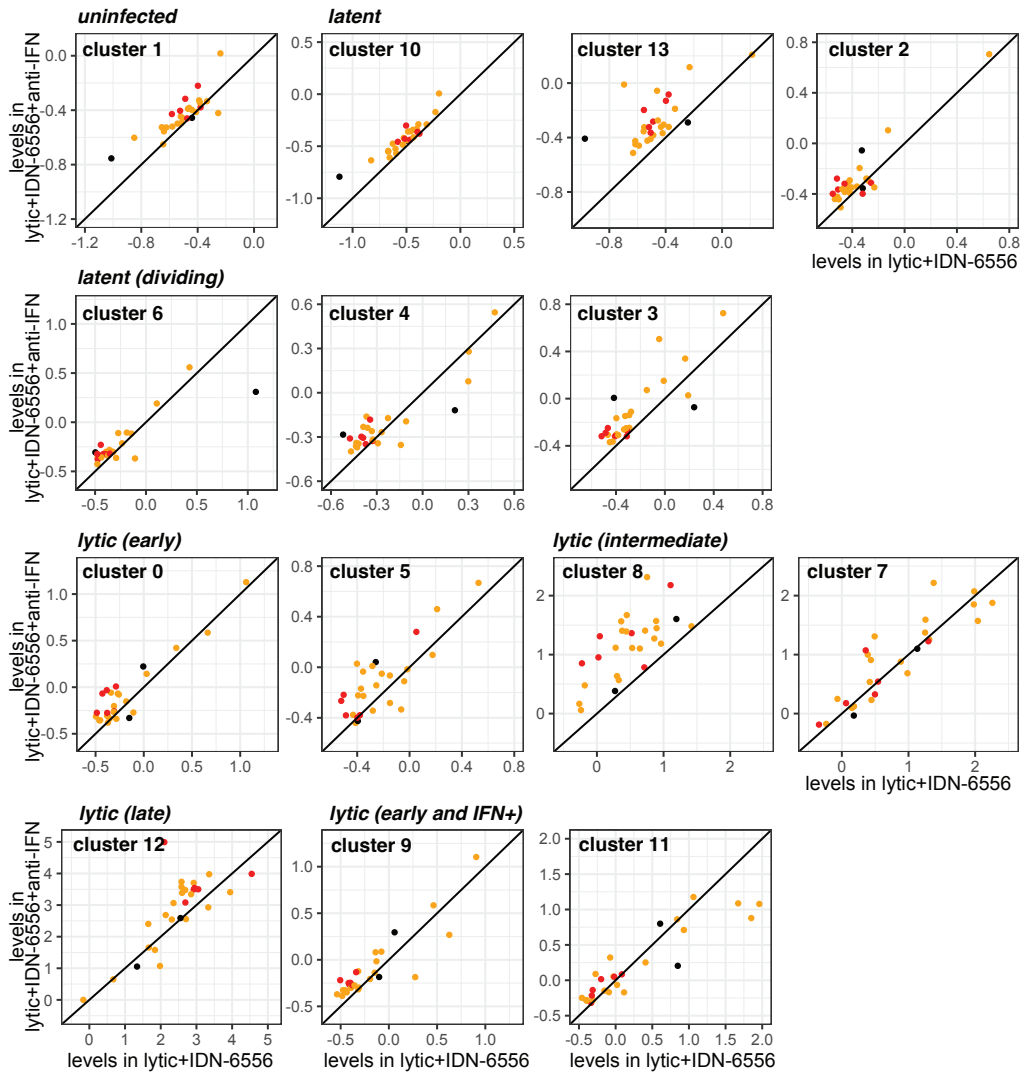

Figure 3.7: Changes in viral gene expression between lytic iSLK.219 cells treated with caspase-inhibitors and lytic iSLK.219 cells treated with caspase inhibitors and anti-IFN antibodies.

Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. Scatter plots show the average expression of viral genes in each of the clusters (ordered based on our classification) in iSLK.219 cells treated with doxycycline and caspase inhibitors (lytic + IDN-6556, x-axis) and iSLK.219 cells treated with doxycycline, caspase inhibitors, and anti-IFN antibodies (lytic + IDN-6556 + anti-IFN, y-axis). Black dots represent latent genes, yellow dots early genes and red dots late genes. The diagonal line represents equal levels.

3.2.5. Only a small subset of infected cells expresses IFNs to confer a potent antiviral state

An outstanding question from our bulk analysis was the identity of the cells that

produce IFN-β in the caspase inhibitor-treated samples. We had hypothesized that lytically reactivating cells, rather than latent ones, produce IFNs. Indeed, our scRNA-Seq data showed that type I IFN expression is limited to cells that express viral lytic genes (**Fig. 3.4H**). However, the scRNA-Seq also surprisingly revealed that only 3.8% of the cells in the caspase inhibitor-treated samples expressed IFNs, particularly IFN-β (**Fig. 3.8B**). The IFN-β+ cells were concentrated in clusters 9 and 11, which only appeared in the +IDN-6556 samples (**Fig. 3.8A, B**). We did not detect expression of IFN-αs, but we saw that cells in clusters 9 and 11 also expressed the type III IFN IFN-l1 (**Fig. 3.8C, Fig 3.6A**). The IFN coming from this small fraction of cells was sufficient to elicit a strong IFN response in the entire population of cells, as indicated by the IFN-dependent increase in expression of IFN-stimulated genes (ISGs) after IDN-6556 treatment across almost all clusters (**Fig. 3.8D, E, Fig. 3.9B**). The only exception was cluster 12, which contains cells expressing late lytic gene. These cells are likely undergoing host shutoff, and generally express reduced levels of host genes[82]. To ensure that the variability in IFN-β induction is not an artifact of a limit of detection by scRNA-Seq, we constructed iSLK.219 and BC3-based cell lines that expressed the TagBFP or tdTomato proteins under the control of the IFN-β promoter. When the lytic cycle was induced in these cells concomitantly with caspase inhibitor treatment, we found that ~1-2% of the cells became TagBFP+ (**Fig. 3.8E**) or tdTomato+ , in line with our scRNA-Seq results (**Fig. 3.8F**). In addition, we found that caspase inhibitor treatment of latent BC3 cell lines did not increase the percentage of tdTomato+ cells (**Fig. 3.8F**), consistent with our previous results that lytic induction is also required for the type I IFN induction (**Fig. 3.3B, 3.3D**,[139]). Overall, these data show that an exceedingly small fraction of cells induce IFNs even under caspase inhibition, yet they can elicit a strong anti-viral response in the entire cell population. Moreover, this heterogeneous induction of type I IFN occurs in both cell types we tested.

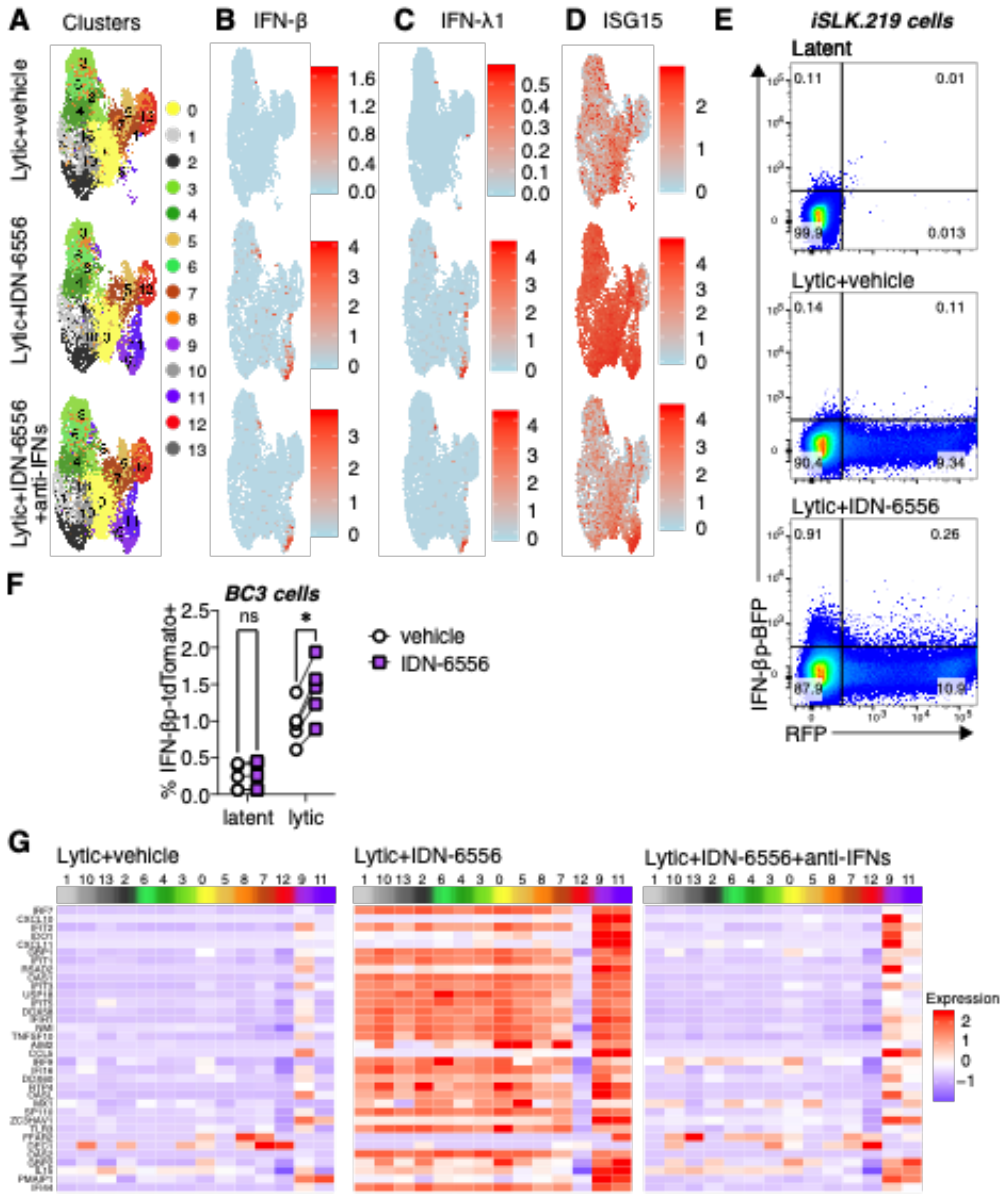

Figure 3.8: Only a small fraction of KSHV lytic cells express IFN-β.
*(A-D) Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. (A) UMAP diagram of the clusters unbiasedly defined by Seurat in the 3 lytic samples (same as Fig. 3.4B). (B-D) UMAP diagram of the level of expression of IFN-β (B), IFN-l1 (C) or ISG15 (D) in each cell for the 3 lytic samples. The legends on the right define what expression level the colors represent (arbitrary units). (E) iSLK.219 expressing TagBFP from a human IFN-β promoter were treated with doxycycline to reactivate the lytic cycle ("lytic") and IDN-6556, where indicated. Flow cytometry was carried out on the cells 4 days after doxycycline addition. RFP axis reflects the RFP signal in lytic cells and BFP axis reflects activity of the IFN-β promoter. Percentage of cells mapping to each quadrant are listed. Flow cytometry plots are representative of 3 repeats. (F) BC3 expressing tdTomato from a human IFN-β promoter were treated with TPA to reactivate the lytic cycle ("lytic") and the caspase inhibitor IDN-6556, where indicated. Flow cytometry was carried out on the cells 2 days after TPA addition. The percentage of cells that were tdTomato positive is plotted for 5 biological replicates. ^{ns}, *: p value > 0.05, <*

0.05, Šidák's multiple comparisons test after two-way ANOVA. (G) Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. Heat maps of average expression of ISGs in each cluster for the three lytic samples. The legends on the right define what expression level the colors represent (arbitrary units). Corresponding heatmaps with gene expression in each individual cell can be found in Fig. 3.9B.

