

**KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS DEGRADES
TRIF PROTEIN FOR DOWN-REGULATION OF INNATE
IMMUNITY**

An Undergraduate Thesis

By

HUMERA AHMAD

Submitted to the College of Arts and Sciences
University of Nebraska-Lincoln

In partial fulfillment of requirements for a Bachelor of Science with Highest Distinction
and completion of the Honors Program

March, 2010

Major: Biochemistry

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus degrades TRIF protein for down-regulation of innate immunity. (March 2010)

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Kaposi's sarcoma-associated herpesvirus (KSHV), a virus similar to the Epstein-Barr Virus (EBV), is associated with various cancers including Kaposi's sarcoma and body cavity-based lymphoma (BCBL). The production of this herpesvirus is under the control of the KSHV replication and transcription activator (K-RTA) gene. The expression of K-RTA is responsible for the conversion from latent to lytic cycle possibly leading to oncogenic transformation. Sodium butyrate is known to induce the expression of K-RTA and enhance the virus production. Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon (TRIF) is a cellular gene that is critically involved in innate immunity against viral infection. We have found a new mechanism by which K-RTA blocks interferon production by degrading cellular TRIF. This degradation may partially explain why K-RTA down-regulates an innate immune response to viruses, thus facilitating their survival.

ACKNOWLEDGEMENTS

I would like to thank Dr. Luwen Zhang for serving as my research advisor for this project. He provided continuous guidance and assisted with the experimental designs and cell preparations for my research. In addition, I would like to thank Dr. Madhavan Soundararajan and Dr. Cheryl Bailey for serving as my co-advisors and for all the support and feedback they provided. I would like to thank Dongsheng Xu for his extensive training and help with cell preparations. I also appreciate the help of Rachel Gubbels in preparing cell cultures and performing transfections. I thank other members of the Zhang Lab for help with various aspects of my research. I would also like to thank my parents for their constant support and encouragement.

This work was supported primarily by grants from Undergraduate Creative Activities and Research Experience (UCARE) at the University of Nebraska-Lincoln.

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ABBREVIATIONS

BCBL	Body Cavity-Based Lymphoma
DMEM	Dulbecco's Modified Eagle Medium
EBV	Epstein-Barr Virus
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HHV-8	Human Herpesvirus 8
IFN	Interferon
IRF-3	Interferon Regulatory Factor 3
IRF-7	Interferon Regulatory Factor 7
K-RTA	KSHV Replication and Transcription Activator
KSHV	Kaposi's Sarcoma-Associated Herpesvirus
LB	Luria-Bertani
ORF-50	Open Reading Frame 50
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PEL	Primary Effusion Lymphoma
PVDF	Polyvinylidene Fluoride
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium Dodecyl Sulfate
TBST	Tris-Buffered Saline + Tween-20 (0.1%)
TLR	Toll-like Receptor
TRIF	Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon

CHAPTER 1: INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gamma herpesvirus that is the etiological agent of Kaposi's Sarcoma (5,6). Kaposi's sarcoma is a highly vascular neoplasm that is characterized by the appearance of red and purple patches of tissue under the skin (6). KSHV DNA has been found in all forms of Kaposi's Sarcoma (9). KSHV also plays a key role in the development of body cavity-based lymphoma (BCBL), a type of primary effusion lymphoma (PEL), and multicentric Castleman's disease (1). Immunocompromised patients who have contracted KSHV are more susceptible to these diseases (13).

Herpesviruses are DNA viruses that can infect the host in two different ways. During latent infection, the virus remains in the host but does not cause any cellular harm and does not replicate itself. Cells in the latent cycle can enter the lytic cycle via cell signaling by specific proteins. The lytic stage is characterized by greater viral gene expression and replication, eventually leading to cell death (3). Latent KSHV cells are found within B lymphocytes and only spread to surrounding endothelial cells if the virus is reactivated. The small subset of cells that contain KSHV replicate along with host cells, but a new virus is not produced. Therefore, KSHV cells can remain in the host and avoid detection from the host's immune system (3,6). During lytic replication, KSHV genes produce proteins that are utilized for the successful replication of the viral DNA and help to regulate the host's immune response while a new virus is produced (6). The closest relative to KSHV in humans is the Epstein-Barr Virus (EBV) (9). KSHV and EBV both contain genes that encode specific proteins that play a vital role in the transition from latent to lytic cycles in the respective viruses (12).