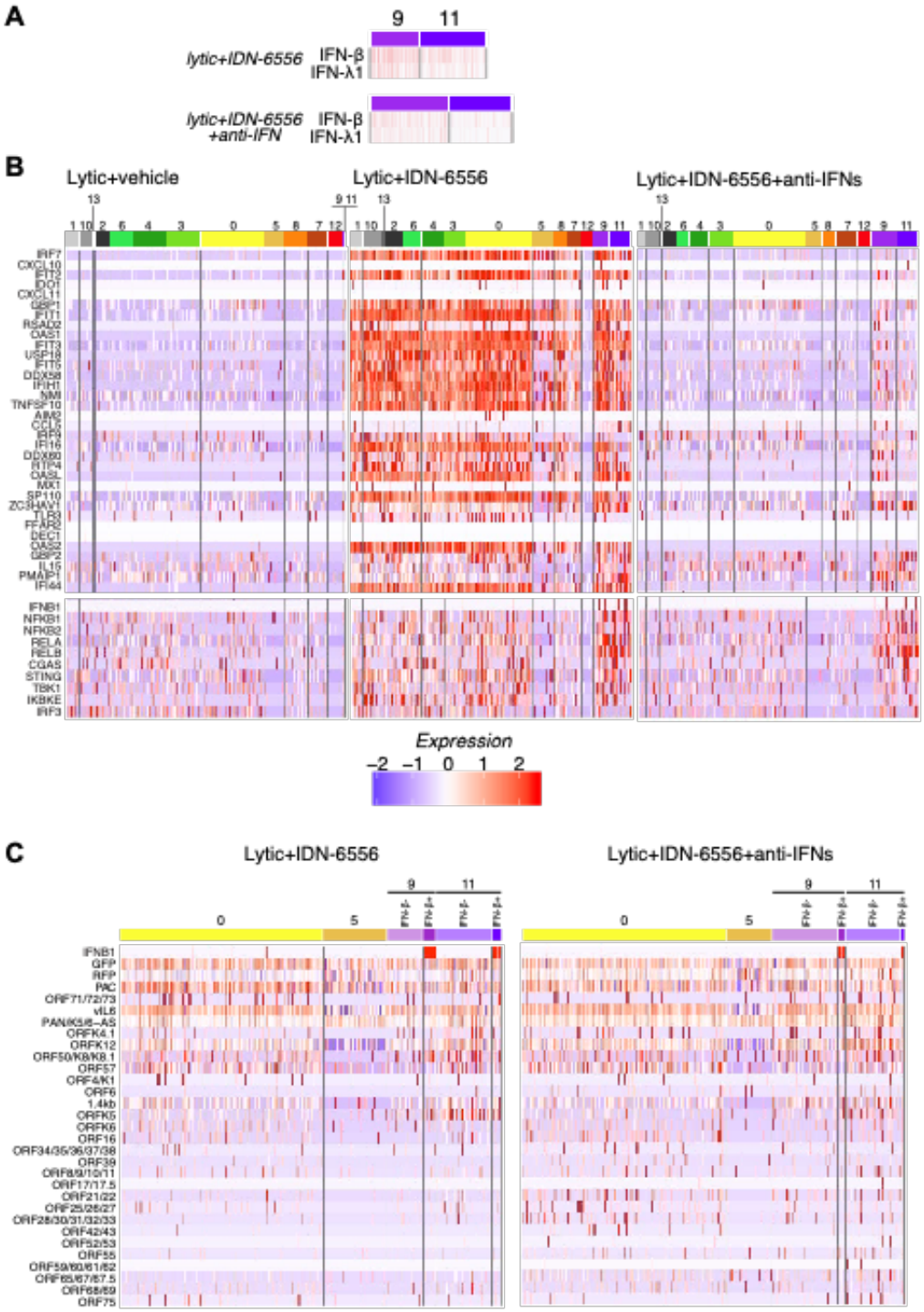

Figure 3.9: Expression patterns of IFNs, ISGs and genes in the type I IFN induction pathway.
Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. (A) Heatmaps of expression of IFN-b and IFN-l1 in each cell in clusters 9 and 11 in the two IDN-6556 treated samples. (B) Heatmaps of expression of ISGs (top) and select genes from the type I IFN induction pathway (bottom) in each cell in the three lytic samples, sorted by cluster. (C) Heatmaps of viral gene expression in each cell in clusters 0, 5, 9, and 11, with cells in 9 and 11 divided by IFN-b status, in the indicated samples. The legend defines what expression level the colors represent (arbitrary units).

3.2.6. The heterogeneity of type I IFN induction is not easily explained by differential viral gene expression

We considered the possibility that only a subset of KSHV-infected cells makes IFN-β due to differential expression of viral genes. For example, IFN-β+ cells could lack expression of KSHV-encoded anti-IFN factors. Consistent with this idea, cells in the IFN-β+ clusters (clusters 9 and 11) expressed only early lytic genes and low levels of the RFP lytic marker (**Fig. 3.4H, 3.10A, Fig. 3.3B, 3.9C**). However, their viral gene expression pattern was similar to that of other clusters that do not express IFNs, especially cluster 0 (**Fig. 3.4H, 3.10A, Fig. 3.5B, 3.9C**). Moreover, the viral gene expression pattern is also similar between IFN-β- and IFN-β+ cells within cluster 9 and 11 (**Fig. 3.10A, Fig. 3.9C**). One notable exception was the K5 mRNA, which was differentially expressed depending on IFN status. However, expression of K5 was actually higher in the IFN-β+ cells, rather than lacking in these cells. These observations suggest that lack of expression of viral anti-IFN regulators does not explain the restricted IFN-β induction. Another possibility is that some cells express higher basal levels of genes in the type I IFN induction pathway and are thus more sensitive to IFN-inducing cues. This model was proposed in the case of other viral infections by Zhao *et al.*[199]. Analyzing these genes is complicated by the fact that many of them are themselves ISGs. Indeed, their expression was increased in all clusters (except cluster 12) in the

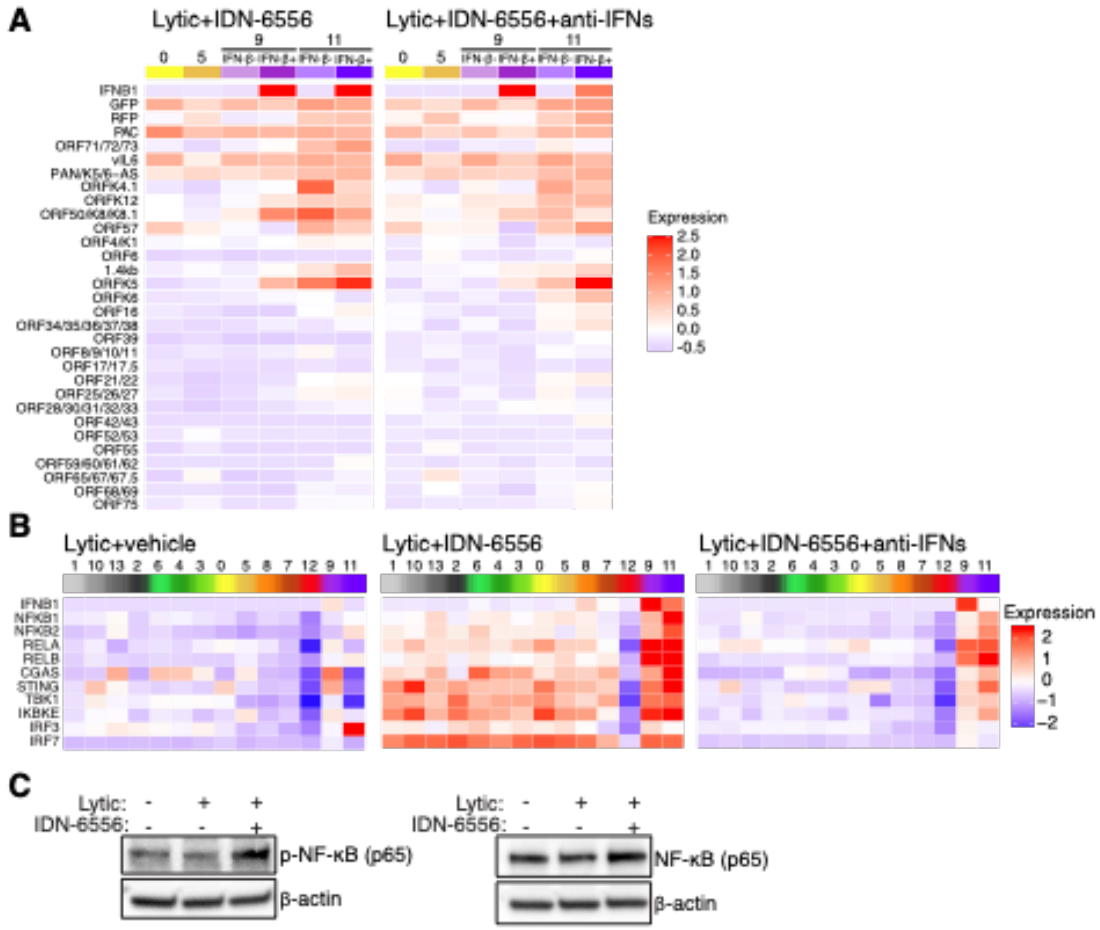

Figure 3.10: IFN-β-producing cells express high levels of NF-κB transcripts. *(A-B) Analysis of data from the scRNA-Seq experiment presented in Fig. 3.4A. Heatmaps of average expression of KSHV genes in clusters 0, 5, 9, and 11, with 9 and 11 divided based on IFN-b status (A) and of average expression of select genes from the type I IFN induction pathway in all clusters (B) for the indicated samples. The legends on the right define what expression level the colors represent (arbitrary units). Corresponding heatmaps with gene expression in each individual cell can be found in Fig. 3.9B and 3.10C. (C) iSLK.219 were treated with doxycycline to reactivate the lytic cycle ("lytic") and with IDN-6556 where indicated. Protein lysates were collected at day 4 and analyzed by western blotting for NF-κB, phospho-NF-κB (p-NF-κB) and β-tubulin as a loading control. Blots are representative of 3 replicates.*

caspase inhibitor-treated samples by IFN signaling, and was reduced back to basal levels by anti-IFN antibody treatment (**Fig. 3.10B, Fig. 3.9B**). Nonetheless, there was a clear enrichment of transcripts for the NF-κB transcription factor family members (NFKB1, NFKB2, RELA, RELB) in the IFN-β+ clusters 9 and 11 (**Fig. 3.10B, Fig. 3.9B**). NF-κB is also needed for IFN-β transcription downstream of pathogen sensors, together

with IRF3[137]. Indeed, levels and phosphorylation of NF-κB were increased in lytic cells treated with IDN-6556, showing that caspase activity also modulates NF-κB activation (**Fig. 3.10C**). It is thus possible that high NF-κB expression is needed to induce type I IFN in these cells, perhaps as a feed-forward mechanism to ensure commitment to IFN production

3.3. Discussion

This study provides mechanistic insights into multiple levels of type I IFN regulation during KSHV infection. We find that caspases regulate cGAS activity during KSHV lytic infection, likely indirectly, either through a cGAS regulator/cofactor or by modulating access of cGAS to its stimulating DNA (**Fig. 3.1, Fig. 3.3**). Moreover, we find that although caspase inhibition results in a strong type I IFN induction, type I and III IFNs are only turned on in a very small percentage of cells. IFN expression appears to correlate with strong induction of NF-kB transcripts, rather than differences in viral gene levels.

The cGAS/STING pathway has emerged in recent years as a crucial component of the innate immune response to DNA viruses and various other pathogens[200]. This is highlighted by the multitude of strategies employed by pathogens[201], including herpesviruses[202], to disrupt the cGAS/STING pathway. While this immunosuppression is often driven by virus-encoded proteins, we have uncovered an intriguing mechanism in KSHV infection, which involves the hijacking of the host cell caspases to subvert immune activation and viral clearance[139]. During apoptosis and other viral infection, caspase-3 and -7 have been reported to directly cleave cGAS[186]. However, our previous results suggest that caspase-8 activity mediates type I IFN suppression in KSHV-infected cells without triggering apoptosis, whereas caspase-3/7 activity is

dispensable[139]. Moreover, we have been consistently unable to detect cleavage fragments or downregulation of cGAS in infected cells. Together, our previous report and the current study suggest an unexpected type I IFN regulatory function of caspase-8 activity in targeting the cGAS/STING pathway that is separate from its apoptotic activity. Perhaps caspases like caspase-8 cleave regulators of the cGAS/STING pathway. Alternatively, they may affect access of the DNA to cGAS. The latter is hard to test, because we do not know what the cGAS stimulating DNA is during KSHV reactivation. We have previously reported that viral DNA replication is not required for IFN induction[139]. Moreover, we have found that depletion of mitochondrial DNA, a common stimulus of cGAS, reduced reactivation of the lytic cycle, confounding analysis of IFN induction (data not shown). Identifying potential relevant caspase targets will be important to determine how they regulate cGAS activity. Nonetheless, caspases are an attractive therapeutic target that could be exploited to potentiate cGAS/STING-mediated immune responses for viral clearance. This could have far reaching implications for other pathologies, as regulation of the cGAS/STING pathway is fundamental in various inflammatory diseases and in tumor immunity[203,204], and thus is a current focus of intensive drug discovery research efforts.

The notable cellular heterogeneity of type I IFN production reveals an additional layer of complexity and regulation of innate immune responses during KSHV infection. We detect an exceptionally small fraction of IFN-β+ cells upon caspase inhibitor treatment (< 5%, **Fig. 3.8B**). Remarkably, this small group of IFN-β+ cells still induces a robust IFN response in the whole population (**Fig. 3.8D, F**). On a practical level, the high heterogeneity in IFN-β expression complicates our analysis of caspase regulation of cGAS. It is possible that the upstream portions of the pathway (i.e. cGAS) are also only active in the IFN-β+ cells, and that cell sorting may be needed to detect differences in the levels of the direct target(s) of caspases in IFN regulation. Alternatively, it is possible

that only a subset of the cells in which the IFN induction pathway is activated end up expressing IFN-β. In support of this idea, we were able to easily detect phosphorylation of many of the components of the IFN induction pathway using bulk analysis (**Figs. 3.1E, 3.3C, 3.10C**).

Single-cell RNA-Seq studies in herpesviruses have started to come out in the last few years, but many have not analyzed IFN responses, focusing instead on latency and reactivation[205–210]. However, several studies in multiple viruses have examined IFN induction, and have also showed that only a small number of cells respond to infection in a population during infection with a number of other viruses[201,207,211–217]. Interestingly, studies that investigated infections in innate immune cells like macrophages and dendritic cells or fibroblasts saw higher percentages of responding cells (10-30%)[199,211,212]. In contrast, iSLK.219 cells are of epithelial origin[218]. Epithelial cells are often the first exposed to invading pathogens and mount responses that alert and prime surrounding cells to counteract viral spread. Therefore, it is possible that epithelial cell responses are even more tightly regulated to prevent inadvertent activation of the pathway. Consistent with this idea, influenza A virus and SARS coronavirus 2 infection of epithelial cells also elicit responses in a small minority of cells[213–215]. Moreover, while we did not perform scRNA-Seq on BC3 cells, flow cytometry analysis of BC3 cells encoding an IFN reporter also indicate that IFN-β is induced in less than 2% of these cells (**Fig 3.8F**). Taken together, our findings suggest differences in IFN responsiveness depending on the cell type as well as the virus infecting the cells.

It is unclear what the source of interferon heterogeneity is in KSHV-infected cells. Viral gene expression, particularly expression of viral immune regulators, is often considered the reason for limited type I IFN responses[215]. A previous study in herpes simplex virus 1 reports that type I IFN signaling only occurs in a small percentage of cells that are all abortively infected[207]. This is also a potential explanation for our results,

since we find that the cells that make IFNs only express a subset of viral genes, mostly early genes (**Fig. 3.4H, 3.10A**). However, this pattern of gene expression is not unique to the small percentage of IFN-β+ cells. Multiple clusters of cells do not express high levels of late lytic genes. Indeed, the pattern of viral gene expression is generally similar in clusters 0, 5 (IFN-β negative) and 9, 11 (IFN-β positive) (**Fig. 3.10A, Fig. 3.9C**). Moreover, when we compare IFN-β positive and negative cells within the same clusters (clusters 9 and 11), we find that they also have a similar viral gene expression pattern (**Fig. 3.10A, Fig. 3.9C**). We also do not know whether this gene expression pattern represents abortive infection, or simply an early stage of reactivation that facilitates IFN induction, since we know that iSLK.219 reactivation is heterogeneous. This also means that we cannot distinguish a situation whereby progression to the late stage of the lytic cycle prevents IFN-β induction from one where IFN-β induction prevents progression through the lytic cycle. It thus appears that abortive or early-stage infection alone does not explain the restricted pattern of IFN gene expression in our experiments. Our results also do not support the idea that some cells express IFN because they are missing expression of a viral negative regulator of type I IFN, although we cannot fully exclude this model because we do not reliably detect all viral genes. However, we did find that the KSHV gene K5 displayed much higher expression in the IFN-β-positive cells (**Fig. 3.10A, Fig. 3.9C**). This was surprising to us as we would not expect viral genes to exhibit positive regulation on IFN induction. There is no known role of K5 in regulating type I IFN signaling, so it is unclear why its expression correlates with IFN-β expression at present. Future studies will hopefully elucidate this correlation.

Our results point to the possibility that inherent differences in the cells govern the heterogeneity of the interferon response. Stochasticity in gene expression, which is common among cytokines, has previously been proposed as driver of type I IFN expression heterogeneity. Zhao *et al*. suggested some of this stochasticity may be

epigenetically encoded[199], reducing the number of cells that are able to express type I IFN, perhaps to prevent over-activation and inflammation. Paracrine IFN signaling is also thought to influence and modulate heterogeneity[216]. This cell-cell communication may fine tune immune signal amplification in a cell density-dependent manner. Indeed, we observe 3.8% IFN-β+ cells in the IDN-6556 treated sample, but only 1.6% IFN-β+ cells in the sample treated with IDN-6556 and anti-IFN antibodies that block IFN signaling. Moreover, while cells in cluster 9 retain high average IFN-β expression even when IFN signaling is blocked, cluster 11 cells have lower IFN-β without IFN signaling (**Fig. 3.10A, Fig. 3.9A, C**), suggesting two different modes of IFN induction in these groups of cells. This reinforces the notion of self-amplification of the type I IFN response as a mechanism to ensure robust responses only in the appropriate contexts for infection control, while avoiding tissue damage from unnecessary inflammation.

3.4. Acknowledgments

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3.5. Declaration of interests

The authors declare no competing interests.

3.6. Methods

3.6.1. Cell lines, constructs, reagents, and treatments

All cells were cultured at 37 °C and 5% CO_2 conditions. iSLK.219, iSLK.219-IFNBp-BFP, iSLK.RTA[147], and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS) (HyClone). BC3 cells were grown in RPMI supplemented with 20% FBS, 2 mM GlutaMAX supplement, and 50 μM β-mercaptoethanol (Gibco/Thermo Fisher). iSLK.219 cells were reactivated with 1 μg/ml of doxycycline (Thermo Fisher). Reactivation was confirmed visually for all iSLK.219 samples by examining the appearance of RFP-positive cells (RFP is driven by the lytic PAN promoter). BC3 cells were reactivated with 20 ng/ml of TPA for 48 h. iSLK.219-IFN-bp-BFP cells were generated by transducing iSLK.219 cells with lentiviruses containing pJP1_IFNBp-tagBFP and BC3-IFN-bp-tdTomato by transducing BC3 cells with pLJM1_2xIFNBp-tdTomato. All pLJM and pJP1 are based on pLJM1-GFP, a gift from David Sabatini (Addgene plasmid # 19319 ; http://n2t.net/addgene:19319 ; RRID:Addgene_19319)[219]. For pJP1_IFNBp-tagBFP, the CMV promoter of pLJM1 was replaced with a human IFN-b promoter fragment subcloned from a luciferase reporter construct (kind gift of Michaela Gack) and the GFP sequence was replaced with a TagBFP sequence. In addition, the antibiotic resistance gene was changed from a puromycin resistance to a zeocin resistance gene. For pLJM1_2xIFNBp-tdTomato, the CMV promoter and multiple cloning sites were replaced with two copies of a human IFN-b promoter 303 bp fragment cloned from IFN-Beta_pGL3, a gift from Nicolas Manel (Addgene plasmid # 102597 ; http://n2t.net/addgene:102597 ; RRID:Addgene_102597)[220]. To add the 54 bp 5' untranslated region of human IFN-b, sequences were added to the primers used to assemble the construct. Lentiviral packaging was carried out using packaging plasmids pMDLg/pRRE (Addgene plasmid # 12251 ; http://n2t.net/addgene:12251 ;

RRID:Addgene_12251), Addgene plasmid # 12253 ; http://n2t.net/addgene:12253 ; RRID:Addgene_12253) and pMD2.G (Addgene plasmid # 12259 ; http://n2t.net/addgene:12259 ; RRID:Addgene_12259), kind gifts from Didier Trono[221]. Where indicated, cells were treated with vehicle (dimethyl sulfoxide [DMSO]; Sigma-Aldrich), 10 μM IDN-6556 (MedChem Express or Selleck Chemicals), 24.1 μM RU.521 (Invivogen), 1:50, 1:100, or 1:500 dilutions of a mixture of neutralizing antibodies against type I IFNs (PBL Assay Science, catalog # 39000-1). The concentrations of all drug treatments were chosen based on common concentrations used in previously published studies.

3.6.2. siRNA knockdown

iSLK.219 cells were transfected while in suspension (reverse transfection) with 10 nM siRNAs (purchased from Thermo Fisher) against STING (HSS139156), MAVS (HSS148537), cGAS (siRNA#1, HSS132955; siRNA#2, HSS132956), IFI16 (siRNA#1, HSS105205; siRNA#2, HSS105206), RIG-I (siRNA #1, s24144; siRNA #2, s223615), or a nontargeting siRNA (12935300) at a density of 16,700 cells/cm^2 using the Lipofectamine RNAiMAX transfection reagent (Life Technologies/Thermo Fisher) per the manufacturer's protocol. Six hours later, the medium was replaced. The lytic cycle was induced two days later. For cGAS and RIG-I knockdowns, the reverse transfection process was done twice, the first time two days prior to induction and a second time on the day of lytic cycle induction. All siRNAs were obtained from Life Technologies/Thermo Fisher.

3.6.3. Realtime quantitative polymerase chain reaction (RT-qPCR)

For RNA analysis, iSLK.219 cells were seeded at a density of 33,000 cells per well in a 24-well plate, and the lytic cycle was induced by the addition of doxycycline (1 μg/ml). RNA samples were collected at the indicated time points in RNA lysis buffer (Zymo Research). BC3 cells were seeded at 500,000 cells per ml and induced using TPA for 48 h. One ml of the cells was collected, and the cell pellet was lysed in RNA lysis buffer (Zymo Research). Total RNA was extracted using the Quick-RNA MiniPrep kit (Zymo Research) by following the manufacturer's protocol. For mRNA measurements, cDNA was prepared using an iScript cDNA synthesis kit (Bio-Rad) per the manufacturer's protocol. In all cases, 18S rRNA levels were used as an internal standard to calculate relative mRNA levels. Real-time quantitative PCR (RT-qPCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in a CFX Connect real-time PCR detection system (Bio-Rad). No-template and no-RT controls were included in each replicate. CFX Manager software was used to analyze the data. Primers are listed in Table S3.

3.6.4. Protein analysis

iSLK.219 cells were seeded at a density of 167,000 cells per well in a 6-well plate and treated with doxycycline (1 μg/ml). Lysates were collected at the indicated time points. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) or an NP-40-only buffer for caspase blots and phospho-protein blots (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with 50 μg/ml phenylmethylsulfonyl fluoride (PMSF; G-Biosciences) and cOmplete protease cocktail inhibitor (Roche). Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The following Cell Signaling Technologies antibodies were used on PVDF membranes at 1:1,000 dilution in 5% milk in in Tris-

buffered saline with 0.1% Tween 20 (TBST): anti-cGAS (D1D3G) (no. 15102). The following Cell Signaling Technologies antibodies were used with PVDF at 1:1,000 dilution in 5% BSA in TBST: anti-caspase-8 (no. 9746), anti-cleaved caspase-8 (no. 9496), anti-IRF3 (D83B9) (no. 4302), anti-phospho-IRF3 Ser396 (4D4G) (no. 4947), anti-TBK1 (D1B4) (no. 3504), anti-phospho-TBK1 Ser172 (D52C2) (no. 5483), anti-STING (D2P2F) (no. 13647), anti-phospho-STING Ser366 (D7C3S) (no. 19781), anti-RIG-I (D14G6) (no. 3743), anti-NF-kB p65/RelA (D14E12) (no. 8242S), anti-phospho-NF-kB p65 Ser536/RelA (93H1) (no. 3033) and anti-β-tubulin (9F3) (no. 2128). Anti-IFI16 (ab169788; 1:1000, Abcam) and anti-β-actin (ab8229; 1:500, Abcam) were diluted in 5% BSA in TBST and used with PVDF membranes blocked in 5% BSA in TBST. Anti-MAVS (sc-166583, 1:1000 dilution; Santa Cruz Biotechnology) was diluted in 0.5% milk in phosphate-buffered saline with 0.1% Tween 20 (PBST) and used with PVDF membranes blocked in 5% milk in PBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (both 1:5,000 in blocking buffer) were purchased from Southern Biotechnology. HRP-conjugated donkey anti-goat IgG (1:5,000 in 0.5% milk in PBST) was purchased from Santa Cruz Biotechnology. All membranes were exposed using Pierce ECL Western blotting substrate (Thermo Fisher) and imaged with a Syngene G:Box Chemi XT4 gel doc system.

3.6.5. cGAMP ELISA

Cell lysates were harvested 4 days post reactivation in RIPA and subjected to enzyme-linked immunosorbent assay (ELISA) analysis for 2’3’-cGAMP (Cayman Chemical) according to the manufacturers' protocols. Each biological replicate consisted of three technical replicates per condition.

3.6.6. Flow cytometry analysis

10^6 IFN-bp-TagBFP cells were treated with DMSO, IDN-6556 (10 μM), docycline (1 mg/ml) or doxycycline and IDN-6556 for 4 days to induce KSHV reactivation. 10^6 BC3-IFN-bp-tdTomato cells were treated with DMSO, IDN-6556 (10 μM), TPA (20 ng/mL), or TPA and IDN-6556 for 48 hours to induce KSHV reactivation. Cells were fixed in 4% PFA for 15 minutes at room temperature and washed in PBS before being analyzed on a BD Biosciences LSR II flow cytometer in the Tufts Laser Cytometry Core facility. 500,000 events were collected per sample. TagBFP, RFP, and tdTomato expression was gated based on the corresponding latently infected and vehicle (DMSO) treated cells.

3.6.7. Single cell RNA sequencing

iSLK.219 and iSLK.RTA cells were seeded onto T25 flasks at a density of 16,700 cells/cm^2 and induced with doxycycline in the presence or absence of IDN-6556 (10 μM) and a mixture of neutralizing antibodies against type I IFNs at a 1:500 dilution (39000-1; PBL Assay Science). The media was replaced at day 2 post reactivation with fresh doxycycline, IDN-6556, and type I IFN neutralizing antibodies. Cells were harvested at day 4 post reactivation, washed twice with PBS, and strained to create single cell suspensions at a concentration of 1,000,000/ml. Single cell samples were generated according to 10X Genomics' sample preparation protocol for cultured cell lines (CG00054) and Gel Beads-in-emulsion (GEM) generation protocol (CG000183). Briefly, cells were trypsinized and washed twice with Dulbecco's phosphate buffer saline (DPBS, Life Technologies). Cells were resuspended in DPBS and passed through a 30 μm strainer to remove clumps. Cells were counted and diluted to a concentration of 1,000,000 cells/mL. Cells were mixed with a reverse transcription mastermix (including a primer with polydT anchor) and loaded onto a Chromium B microfluidic chip. Gel beads

and partitioning oil were loaded onto the chip and the chip was run through the chromium controller device to generate the GEMs. GEMs were incubated in a thermal cycler for 1 cycle to generate cDNA. The emulsion was broken, and cDNA was isolated with magnetic beads, amplified for 12 cycles, and then cleaned up with SPRIselect reagent.

Libraries were prepared according to 10X Genomics library construction protocol (CG000183). Briefly, cDNA was fragmented and then end-repaired and A-tailed with the enzymes in the provided 10X Genomics kit (version 3, PN-1000092). cDNA was size selected using SPRIselect and adaptor oligos were ligated onto the cDNA and further cleaned up with SPRIselect. Paired-end sequencing with 75 cycle read length was performed on an Illumina Nextseq 550 at Tufts Genomics Facility. Raw sequencing data (.bcl files) were processed using the 10X Genomics CellRanger (3.0.0) software. Fastq files were generated for the reads using the mkfastq function as described on the 10X Genomics website (https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/mkfastq). Also using CellRanger, the count function was used to align the reads to a reference genome including the human genome (GRCh38), the KSHV genome (GQ994935.1), and the additional sequence of the RFP/GFP/PAC insertion cassette (Genbank MZ617352). This third reference was added because the GQ994935.1 reference genome corresponds to the KSHV BAC16 genome, whereas our cells contain the rKSHV.219 virus[147,222]. To our knowledge, a full reference for rKSHV.219 is not available. We thus amplified the missing region and sequenced it using Sanger sequencing. The matrix table of expression for each gene in each single cell from CellRanger was used as an input for integration and clustering analyses using the R-based Seurat package (version 3.1.1.9021, R version 3.6.1)[192,193]. Cell cycle analysis was done according Tirosh *et al.*[223]. Raw and processed data are available on NCBI GEO, record # GSE190558 (reviewer token: gzqbaqcqfpofdqd).