The KSHV gene, open reading frame 50 (ORF-50), encodes the protein KSHV replication and transcription activator (K-RTA), which is the primary viral protein responsible for converting KSHV from its latent cycle to its lytic stage (1,3,5,9). Similarly, the EBV gene BRLF1 produces the protein E-RTA, a homologue of K-RTA that acts as a transcriptional activator during the switch from latency to the lytic cycle in EBV (3,7, 9). K-RTA is a sequence-specific DNA-binding protein that causes lytic reactivation in B lymphocytes through regulating gene expression at the transcriptional level of viral genes (1,3). K-RTA can activate the transcription of viral genes either by directly binding to DNA or by binding to other cellular factors and mediating activation of the target promoter (1,3,5). Several other viruses and mammalian cells contain transcription factors with activation domains, such as the N-terminal basic domain and the C-terminal acidic domain, which are homologous to those found in K-RTA (9). Sodium butyrate can induce the conversion of KSHV from its latent to lytic cycle by activating the promoter of ORF50, thus enhancing the gene's expression. Therefore, the expression of K-RTA can be induced by treating cells with sodium butyrate (7,9,12).

The immune system utilizes toll-like receptors (TLRs) to recognize microbial agents and initiate a series of reactions that activate the body's innate and adaptive immune systems in response to viruses (8). Toll-like receptor-3 (TLR3) is a specific protein that initiates an immune response by inducing the expression of interferons (IFNs) (2). TLRs are aided in pathogenic defense by TLR adaptor molecules, such as Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon (TRIF). TRIF binds to TLR3, and together this complex activates the transcription factors interferon regulatory factor 3 (IRF-3) and interferon regulatory factor 7 (IRF-7) (2,8).

This interaction between TRIF, IRF-3, and IRF-7 leads to the production of IFN- β (2). IFN- β is a member of the type I IFN family, which consists of a group of cytokines that function antivirally by inhibiting viral replication (4,10). IFN production is stimulated in response to infection of cells by viruses, which eventually leads to IRF-7 activation (10).

IRFs are a family of transcription factors that serve many functions in the cell involving antiviral defense and the regulation of the transcription of IFNs. A reduction in expression of IFNs may result in a compromised immune system that is vulnerable to viruses, such as KSHV (4). IRF-7 and IRF-3 mediate IFN- α and IFN- β mRNA production. IRF-3 is expressed constitutively and plays a key role in the early stages of IFN production. IRF-7 is more dominant in the later phase of IFN production and works together with IRF-3 to enhance the host's antiviral response by inducing the transcription of other IFN subunits (4,13).

Endogenously, sodium butyrate can induce the expression of IRF-7. Sodium butyrate is a chemical that can alter the chromosomal structure of DNA, which may regulate the activation of IRF-7 (14). Previous studies have found that the integral protein involved in the transformation process of KSHV, K-RTA, actually degrades IRF-7. When cotransfected with K-RTA, the protein IRF-7 was targeted for proteasome-mediated degradation and thus production of IFN- α and IFN- β was blocked (13).

Current studies have tested these latter results and have come to a different conclusion. When cells were cotransfected with K-RTA and IRF-7, IRF-7 was still expressed. However, K-RTA inhibited the production of IFN- β . This data suggested that K-RTA blocks transcription of IFN- β by targeting a different protein involved in the upstream regulation of IFN factors of the innate immune system. After screening several

different upstream mediators, it was determined that the expression of the protein TRIF was down-regulated by K-RTA.