Scripts used for analysis are available on GitHub, https://github.com/mgaglia81/iSLK.219_scRNAseq. The matrices were converted to Seurat objects and then pre-processed by performing quality control analyses. First, the four objects from the different samples were integrated together into one object using Seurat functions FindIntegrationAnchors() and IntegrateData(). Dying cells with mitochondrial contamination or possible cell doublets were filtered out by eliminating cells that have >20% mitochondrial RNA counts and cells that have unique feature (RNA) counts > 7500. Feature expression measurements for each cell were normalized by the total expression, multiplied by a scale factor (10,000) and log-transformed using the Seurat function NormalizeData(). The expression was scaled so that the mean expression across all cells is 0 and the variance is 1, using the Seurat function ScaleData(). For linear dimensional reduction, principal component analysis (PCA) was performed using the Seurat function RunPCA(). Only the PCs that account for the most variation were included, to overcome technical noise when clustering cells. These were chosen by visualizing each PC with Seurat functions JackStrawPlot() and ElbowPlot(), and determining that variance was negligible after 30 PCs. Therefore, UMAP cluster plots were created using dimensions 1-30.

3.6.8. Statistical analysis

All statistical analysis was performed using GraphPad Prism version 8.4.2 or later (GraphPad Software, La Jolla California USA, www.graphpad.com). Statistical significance was determined by Student's *t*-test or analysis of variance (ANOVA) followed by a *post hoc* multiple comparison test (Dunnett's, Sidak's or Tukey's) when multiple comparisons were required. All data are plotted as mean ± standard deviation.

3.7. Supplemental information

Supplementary Table 3.3 – Primers used for RT-qPCR.

3.8. Author contribution

Tate Tabtieng performed the scRNAseq experiment and conducted all of the scRNAseq analysis, with the exception of Figure 3.9A which was performed by Machika Kaku. Machika Kaku also performed the experiment for Figure 3.8A. Tate Tabtieng contributed some or all replicates for Figures 3.1, 3.2A-C, and 3.3B,D,E. The rest of the experiments and the majority of manuscript writing was performed by Rachel Lent.

Chapter 4. Discussion

4.1. Main conclusions

In the past ~14 years, intensive research on caspases has uncovered non-canonical roles for these enzymes. They are thus now appreciated as more complex, multifunctional signaling molecules. Notably, several classically apoptotic caspases are now recognized for their immunomodulatory functions, particularly in regard to IFN regulation. Before I began my thesis work in 2019, our lab had discovered that caspase-8 activity inhibits type I IFN induction and secretion during KSHV lytic infection without triggering apoptotic cell death[98]. While numerous immunosuppressive functions of KSHV have been described, the vast majority are enacted by viral encoded proteins. Thus, our lab's discovery is not only novel in revealing an immunomodulatory, non-apoptotic function of caspase-8, but also showed how KSHV cleverly hijacks host cellular machinery to dampen an innate immune response and replicate more efficiently. However, two major facets remained unclear about how KSHV usurps caspase-8 to impede the type I IFN response: how caspase-8 becomes non-apoptotically activated during lytic infection and how caspase activity interferes with IFN signaling. My thesis work has focused on elucidating both of these mechanistic aspects to paint a more comprehensive picture of this pro-viral immune regulation by caspase-8.

4.1.1. Caspase-8 is activated through the TLR3-TRIF pathway and not through canonical death receptor signaling during KSHV lytic infection

I was surprised to find that FADD, a key signaling adaptor protein for death receptor signaling that is almost always found to be required for caspase-8 activation, was dispensable for caspase-8 activation during KSHV lytic infection (**Fig 2.1A-B**). This revealed that the IFN-regulatory function of caspase-8 during KSHV lytic infection is

mediated by its non-canonical activation through a death receptor-independent signaling pathway. Moreover, lytic reactivation predominantly triggers only partial processing of caspase-8 to its non-apoptotic, yet still enzymatically active, p43/41 form (**Fig 2.1C**). This partial activation of caspase-8 is in line with the lack of caspase-mediated apoptotic cell death observed during KSHV lytic infection[98]. This is an important aspect that contributes to the pro-viral role of caspase-8 during KSHV infection, as cell death would impede viral replication.

KSHV infected iSLK.219 and BC3 cells are capable of triggering full caspase-8 activation to its apoptotic form when treated with the death receptor ligand TNF (**Fig 2.1C**). To test if any anti-apoptotic conditions induced during KSHV lytic infection can inhibit this death receptor-mediated full caspase-8 activation, I treated both latently and lytically infected cells with TNF to measure the extent of caspase-8 activation (**Fig 4.1**).

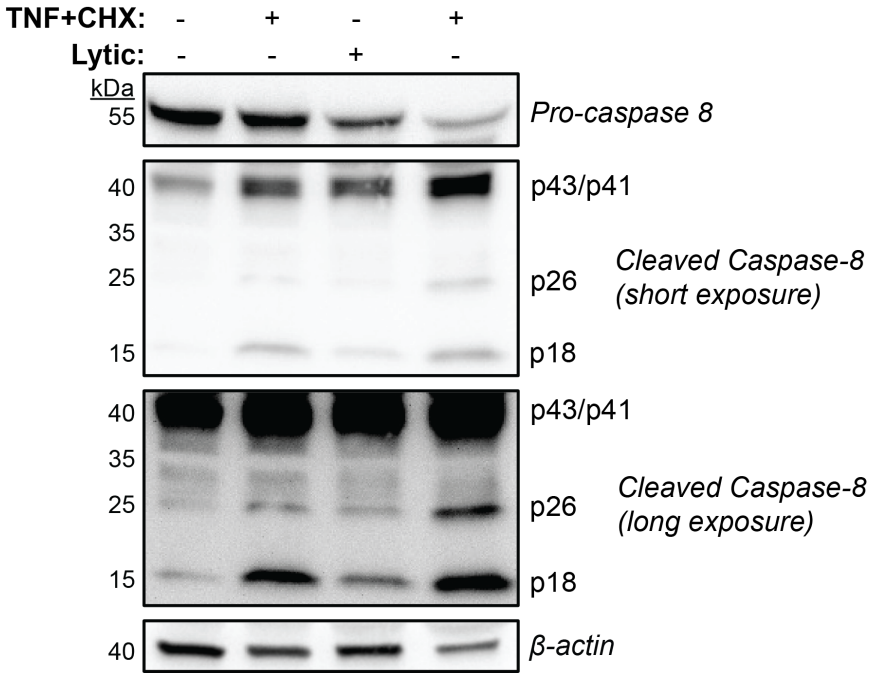

Figure 4.1: Caspase activation upon KSHV lytic reactivation and death receptor stimulation.
(A-B) BC3 cells were either lytically reactivated for 2 days and/or treated with TNF and CHX for 6h where indicated. Samples were collected to assess caspase-8 activation by western blot.

Lytic reactivation of KSHV did not prevent full caspase-8 activation from death receptor activation. This suggests that the death receptor-independent route of caspase-8 activation during KSHV lytic infection is at least partially responsible for its processing to the non-apoptotic p43/41 form and lack of subsequent cell death.

By probing for alternative mechanisms of caspase-8 activation, I revealed that this activation occurs non-canonically through the pathogen sensing TLR3-TRIF pathway (**Figs 2.2, 2.3**). While TLR4/TRIF signaling also facilitates caspase-8 activation[125–127], I found no evidence of TLR4 being active in either iSLK.219 or BC3 cells (**Fig 2.4**), suggesting that it is not driving caspase-8 activation. Although caspase-8 activation through the TLR3-TRIF pathway has been previously reported, it has only been demonstrated via artificial TLR3 stimulation with poly(I:C)[128–131,133–135]. Thus, my findings are the first to our knowledge to reveal TLR3-TRIF-mediated caspase-8 activation during natural virus infection. However, it is likely that other viral infections also trigger this signaling pathway, as many viruses, including other DNA viruses, trigger TLR3 signaling. It is unclear how KSHV and several other DNA viruses activate the RNA-sensing TLR3-TRIF pathway (as discussed below in section 4.2.3). However, the cellular conditions induced during KSHV lytic infection clearly sensitize cells to TLR3-driven caspase-8 activation as I find that lytically infected, but not latently infected, cells have robust caspase-8 activation following poly(I:C) treatment (**Fig 2.5**).

4.1.2. The upregulation of TLR3 during KSHV infected cells is sufficient to activate caspase-8 and potentiates further caspase-8 activation upon TLR3 stimulation

Activation of the KSHV lytic program orchestrates widespread changes in host and viral gene expression that could potentiate TLR3-driven caspase-8 activation. However, a notable change that I observe upon KSHV lytic reactivation is robust upregulation of TLR3 (**Fig 2.5**). This corroborates a previous report of TLR3 upregulation during *de novo*

infection of KSHV in human monocytes[152]. As the upregulation of TLR3 is thought to potentiate its activation in other contexts[154–162], I hypothesized that this upregulation was responsible for the sensitization of lytically infected cells to ligand-induced, TLR3-driven caspase-8 activation. Intriguingly I found that TLR3 overexpression alone in latently infected cells, without any external stimulus, was sufficient to trigger caspase-8 activation. This could possibly explain how caspase-8 is activated through the TLR3-TRIF pathway upon lytic reactivation of KSHV, a DNA virus that has no clear mechanism of engaging TLR3 with an RNA ligand. This is further discussed in section 4.2.3.1.

4.1.3. Caspase activity likely inactivates an unknown cGAS regulator to suppress cGAS-STING, thus creating negative regulatory crosstalk between two pathogen sensing pathways

The cGAS-STING pathway is the best characterized DNA sensing pathway as it is crucial for mounting an immune response to both self and foreign DNA in numerous contexts[44], including during KSHV infection. We revealed that caspase activity impedes IFN induction specifically through the cGAS-STING pathway by inhibiting cGAS activity (**Figs 3.1, 3.3**). These findings suggest that caspase activity either acts directly on cGAS or a regulator of cGAS to restrict its enzymatic activity. In support of caspase activity targeting a cGAS regulator, we do not detect any cGAS cleavage products or a reduction in cGAS protein levels upon lytic reactivation (**Fig 3.1C**). Furthermore, we exogenously co-expressed cGAS and caspase-8 in HEK293T cells to assess if this would enhance the detectability of any caspase-8-mediated cGAS cleavage. Caspase-8 over-expression induces its autoproteolytic cleavage and activation, as demonstrated by its cleavage fragments and caspase-3 and -7 activation (**Fig 4.2**). We found that co-expression of caspase-8 with cGAS still yielded no detectable cGAS cleavage products, but it did reduce protein levels of cGAS (**Fig 4.2A**).

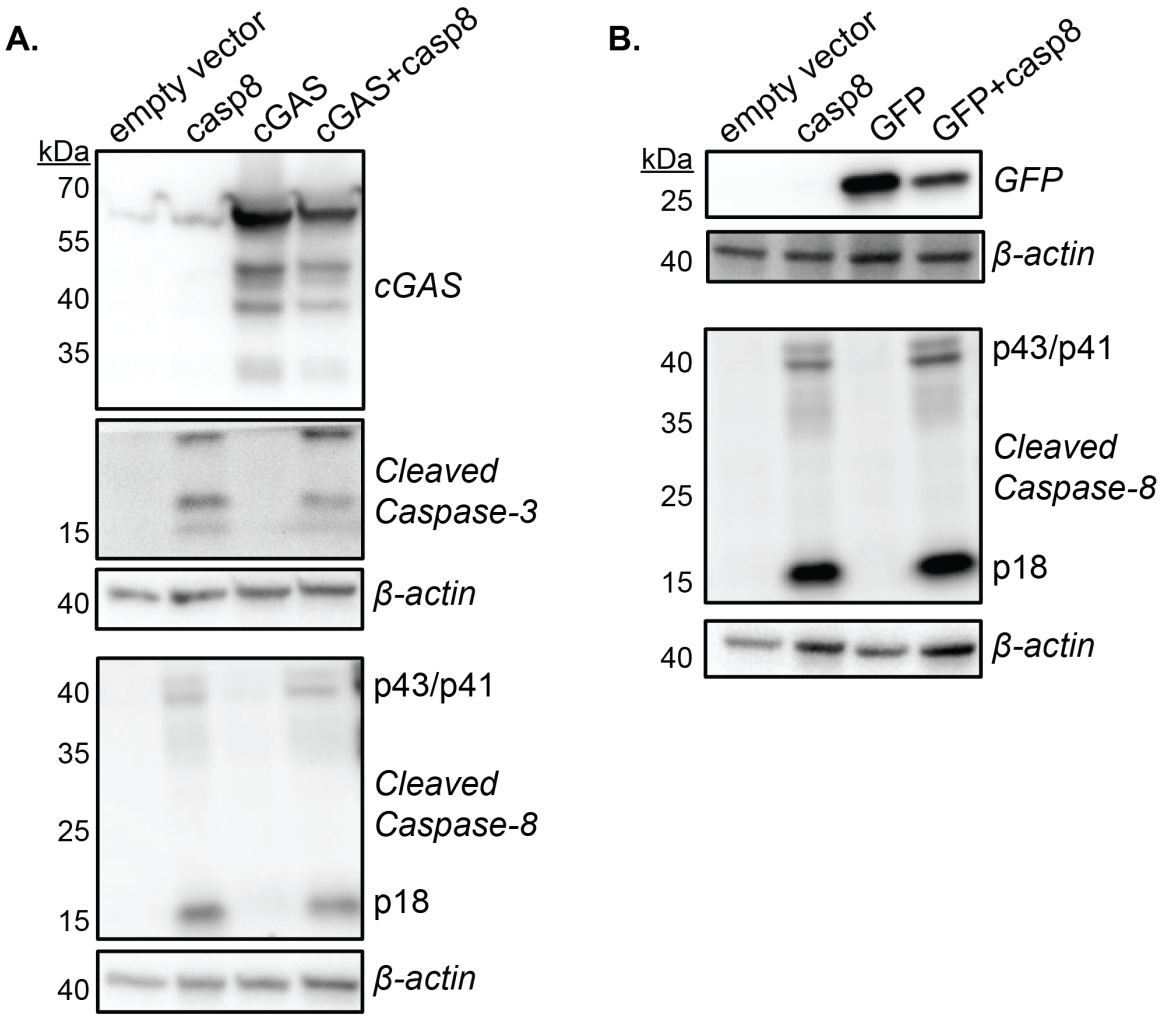

Figure 4.2: Effect of caspase-8 over-expression and activation on cGAS and GFP protein levels.
HEK293T cells were transfected with either an empty vector or a plasmid encoding caspase-8 and/or cGAS (A) or caspase-8 and/or GFP (B). Media was replaced 3h later. After 16h, samples were collected to assess protein expression and caspase activation by western blot.

However, this was likely due to a global reduction in protein expression that resulted from robust caspase-8 activation and subsequent cell death, as co-expression of caspase-8 with GFP similarly reduced protein levels of GFP (**Fig 4.2B**). Taken together, our data suggest that caspase-8 does not directly target cGAS and thus likely cleaves a cGAS regulator to restrict cGAS activity.

4.2. Implications of a virus usurping a pathogen sensing pathway to activate caspase-8

4.2.1. The complex interactions between viruses and TLR3

The importance of TLR3 in mounting antiviral immune responses is exemplified in numerous cell-based studies, mouse models of infection, and human clinical data. Indeed, some TLR3 polymorphisms that impair signaling are associated with increased morbidity in infections with HSV-1 and HCMV, as well as with the EBV-associated nasopharyngeal carcinoma[168–171]. This antiviral potential of TLR3 is also supported by the diverse mechanisms that different viruses have evolved to counteract TLR3-TRIF signaling[224]. However, the TLR3-TRIF pathway has complex and paradoxical roles in viral infections that vary based on external factors such as the virus, cell type infected, and anatomical site of infection[224]. In the case of influenza A virus (IAV)-induced encephalopathy, TLR3 may be protective as a patient with this condition was found to have a TLR3 a loss-of-function mutation[225]. Yet in a mouse model of IAV-induced pneumonia, mice lacking TLR3 have decreased levels of inflammatory mediators and improved survival, despite having higher viral loads[226]. This illustrates that proper balance of host innate immune responses, including TLR3-mediated responses, is crucial to facilitate viral clearance without inducing detrimental hyperinflammation. Indeed, immunopathology-associated damage to host tissues is often the cause of severe morbidity and mortality for IAV and other viruses that present immense public health threats, such as SARS-CoV-2[227,228]. Morbidity and mortality from immunopathology is likely an unintended consequence of viral infections, as viruses require live hosts in order to propagate and thus do not benefit from killing their hosts. Indeed, part of why herpesviruses are such successful pathogens is that they establish

lifelong latent infections in their hosts, which often allows them to persist without triggering immune responses that could lead to their clearance and damage to the host. Intriguingly, some viruses have evolved to utilize TLR3-dependent inflammation to their benefit. For example, West Nile virus takes advantage of the TLR3-dependent inflammation triggered during infection since it permeabilizes the blood brain barrier and in turn facilitates viral entry into the brain[229]. In the case of KSHV, my findings suggest that this virus circumvents innate immune signaling by using TLR3 signaling to trigger caspase-8-mediated negative crosstalk with the DNA sensing cGAS-STING pathway. This crosstalk may have initially evolved to prevent excessive immune activation, as discussed in section 4.3. Nevertheless, KSHV has cleverly hijacked a pathway designed for the protection of the cell to benefit itself. While this control of IFN induction could confer some benefit to the host by preventing potential immunopathology, it would likely still be disadvantageous, as it allows the virus to evade immune detection and clearance. For KSHV, replication in the lytic cycle promotes tumorigenesis by reseeding the pool of latent cells and triggering the secretion of tumor-promoting signals, such as pro-inflammatory cytokines[71]. Thus, the augmentation of KSHV replication by caspase activity may bolster the tumorigenic potential of KSHV infection.

4.2.2. TLR3 and caspase-8-mediated cell death

As discussed in section 1.5.3, several previous studies have described caspase-8 activation through the TLR3-TRIF pathway. While none have demonstrated this during viral infection, it seems likely that other viruses besides KSHV that activate TLR3 would induce caspase-8 activation through this pathway. Nevertheless, all previous studies use poly(I:C) to artificially activate TLR3, which led to apoptosis. This has spurred great interest in manipulating this pathway for destruction of cancer cells. Activation of TLR3

on immune cells had already been widely used in experimental models to promote an antitumor response as it stimulates APCs that trigger tumor-specific T cell responses[230]. However, more recent appreciation for the apoptotic effect of TLR3 stimulation has led to efforts to trigger apoptosis in many types of cancer cells that express TLR3[230]. Indeed, TLR3 expression in Hepatocellular Carcinoma is associated with greater prognosis[231], and *in vitro* evidence supports a role for TLR3-induced apoptosis in this difference[232]. As such, a proposed anti-cancer strategy is manipulating regulators of TLR3-mediated apoptosis[230]. Several cellular and/or viral factors during KSHV lytic infection may prevent apoptotic processing of caspase-8 or downstream apoptotic signaling events that follow its activation. KSHV itself encodes proteins that inhibit apoptosis. One protein known to inhibit caspase-8-mediated apoptosis through death receptor signaling is vFLIP. However, it is unclear whether this is due to direct caspase inhibition or activation of other anti-apoptotic factors[233–235]. Indeed, vFLIP upregulates its cellular homolog cFLIP through NF-kB signaling. cFLIP is a well-known regulator of caspase-8 activation that directly binds caspase-8 and controls the extent of its processing and apoptotic activity. The short isoform of cFLIP ($cFLIP_S$) is known to completely inhibit caspase-8 processing, while the long isoform of cFLIP ($cFLIP_L$) limits caspase-8 processing to the non-apoptotic, partially cleaved p43/p41 form[113,116,182]. It thus seems likely that $cFLIP_L$ would be involved in caspase-8 activation during KSHV lytic infection to facilitate the partial processing that we observe. Interestingly, I observe a robust upregulation of $cFLIP_S$ upon lytic reactivation of iSLK.219 cells. However, this was not the case in BC3 cells. In addition, neither cell type has any clear upregulation of $cFLIP_L$ (**Fig 4.3**). It is surprising that any caspase-8 processing occurs in lytically reactivated iSLK.219 cells with such a potent induction of $cFLIP_S$. One possibility is that $cFLIP_S$ is only binding to DISC-associated caspase-8 to prevent any apoptotic caspase-8 activation through death

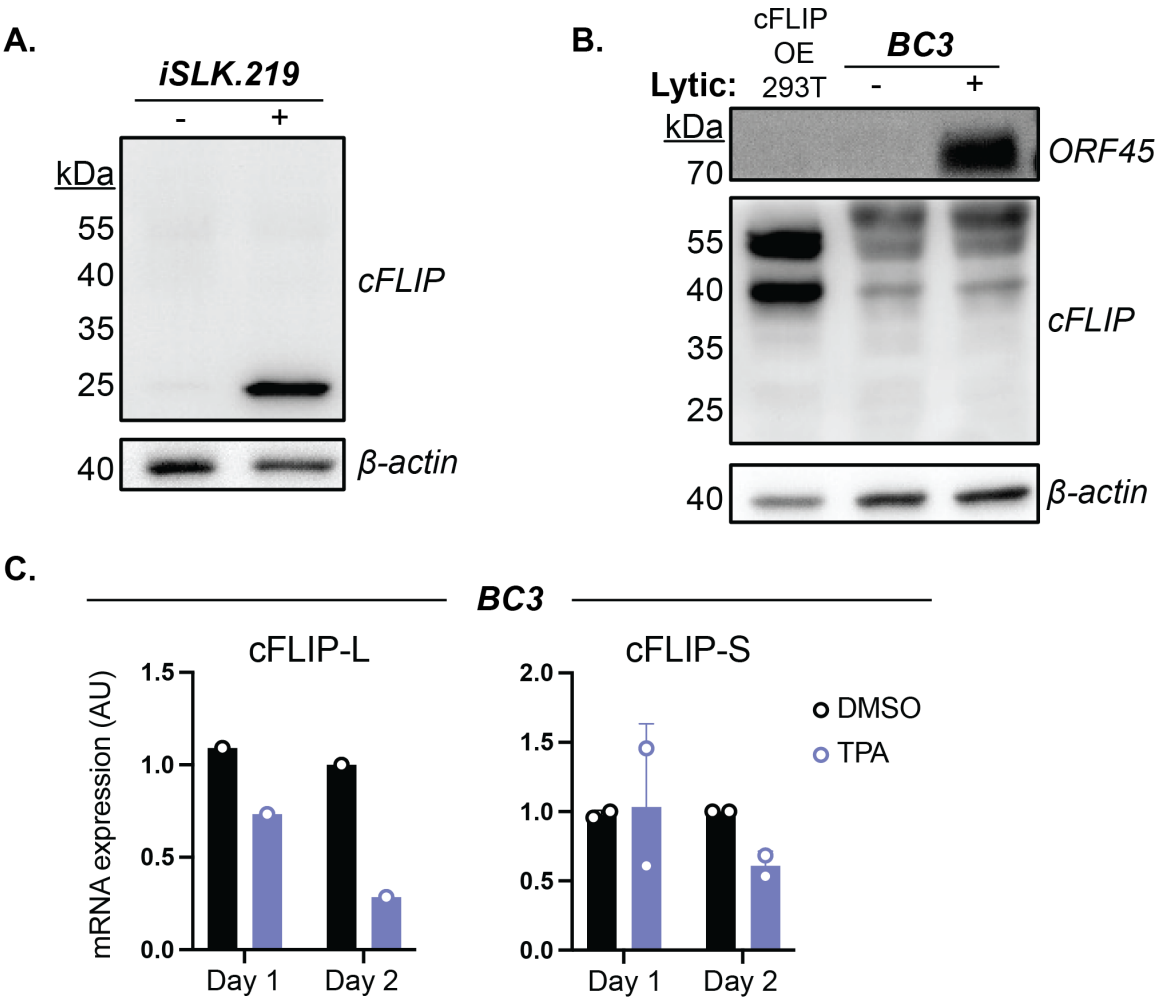

Figure 4.3: cFLIP protein and mRNA levels in latent and lytic cells.
(A) iSLK.219 cells were lytically reactivated for 4 days and samples were collected to assess cFLIP protein levels by western blot. (B) BC3 cells were lytically reactivated for 2 days and samples were collected to assess cFLIP protein expression by western blot. As a positive control for cFLIP expression, lysates from HEK293T cells over-expressing cFLIP were analyzed on the same blot. The KSHV lytic protein ORF45 protein levels were measured as an indicator for lytic cycle induction. Blot is representative of two replicates. (C) BC3 cells were lytically reactivated for 2 days. Total RNA was extracted and the levels of cFLIP-L and cFLIP-S mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA.

receptor signaling. Alternatively, $cFLIP_S$ could also incompletely inhibit caspase-8 activation through the TLR3-TRIF pathway, which may also help to prevent a threshold level of activation that could lead to cell death. It is also worth noting that the lack of $cFLIP_L$ upregulation does not rule out its involvement in controlling the extent of caspase-8 activation. Basal $cFLIP_L$ levels may be sufficient for this function, and

selective depletion of these isoforms will be necessary to determine their precise roles in regulating caspase-8 activation during KSHV infection. Cellular inhibitor of apoptosis proteins (cIAPs) can also prevent both death receptor and TLR3-triggered apoptosis, although the mechanism is unclear[128,132,183]. It would be interesting to test whether these regulate caspase-8 activation and/or apoptosis during KSHV lytic infection using Smac mimetics, small molecules that induce cIAP degradation. Inhibition of apoptotic signaling downstream of caspase-8 activation may also prevent cell death. KSHV K7, for example, prevents apoptosis by binding caspase-3 to block its activity[146]. Further investigation is needed to decipher which viral or cellular factors prevent the antiviral effects of caspase-8-mediated apoptosis. Moreover, future studies should also examine the possibility that the level of TLR3 stimulation dictates its apoptotic potential.

4.2.3. Activation of TLR3

It is unclear how lytic reactivation of KSHV activates the TLR3-TRIF pathway, because this pathway is typically viewed as a sensor for dsRNA in the endosomes. Interestingly, KSHV is not the only herpesvirus to activate TLR3. As discussed in section 2.3, HSV1, HSV2, and EBV also activate TLR3-TRIF signaling during infection, although studies have focused on induction of IFN as a consequence of signaling, rather than caspase activation and/or cell death. Multiple mechanisms have been proposed to explain how these DNA viruses trigger RNA-sensing pathways like TLR3-TRIF, as well as cytosolic RNA sensors like RIG-I and MDA-5. In the case of HSV-1, dsRNA intermediates generated during replication due to bidirectional transcription are thought to trigger RNA sensors including TLR3. However, it is unclear how those dsRNA species would physically interact with TLR3 on the cell surface or in endosomes to activate it. The proposed mechanism for EBV infection is better defined: a small non-coding EBV RNA species, known as EBER, is released from infected cells to activate TLR3 signaling[176]. EBER is produced by RNA polymerase III and contains double stranded

moieties within its stem loop structure, which also enables its stimulation of cytosolic RIG-I[236]. In general, RNA polymerase III facilitates detection of several DNA viruses as it transcribes AT-rich regions of their genomes that end up in the cytosol[236–241]. The resultant transcripts activate RIG-I, as RNA polymerase III transcripts often contain a 5'-triphosphate and RNA secondary structures[177]. Thus, dsRNA species produced during DNA virus infection, either as functional viral transcripts like EBV EBER RNA or through other RNA polymerase III transcription of the genome, have the potential to activate RIG-I and TLR3 depending on their spatial dynamics. This potential redundancy of RNA sensors is a common feature of pathogen sensing pathways that underscores the importance of innate immune detection of viral infections. Redundancy in RNA sensing of viral infections could explain why a particular patient found to have complete TLR3 deficiency was hyper-susceptible to childhood herpes simplex virus 1 encephalitis yet otherwise did not seem to have impaired resistance to other viral pathogens[242].