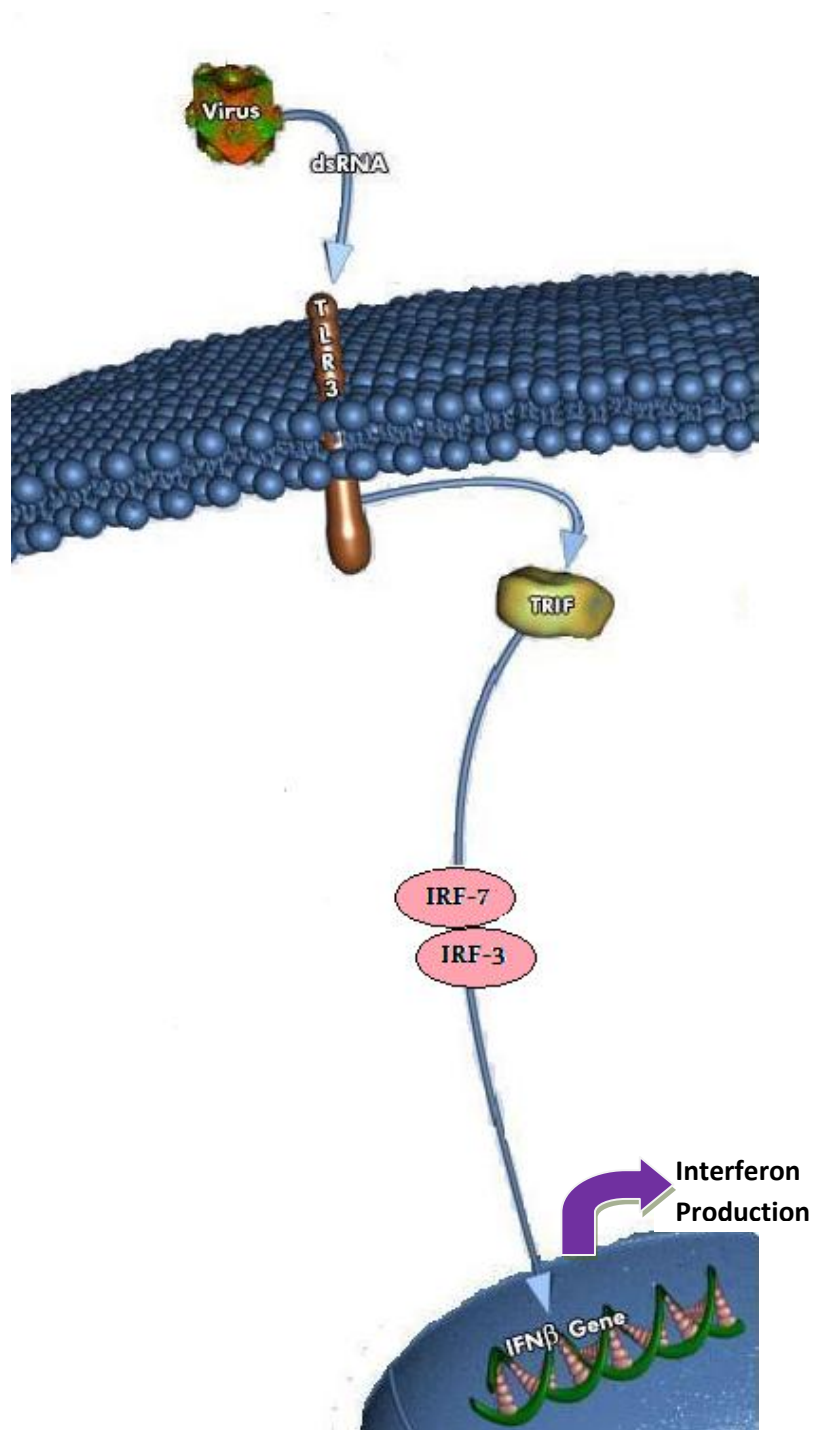
FIGURE 1: TRIF INTERFERON REGULATORY PATHWAY

Figure 1. Depiction of a virus infecting the cell and activating an innate immune response. Introduction of viral RNA activates TLRs. TLRs then initiate a signal transduction pathway involving TRIF that leads to IFN production. Figure is modified from Qiagen

(<https://www1.qiagen.com/Geneglobe/PathwayView.aspx?pathwayID=436>).