Some immunostimulatory RNAs are known to be produced during KSHV infection and activate RIG-I signaling, presumably in the cytosol. One study found that certain KSHV viral RNAs (transcripts from ORF8, Repeat region (LIR1), and ORF25) bind to and activate RIG-I in an RNA polymerase III-independent manner in iSLK.219 cells[243]. Another study found that cellular RNAs produced from RNA polymerase III trigger RIG-I-dependent interferon signaling and thereby restrict KSHV lytic reactivation in BC3 cells[244]. The presence of these immunostimulatory cellular RNAs was due to KSHV infection impairing DUSP11-dependent host RNA processing. This is one of many possible sources of cellular RNAs that can trigger RNA sensing pathways to induce innate immune signaling in infectious and sterile contexts[177]. Thus, it is possible that during KSHV infection, TLR3 becomes activated by either these known viral and cellular RIG-I-inducing RNAs or other unidentified RNA species. TLR3 upregulation during other viral infections, as well as in several sterile contexts, potentiates signaling through the

TLR3-TRIF pathway[154–162]. Thus, if TLR3 is activated by any RNA ligands during KSHV infection, my data suggest that upregulation of TLR3 during lytic infection would facilitate its recognition of these RNA species.

4.2.3.1. Potential ligand-independent TLR3 activation

Since lytic reactivation significantly alters the cellular and viral transcriptional landscapes, numerous factors could contribute to this potentiation of TLR3-driven caspase-8. However, upregulation of TLR3 is thought to potentiate its activation in other contexts, and I observed TLR3 upregulation during KSHV lytic infection This observation corroborated a previous report that TLR3 is upregulated upon *de novo* infection of KSHV in THP-1 monocytes and primary human monocytes[152]. My results not only reveal that over-expressing TLR3 indeed potentiates TLR3 ligand-induced caspase-8 activation, but also support the novel notion that no ligand is necessary for TLR3 activation upon TLR3 upregulation. Indeed, TLR3 over-expression alone was sufficient to induce caspase-8 activation in latently infected cells (**Fig 2.7**). This is the first demonstration to our knowledge of TLR3 activation in the absence of an external stimulus. This intriguing finding presents a potential mechanism by which TLR3 can be activated during KSHV infection, and perhaps infection with other DNA viruses, without physically associating with a specific viral or cellular dsRNA species. TLR3 activation is known to occur via RNA ligand-induced clustering, which facilitates TRIF recruitment and downstream signaling[181]. Consequently, RNAs must be at least 90 bp to induce sufficient clustering to activate a potent TLR3-mediated immune response[181]. Furthermore, TLR3 clustering and activation can also be facilitated by antibody-mediated crosslinking of TLR3 in the absence of an RNA ligand[181]. It is possible that increased membrane density of TLR3 from its over-expression facilitates its clustering to trigger downstream signaling events.

This is a potential alternative mechanism to TLR3 activation that may occur instead of or concurrently with the possible ligand-dependent mechanisms discussed above.

4.2.4. How does TLR3 become upregulated?

It is unclear how KSHV lytic reactivation augments TLR3 expression. Other viruses have been reported to upregulate TLR3, including hepatitis C virus, influenza A virus, respiratory syncytial virus, rhinovirus, human immunodeficiency virus, simian immunodeficiency virus, and measles virus[159,174,245–248]. In some cases, virus-induced TLR3 upregulation occurs specifically on the cell surface, which influences how TLR3 can become activated[247,248]. Thus, it would be interesting to determine if this similarly occurs in KSHV lytic infection. TLR3 upregulation occurs through different mechanisms in different contexts. It is unknown how TLR3 becomes upregulated in some sterile contexts such as in senescent human umbilical cord mesenchymal stromal cells, intestinal epithelial cells from aged humans and mice, and a rat arthritis model[155,158,160]. For virus-induced TLR3 upregulation, some cases are IFN-dependent[174,246]. For example, IFN-dependent TLR3 upregulation during measles virus infection is mediated by an interferon sensitive response element (ISRE) in the TLR3 promoter region[174]. This study found that TLR3 is acting as an ISG since ISREs are found in regulatory regions of ISGs, and transcription factors with IFNAR-dependent activation like the ISGF3 trimer (STAT1, STAT2, and IRF9) or the STAT1-regulated IRF1 bind to ISREs to activate transcription[249,250]. Indeed, IRF-1 has been shown to bind the ISRE of human TLR3 in response to type I IFN stimulation[251,252]. However, in other cases induction occurs through unknown IFN-independent mechanisms[161,174,248]. It seems likely that TLR3 upregulation upon KSHV lytic reactivation is IFN-independent. TLR3 upregulation occurs upon lytic reactivation without the need for caspase inhibition (**Fig 2.5**), but we only detect IFN expression with concomitant caspase inhibition (**Fig 3.1**). I do not yet know

what this IFN-independent mechanism may be. As RTA is a key transcription factor that controls KSHV lytic gene expression, I tested whether its doxycycline-driven expression upregulated TLR3 in iSLK.RTA cells, SLK cells that contain the RTA transgene but are not infected with KSHV. Doxycycline treatment and resultant RTA expression do not upregulate TLR3 (**Fig 2.6**), indicating that other conditions induced during KSHV lytic infection are responsible. I similarly tested the effect of TPA, which we use to activate the KSHV lytic program in BC3 cells, on TLR3 expression in BJAB cells, a B-cell line that is not infected with KSHV. TPA treatment appeared to downregulate TLR3 protein levels in BJAB cells (**Fig 2.6**), suggesting that TPA does not induce TLR3 upregulation in lytic BC3 cells.

Some hypotheses have been proposed for IFN-independent TLR3 upregulation in other virus infections. The study that found IFN-dependent TLR3 upregulation during measles virus infection also revealed a potential concurrent IFN-independent mechanism[174]. A non-ISRE regulatory sequence, the Sp1 binding site, also contributed to TLR3 upregulation[174]. This suggests that an IFN-independent regulatory site for TLR3 could mediate virus-induced TLR3 upregulation. NFκB2 (p100/p52) may regulate expression of the Sp1 transcription factor as an NFkB2 binding site was found in the Sp1 promoter region, and this binding site is required for Sp1 gene transcription in response to poly(I:C)[253].Thus, it would be interesting to determine if Sp1 and/or NFkB2 contribute to TLR3 upregulation upon lytic reactivation of KSHV. Our scRNA seq analysis of iSLK.219 cells revealed that NFκB2 is only notably upregulated in lytic samples that are concomitantly treated with a caspase inhibitor (**Fig 3.10**). This was validated via RT-qPCR by a current lab member, Machika Kaku (data not shown). Thus, transcriptional upregulation of NFκB2 is not likely driving TLR3 upregulation, which I observe in lytically infected cells without caspase inhibition (**Fig 2.5**). We did not measure any post-transcriptional regulation of NFκB2, such as total protein levels or activation via p52

phosphorylation. Thus, we cannot rule out that this regulation occurs during lytic infection to induce Sp1 expression and promote TLR3 transcription. NFkB (p65)-dependent upregulation of TLR3 is known to occur via LPS induction of TLR4-MyD88 signaling[254,255]. We have examined NFkB (p65) expression at the mRNA and protein level, as well as its activation via p65 phosphorylation during KSHV lytic infection. Machika Kaku found by RT-qPCR that NFκB is upregulated in lytic samples and is not dependent on caspase inhibition (data not shown). This suggests that NFκB mRNA upregulation is not IFN-dependent and thus could potentially regulate TLR3 expression. However, upregulation of NFkB protein levels and p65 phosphorylation also only occurred in caspase inhibitor treated samples (**Fig 3.10C**), which may support that NFkB does not regulate TLR3 expression during KSHV lytic infection.

The transcription factor and tumor suppressor gene p53 also upregulates TLR3 expression[256,257]. The TLR3 promoter contains a consensus sequence for p53, therefore the hinderance of p53 expression in certain cancer cells results in decreased TLR3 expression and reduced responsiveness to dsRNA agonists. However, expression of LANA during KSHV infection promotes p53 degradation[258], so this may prevent the ability of p53 to regulate TLR3 expression during KSHV infection. Additionally, other unknown regulatory sequences within the coding region of TLR3 could confer its transcriptional regulation during KSHV infection. Expression of TLR3 is also regulated by the cellular miRNA miR-155, as well as the ortholog miR-M4 that is expressed by Marek's disease virus, a chicken herpesvirus[259]. Interestingly, KSHV also encodes a miR-155 ortholog, miR-K12-11, which shares the same seed sequence (the 5' end nucleotides that base pair to the target mRNAs) as miR-155 and miR-M4 and is thought to largely phenocopy miR-155[260]. While generally, miRNAs are known for impeding expression of their mRNA targets via RNA-induced silencing complex (RISC)-dependent mRNA cleavage or translational repression[261], there are reports of specific miRNAs

upregulating expression of certain genes under select conditions in a cell-type dependent manner[262]. Thus, there is potential for miRNAs, such as miR-K12-11, to upregulate TLR3 expression during KSHV lytic infection.

It should also be noted that significant discrepancies exist between pathogen sensing pathway activation by artificial ligands, which are often used to mimic infections, versus natural virus infections. Indeed, poly(I:C)-mediated TLR3 upregulation is only reported to be IFN-dependent[160,263], while viral infections can induce IFN-independent TLR3 upregulation, as discussed above. To this point, a paper that compared the downstream signaling effects of TLR3 activation in human bronchial epithelial cells found significant differences between rhinovirus infection and treatment with poly(I:C)[247]. While both induced expression of pro-inflammatory cytokines and IFN-β, pro-inflammatory cytokine expression was blunted by TLR3 inhibition during poly(I:C) treatment, yet it was potentiated by TLR3 inhibition during rhinovirus infection. The lack of TLR3-mediated IFN-β expression resulted in increased viral replication, which was thought to in turn trigger more potent inflammatory signaling through other pattern recognition receptors. As poly(I:C) does not replicate and lacks other immunostimulatory components of a virus, the lack of antiviral IFN-β expression was likely inconsequential. These and other noteworthy differences between poly(I:C) treatment and virus infection underscore the significance of my findings being the first to our knowledge to demonstrate TLR3-TRIF mediated caspase-8 activation during natural virus infection.

4.3. Caspase-8 activation through the TLR3-TRIF pathway likely inhibits a regulator of cGAS

A major unresolved question of my work, as well as other people's work in my laboratory, is the mechanism underlying caspase-8 inhibition of cGAS activity during KSHV lytic infection. Despite other studies showing direct cleavage of cGAS by the

apoptotic caspase-3 and the inflammatory caspase-1[186,264], our lab's findings strongly suggest that caspase-8 activity during KSHV lytic infection targets a regulator of cGAS, rather than cGAS directly, to prevent its activity and IFN induction. The nature of this regulator remains unknown. The laboratory and I have taken a few approaches to identify this regulator.

First, we tested a set of candidate proteins. A few proteins that are cleaved by caspase-8 also have known implications in cGAS-STING signaling. This includes the KSHV protein LANA, as well as the cellular proteins G3BP1 and PKR[90,265–268]. A former MBS student in the lab, Timothy Bond, examined these proteins as candidates that may be targeted by caspase-8 during KSHV lytic infection to inhibit cGAS. His data suggest that G3BP1 and PKR are not found important for cGAS function and IFN induction during KSHV lytic infection, and the role of LANA remains unclear as its cleavage fragments were not reproducibly detected during lytic infection (data not shown).

As a second approach, we employed an unbiased screening approach to identify the relevant caspase-8 targets. We performed a proteomics screen to identify proteins cleaved by caspases using the Cell Signaling Technologies PTMScan service for caspase fragments. We considered hits proteins whose cleavage fragments were enriched in lytic BC3 cells compared to control conditions that lack caspase activity (lytic samples treated with the caspase inhibitor IDN-6556 and latent samples). A former MBS student in the lab, Timothy Bond, tested eight hits from the screen that were previously reported to physically interact with cGAS in a separate screen[269]. Timothy used RNAi-mediated depletion to assess whether these hits were important for the cGAS-mediated IFN induction upon caspase inhibition. Depletion of three of these proteins prevented IFN induction upon caspase inhibition: CHD1, SQSTM1, and DDX27 (data not shown).