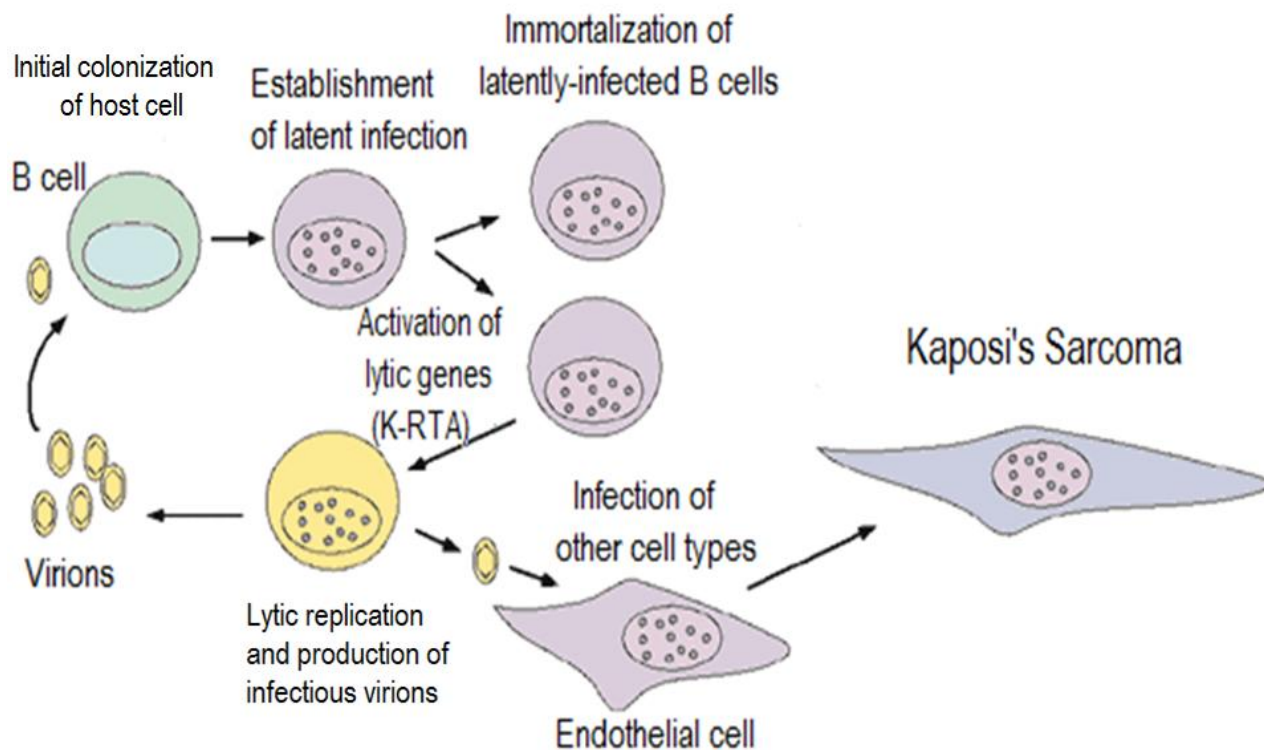
FIGURE 2: KSHV LIFE CYCLE

Figure 2. Mechanism of KSHV infection of B lymphocytes. KSHV virions infect B lymphocytes and establish latent infection. Latently-infected B lymphocytes evade the host's immune response by not producing a new virus. Lytic replication is activated by K-RTA and new infectious virions are produced. The virus spreads to surrounding cells and Kaposi's sarcoma, or other diseases are formed. Figure is modified from NYU School of Medicine

(http://www.med.nyu.edu/microbiology/faculty/wilson/wilson_res.html)