Timothy and I both validated caspase-mediated cleavage of SQSTM1 in HEK293T cells (**Fig 4.4A**), and Timothy also showed SQSTM1 cleavage during KSHV lytic

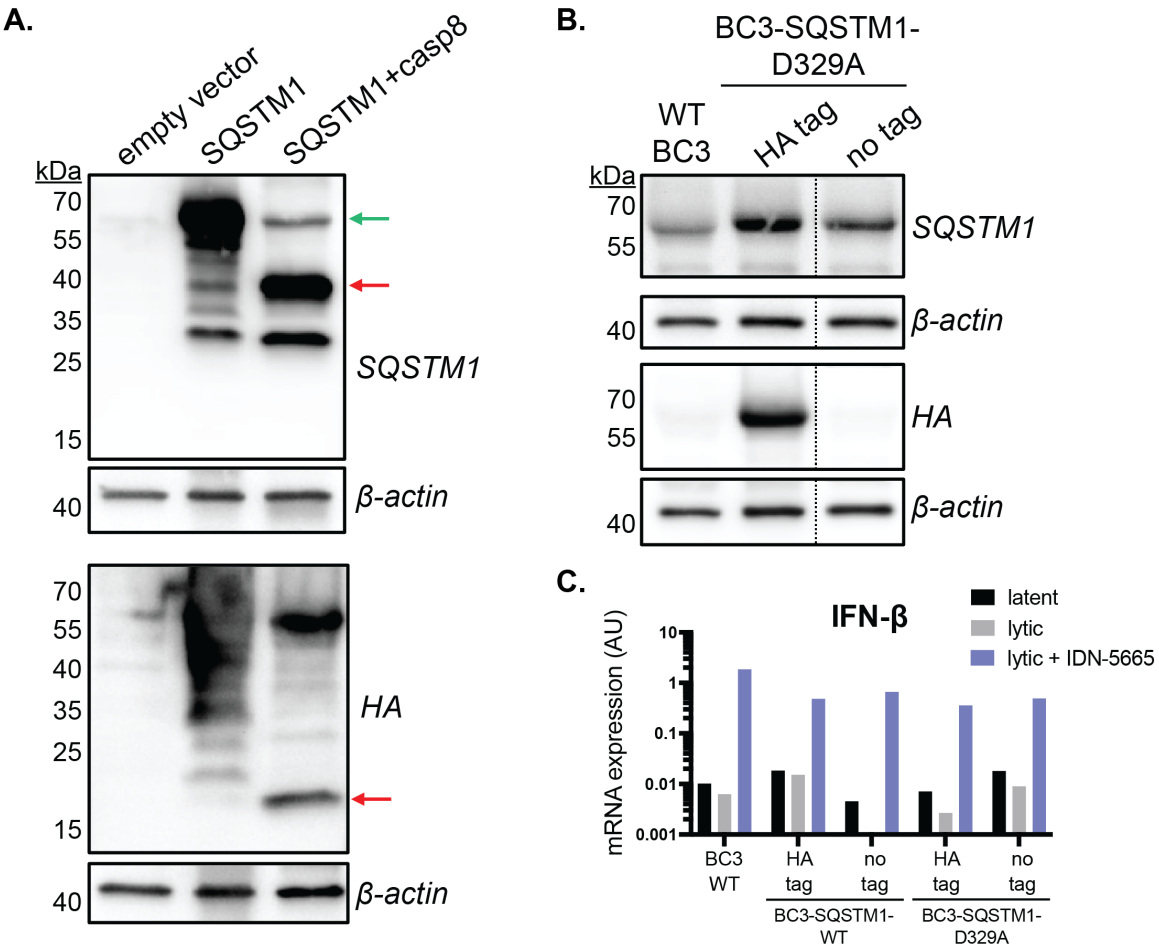

Figure 4.4: SQSTM1 is cleaved by caspase activity but does not promote IFN signaling during KSHV lytic infection.
(A) HEK293T cells were transfected with either an empty vector or a plasmid encoding caspase-8 and/or SQSTM1. Media was replaced 3h later. After 16h, samples were collected to assess protein expression and caspase activation by western blot. Full length SQSTM1 indicated with green arrow and predicted caspase cleavage products indicated with red arrows. Blot is representative of 2 replicates. (B) BC3 cells were BC3-SQSTM1-D329A cells were generated by transducing BC3 cells with lentiviruses containing pLJM1-SQSTM1-D329A (with and without HA tag). Samples were collected to assess protein levels by western blot. (C) WT BC3, BC3-SQSTM1-WT and BC3-SQSTM1-D329A were lytically reactivated for 2 days in the presence or absence of the caspase inhibitor IDN-6556. Total RNA was extracted and the levels of IFN-β mRNA were measured by RT-qPCR and normalized to levels of 18S rRNA.

infection. As this suggested SQSTM1 was a promising candidate, I examined whether SQSTM1 cleavage by caspase-8 inhibits cGAS activity and IFN induction during KSHV lytic infection. I expressed both WT and a non-cleavable SQSTM1 mutant (D329A) in lytic BC3 cells to test whether it could stimulate cGAS and induce IFN expression,

compensating for active caspases (**Fig 4.4B**). To ensure that the HA-tag on SQSTM1 did not impede its function, I also expressed an untagged SQSTM1 D329A mutant. Unfortunately, stable expression of both WT and non-cleavable SQSTM1 failed to induce IFN expression in lytically infected BC3 cells (**Fig 4.4C**). In fact, its expression partially inhibited IFN expression in the caspase inhibitor treated samples. This could be due to a previously described role for SQSTM1 as a negative regulator of STING via autophagy[270]. Although our data identifies SQSTM1 as a novel target of caspase-8, my results indicate that degradation of SQSTM1 by caspases is not the reason cGAS is not activated during lytic reactivation.

Using a similar co-expression approach, I also tried to validate caspase-mediated cleavage of CHD1 and DDX27, since their depletion had also prevented IFN induction upon caspase inhibition in Timothy's experiments. However, I was unable to detect any cleavage products upon exogenous co-expression of these proteins with caspase-8 in HEK293T cells (**Fig 4.5**). In lytic BC3 cells, Timothy did detect faint protein bands for CHD1 at ~33 kDa, the size of a predicted caspase cleavage product, that disappear with caspase inhibition. Thus, caspase-dependent CHD1 cleavage does seem to occur in BC3 cells. It would be interesting in future studies to assess whether expressing non-cleavable forms of CHD1 and DDX27 in lytic BC3 cells rescues IFN induction during lytic reactivation. Identifying the relevant regulator of cGAS may inform therapeutic strategies that could target it to manipulate cGAS-STING signaling in viral infections and other inflammatory disease states that result from aberrant cGAS-STING signaling.

4.4. Negative regulation of the cGAS-STING pathway may have evolved to prevent detrimental outcomes of its hyperactivation

The dysregulation of cGAS-STING signaling has negative implications for many chronic inflammatory diseases[46,203]. Thus, it is likely that caspase-mediated inactivation

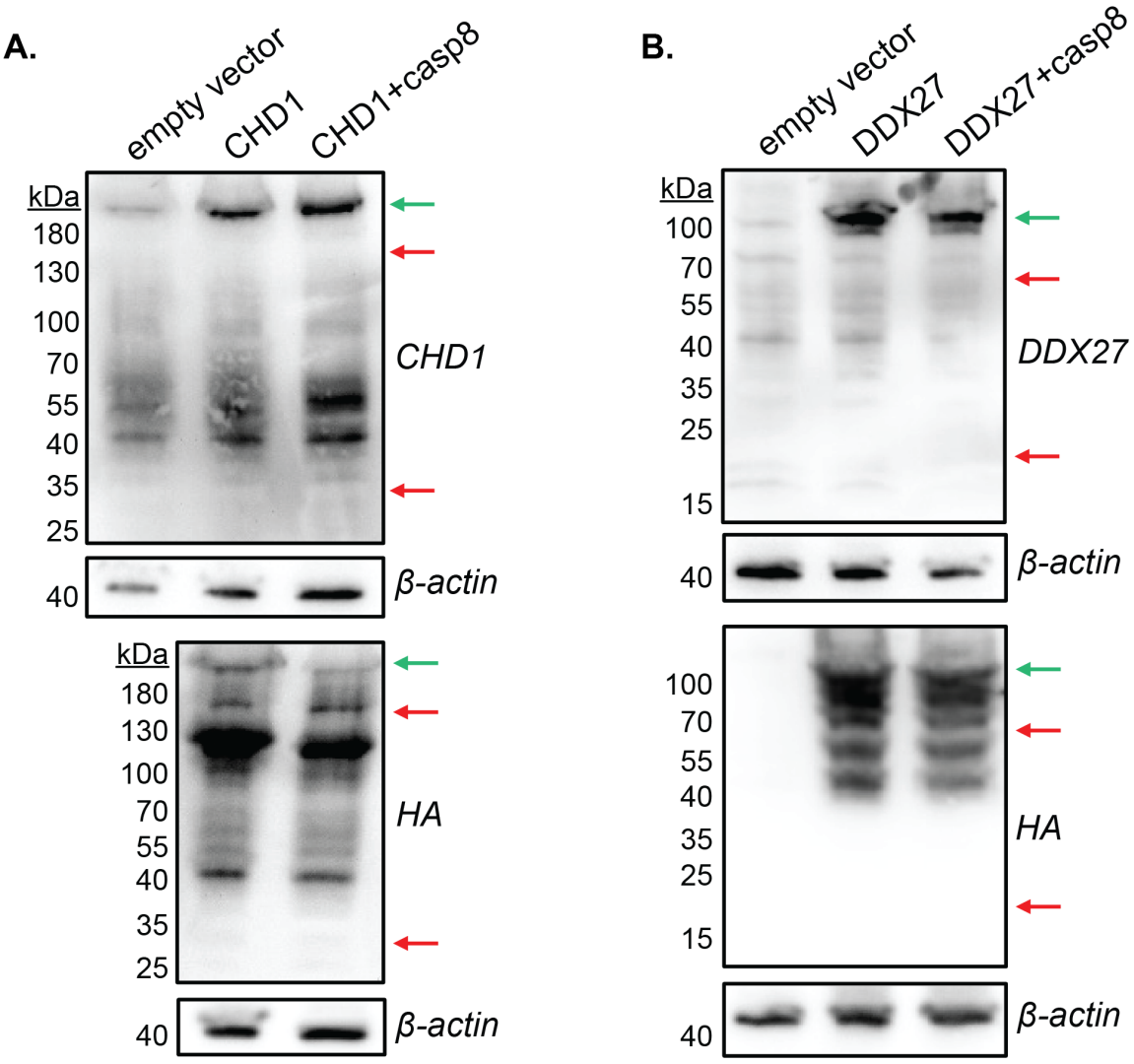

Figure 4.5: CHD1 and DDX27 do not seem to be cleaved by caspase-8 in 293T cells.
HEK293T cells were transfected with either an empty vector or a plasmid encoding caspase-8 and/or CHD1 (A) or caspase-8 and/or DDX27 (B). Media was replaced 3h later. After 16h, samples were collected to assess protein expression and caspase activation by western blot. Full length CHD1 and DDX27 indicated with green arrow and predicted caspase cleavage products indicated with red arrows. Blots are representative of 2 replicates.

of cGAS evolved as a protective mechanism to avoid potential immunopathology. Notably, this inhibition of cGAS results from caspase activation through the TLR3-TRIF pathway. Thus, this negative regulatory crosstalk between these two IFN signaling pathways may have evolved to prevent excessive immune activation when multiple

pathogen sensing pathways are simultaneously triggered. As discussed in section 2.3, there are some other reports of cross-regulation between IFN signaling pathways. However, our lab is the first to demonstrate caspase-mediated negative cross-regulation between the RNA sensing TLR3-TRIF pathway and the DNA sensing cGAS-STING pathway during KSHV infection. KSHV seems to have taken advantage an existing pathway for caspase-8 activation in two different cell types. Indeed, other studies have reported TLR3-driven caspase-8 activation in other cell types as well (discussed in section 1.5.3), but dynamics of cGAS-STING signaling were not examined in those same reports. It seems likely that the caspase-mediated cross regulation between TLR3-TRIF and cGAS-STING would occur in other contexts where both pathogen sensing pathways become simultaneously activated. Many viruses are reported to trigger multiple IFN signaling pathways, even unexpectedly where DNA viruses trigger RNA-sensing pathways and vice versa. Thus future studies should examine whether this caspase-mediated cross regulation occurs in other virus infections. This should also be studied in non-infectious contexts where both pathways may be triggered, such as in various states of cellular stress that can activate both the TLR3-TRIF and cGAS-STING pathway.

4.5. IFN expression in a small fraction of infected cells reveals an additional layer of IFN regulation

Our scRNA seq analyses revealed that when we inhibit caspase activity during KSHV lytic infection, IFN-β is only transcriptionally induced in less than 5% of infected cells. This demonstrates an additional layer of complex IFN regulation during KSHV lytic infection. It remains to be determined what viral and/or cellular factors could be responsible for restricting IFN-β production to such a small fraction of infected cells. The viral gene expression pattern was largely similar between IFN-β-expressing and non-

expressing cells (**Fig 3.10**). However, one exception was that KSHV K5 expression was notably higher in IFN-β-expressing cells. This was surprising as we would expect any viral factors to suppress IFN induction, and K5 has no known functions in IFN regulation. Members of the NFκB transcription factor family were also transcriptionally upregulated in IFN-β-expressing cells, but the functional relevance of this correlation is unclear. Previous reports have found that NFκB cooperatively binds the promoter region of IFN-β in a complex with IRF3 and IRF7[271,272]. Future studies are needed to determine if NFκB upregulation is a determinant of IFN-β induction in KSHV lytic infection, and if so, what limits NFκB expression to such a small fraction of infected cells. Nevertheless, this tight regulation of IFN-β expression is likely an evolutionary strategy to prevent an unnecessary degree of immune activation. Indeed, this minor IFN-β-producing cell population still elicits a potent IFN response as ISG upregulation occurs in nearly the entire cell population (**Fig 3.8**). Moreover, several previous reports show that other virus infections also only result in a small fraction of cells expressing IFN[199,207,211–217]. When studied in innate immune cell types and fibroblasts, the percentage of cells responding to infection was slightly higher than what we observe in iSLK.219 cells, which are epithelial cells[199,211,212]. Thus, epithelial cells have exhibit tighter regulation of IFN induction, perhaps to prevent a premature excess of IFN induction at initial sites of viral infections. Elucidating factors that limit excessive IFN-β expression may inform targeted therapeutic strategies to counteract IFN hyperactivation in inflammatory disease states.

4.6. Future Directions

As mentioned in the above sections, there are still many unanswered questions about the caspase-mediated immune evasion mechanism used by KSHV. Many of these questions related to potential interactions with IFN signaling pathways. Caspases are druggable targets that could be exploited during KSHV infection, and potentially other

contexts, to augment cGAS-STING signaling. However, unraveling molecular details of the caspase-mediated immune evasion during KSHV infection may inform even more targeted therapeutic strategies. In this section, I will discuss the most significant remaining questions for elucidating these interactions.

4.6.1. How is caspase-8 only partially activated in lytic cells to prevent widespread cell death?

Activation of caspase-8 classically occurs through death-receptor signaling in the extrinsic apoptosis pathway and results in cell death. Indeed, I found that iSLK.219 and BC3 cells are capable of inducing full processing of caspase-8 to its apoptotic p18 form upon death receptor stimulation. However, lytic reactivation of KSHV in these cells only induces partial caspase-8 processing to its non-apoptotic p43/p41 form and occurs through the TLR3-TRIF pathway rather than through death receptor signaling. This is presumably critical for caspase-8 activity to be pro-viral, as I would expect caspase-mediated cell death to counteract the enhanced viral replication conferred by caspase-mediated IFN suppression. Thus, to fully understand the pro-viral role of caspase-8 during KSHV infection, future studies should elucidate how caspase-8 becomes only partially activated in lytic cells to prevent widespread cell death. Many cellular and/or viral factors could account for this partial processing and lack of cell death, and multiple factors may work in tandem to achieve this. KSHV encodes several anti-apoptotic proteins and miRNAs that may regulate caspase-8 activation and downstream apoptotic signaling events. Of these, vFLIP is the only one known to regulate caspase-8 activation, though evidence points to this occurring via upregulation of cFLIP through NFκB signaling[233–235]. Nevertheless, vFLIP should be examined for its potential role in regulating the extent of caspase-8 processing during KSHV lytic infection and resulting cell death. This could be done using the vFLIP KO iSLK.219 cells that were kindly gifted

to us by the Jung lab, and by knocking out vFLIP using CRISPR/Cas9 in BC3 cells. To determine if any effects from vFLIP are dependent on its regulation of cFLIP, the different isoforms of cFLIP could be selectively depleted using RNAi in WT and vFLIP KO cells. Likewise, other KSHV-encoded anti-apoptotic factors, as well as cellular factors such as cIAPs, should be assessed for their potential role in regulating caspase-8 activation or downstream apoptotic signaling.

Defining the protein complex that forms to activate caspase-8 should also help to identify important factors that both facilitate its activation and regulate the extent of processing and apoptosis. For example, *Estornes et al.* first reported that, upon poly(I:C) treatment, caspase-8 forms a complex with TLR3 and TRIF to facilitate its activation and downstream apoptosis[273]. Additionally, they found that the activation complex contained RIPK1, $cFLIP_L$, and cIAP2. While RIPK1 was essential for recruitment of caspase-8 to this complex and resultant apoptosis, cIAPs negatively regulated the TLR3-driven apoptosis. Thus, cIAPs may also regulate TLR3-driven caspase-8 activation and apoptosis during KSHV lytic infection. Moreover, *Estornes et al.* had found that RIPK1 was ubiquitinated when in the caspase-8 activation complex[273]. Likewise, a former graduate student in our lab, Tate Tabtieng, observed higher molecular weight forms of RIPK1 upon KSHV lytic reactivation that may be ubiquitinated RIPK1 (data not shown). As in *Estornes et al.*[273], these forms increase in the presence of pan-caspase and caspase-8 inhibitors, likely due to complex stabilization (data not shown). This suggests that RIPK1 is indeed involved in caspase-8 activation during KSHV lytic infection. Thus, RIPK1 and all other potential essential components of this activation complex should be assessed in future studies to better understand how this non-apoptotic, TLR3-driven caspase-8 activation occurs during KSHV infection.

It is also possible that the specific route of caspase-8 activation during KSHV lytic infection limits its apoptotic potential. While we do not know exactly how TLR3 becomes

activated during KSHV infection, my data suggest that the upregulation of TLR3 upon lytic reactivation could be sufficient to induce it activation without engagement of a ligand. As discussed in section 4.2.3.1, increased TLR3 expression may induce similar receptor clustering as ligand binding to initiate downstream signaling events. However, as this is a novel route of TLR3 activation, it is unclear how it exactly compares to traditional ligand induced activation of TLR3. Ligand induced TLR3 activation with poly(I:C) stimulation triggers apoptosis in other reports. Thus, it is possible that differences between ligand and non-ligand induced TLR3 activation could influence the apoptotic potential of caspase-8 activation. Future studies should decipher this by comparing levels of caspase-8 processing and apoptosis upon KSHV lytic reactivation (non-apoptotic, partial caspase-8 activation), death receptor stimulation (apoptotic, full caspase-8 activation), TLR3 over-expression, and poly(I:C) treatment. It would be interesting to compare this in both KSHV infected cells as well as uninfected to determine if viral factors, even during latent infection, influence caspase-8 activation and apoptosis through these different routes. Another potential factor that could influence the output of caspase-8 activation is the degree to which TLR3 is stimulated. Poly(I:C) treatment in other studies may have strongly stimulated TLR3 to trigger a threshold level of caspase-8 activation that triggers apoptosis. On the other hand, KSHV lytic reactivation, whether its ligand-dependent or independent, may activate TLR3 less potently to result in non-apoptotic caspase-8 activation. This should be assessed in future studies by titrating levels of TLR3 expression and poly(I:C) treatment to try to alter the potency of TLR3 activation. It should be noted that other studies have found that high cFLIP expression levels can limit the apoptotic potential of poly(I:C) treatment, so the role of cFLIP should also be considered in these experiments. TLR3 can also be activated from the cell surface or from within endosomes, and TLR3 localization during latent and lytic KSHV infection has yet to be examined. Thus, this should be assessed

as an additional factor that could influence the extent of caspase-8 activation and apoptosis.

4.6.2. How can we mimic this non-apoptotic IFN-regulatory caspase-8 activation to regulate cGAS-STING signaling in other contexts?

The diverse non-apoptotic functions, including IFN regulation, of caspase-8 and other classically apoptotic caspases, are becoming more appreciated Moreover, many context-dependent factors influence the outcome of activating these enzymes. Thus, it is important to consider these potential outcomes when manipulating their activity in different settings, especially for any potential therapeutic purposes. In the context of KSHV lytic infection, specific conditions confer caspase-8 activation in a non-apoptotic, IFN-regulatory manner. As discussed above, further investigation is needed to fully understand what factors facilitate this. It would be interesting, and could have therapeutic potential, to mimic these conditions in other settings to regulate cGAS-STING signaling while avoiding cell death. For example, if TLR3 upregulation is the mechanism by which caspase-8 becomes activated in a non-apoptotic manner to block cGAS activity, future experiments should over-express TLR3 to determine its effect on cGAS-STING-dependent IFN induction. Moreover, determining how KSHV lytic infection upregulates TLR3 expression could potentially inform how to induce TLR3 expression in other contexts, which could allow for non-apoptotic IFN regulation by caspase-8. As discussed in section 4.2.4, many possible factors could regulate TLR3 expression. However, it is likely IFN-independent during KSHV lytic infection, since TLR3 gets upregulated in the absence of IFN-β expression[139]. To rule out that any low levels of IFN-β below the limit of detection facilitate TLR3 upregulation, IFN signaling can be blocked in future experiments.

Additionally, it would be interesting to determine if *de novo* infection of KSHV also triggers TLR3-driven activation of caspase-8 to induce its non-apoptotic, IFN-regulatory function. This would reveal a way in which KSHV can evade immune detection when first establishing infection *in vivo*, and when new cells are infected to reseed and maintain the latent pool later in infection. Lytic reactivation and *de novo* infection are important for KSHV to maintain its long-term persistence in a host, so it makes sense for KSHV to evolve mechanisms to impede innate immune signaling common to both of these processes.

4.6.3. Is TLR3 stimulated by dsRNA during KSHV lytic infection? If so, how does this occur *by an infection with a DNA virus?*

As discussed in section 4.2.3, it remains unknown how KSHV lytic infection activates TLR3 signaling to induce caspase-8 activation. The upregulation of TLR3 during lytic infection could activate its signaling with no ligand binding, or it may sensitize the cells to detect viral or cellular dsRNAs that accumulate during lytic infection. Future experiments could examine this by staining for dsRNA and TLR3 during latent and lytic infection to determine if dsRNA accumulates and co-localizes to TLR3 upon lytic reactivation. This will also reveal if lytic reactivation alters TLR3 localization to facilitate its activation, as is the case for some other viral infections[247,248]. Also in these other viral infections, viral replication was necessary to upregulate TLR3[247,248]. This should be examined for KSHV by blocking viral DNA replication during lytic infection, and determining whether it prevents TLR3 upregulation and subsequent ligand-dependent or -independent activation. It should also be noted that there may be minimal basal levels of dsRNA in endosomes and/or extracellular space during latent infection that can activate TLR3 only when it is upregulated during lytic infection, and future studies should consider this possibility.

4.6.4. What are the mechanisms that regulate type I IFN signaling during KSHV lytic infection?

As discussed in section 4.3, caspase activity is likely targeting a regulator of cGAS to block its activity and subsequent signaling though the pathway to induce IFN expression. While I was unable to validate in HEK293T cells that caspase-8 cleaves DDX27 and CHD1 (**Fig 4.5**), two promising cGAS regulator candidates, this does not rule out that they are cleaved during KSHV lytic infection to regulate cGAS-mediated IFN induction. Future studies should examine this by expressing versions of these proteins with mutations at their predicted caspase-8 cleavage sites. These proteins were selected as hits from our proteomics screen since they have a known interaction with cGAS[269]. If neither of these candidates are the caspase targets that regulate IFN during KSHV lytic infection, other strong hits that do not have a known cGAS interaction with could be investigated. Moreover, additional proteomic approaches could be applied to identify proteins that bind and potentially regulate cGAS during KSHV lytic infection in the presence and absence of caspase-8 activity.

Another notable feature of IFN regulation during KSHV lytic infection is the exceptionally small subset of infected cells (<5%) that express IFN-β upon caspase inhibition (**Fig 3.8**). It is unclear what prevents the expression of IFN-β in the majority of infected cells. cGAS may only be activated in the small subset of IFN-expressing cells, which could complicate the detection of a cGAS regulator targeted by caspase activity if it is only targeted in that subset of cells. If so, these cells may need to be sorted by FACS and analyzed separately to identify the caspase target. Conversely, a downstream event of IFN signaling may only occur in less than 5% of cells to facilitate IFN-β expression. In support of this, we can clearly detect phosphorylation of several proteins in the IFN induction pathway using bulk analysis, including IRF3 (**Figs 3.1E, 3.3C**).

Thus, it should be determined if downstream steps, like IRF3 translocation to the nucleus and/or association with the IFN-β promoter, are limiting factors that occur only in the small subset IFN-β-expressing cells. Moreover, as discussed in section 4.5, some viral and cellular genes that are specifically upregulated in IFN-β-expressing cells, such as KSHV K5 and NFκB, may be determinants of IFN-β induction. Future studies should thus interrogate the role of these genes.

Overall, this work has revealed a novel mechanism by which KSHV infection triggers non-apoptotic caspase-8 activation through TLR3-TRIF to impede cGAS activity and downstream IFN signaling (**Fig 4.6**). Moreover, my data suggest that this may occur via

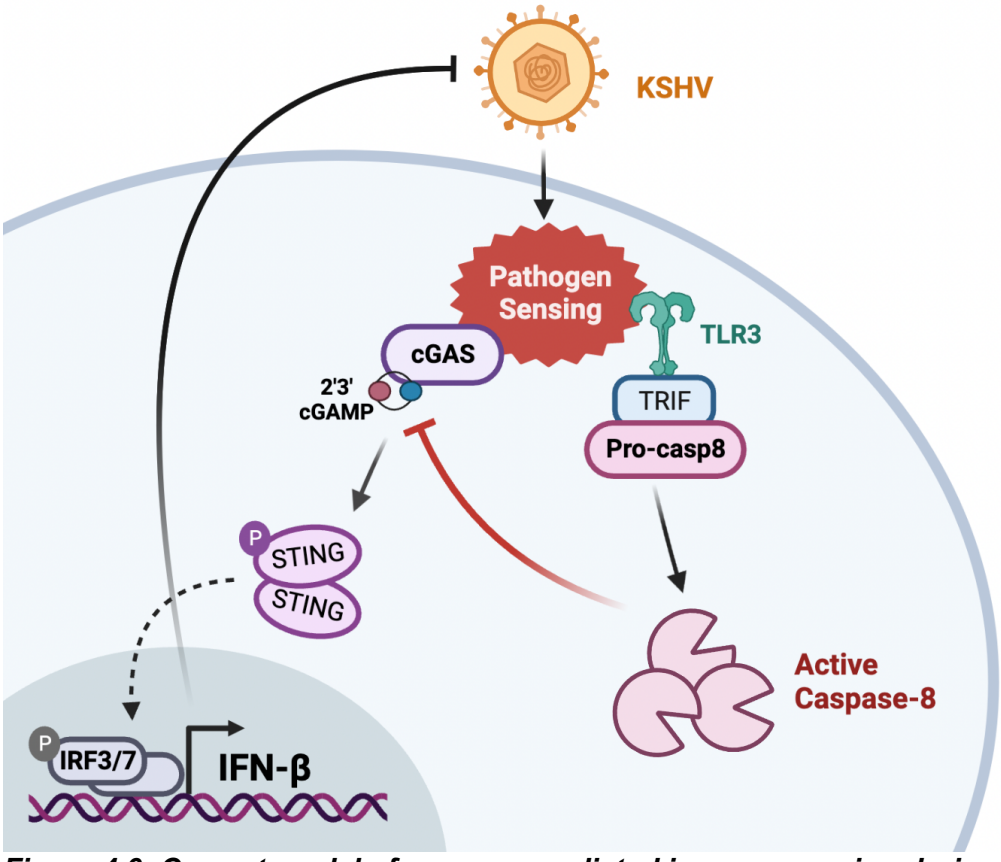

Figure 4.6: Current model of caspase-mediated immune evasion during KSHV lytic infection.
My thesis work suggests a model in which caspase-8 becomes activated through the pathogen-sensing TLR3-TRIF pathway when KSHV is lytically reactivated. Caspase activity then blocks activity of the DNA sensor cGAS to impede signaling through the cGAS-STING pathway, thus inhibiting IFN-β expression. This creates antagonistic cross-talk between two pathogen sensing pathways. Diagram made using BioRender.

a ligand-independent mechanism. Future studies should aim to elucidate further details of this immune regulatory mechanism in KSHV infection and in other contexts.

4.7. Author contribution

All experiments performed in this chapter were conducted by Rachel Lent.

Chapter 5. Bibliography

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