FIGURE 3: K-RTA INHIBITS THE EXPRESSION OF IFN- β

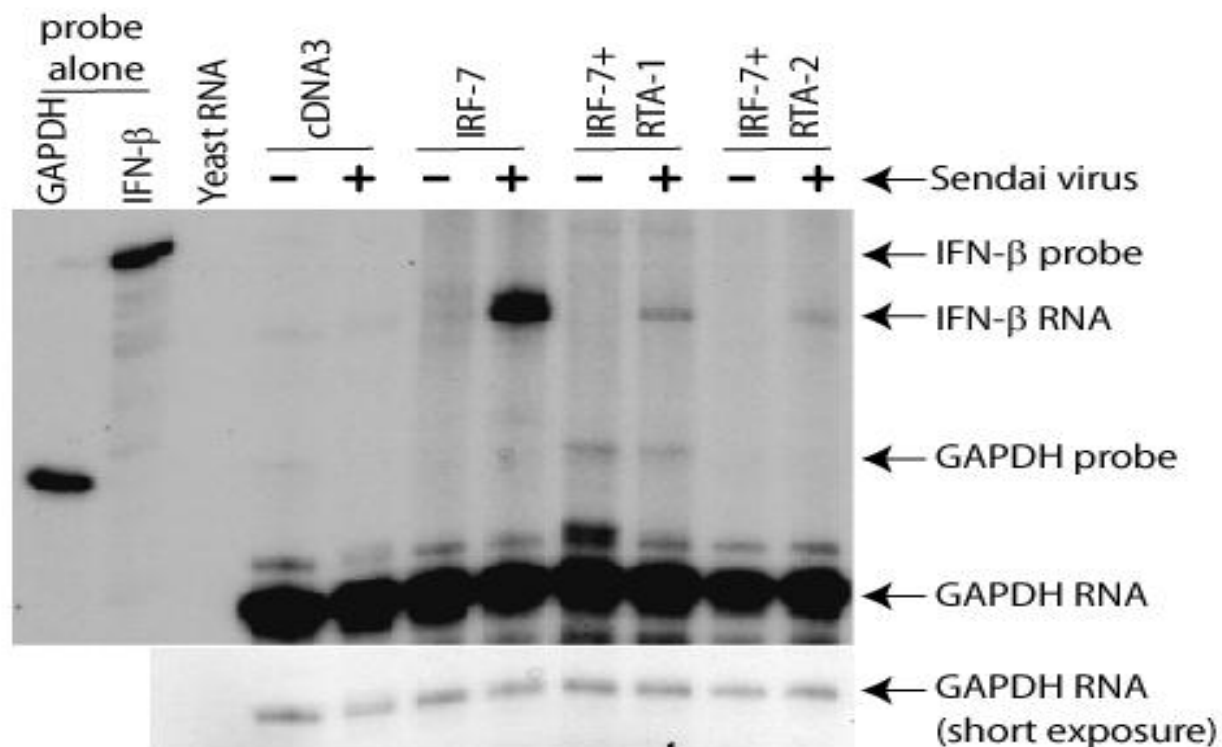


Figure 3. A ribonuclease protection assay (RPA) was done to detect the mRNA expression of IFN- β . 293T cells were transfected with cDNA3, IRF-7, and IRF-7 and K-RTA plasmids. The transfected cells were isolated and half of the cells were infected with Sendai virus for 6 hours. Total RNA were isolated and used for RPA with IFN- β and GAPDH probes. Yeast RNA was used as a negative control. Data indicates that K-RTA significantly inhibits the expression of IFN- β .

CHAPTER 2: MATERIALS AND METHODS

2.1 Plasmids and antibodies.

Colonies from cells on Ampicillin plates were put into LB media and incubated at 37°C overnight. Plasmids were purified using a plasmid kit purchased from Qiagen. The cells were harvested, lysed, and eluted using different buffers then resuspended in solution. Purified plasmids were kept in a -20°C freezer. Antibodies for TRIF were purchased from Cell Signaling Technologies and the IRF-7 and GAPDH antibodies were purchased from Santa Cruz Biotechnology. The ORF-50 antibody was made in Dr. Zhang's laboratory.

2.2 Tissue Culture and Drug Treatment

293T is a human renal epithelial cell line. The 293T cells were maintained at 37°C with Dulbecco's modified eagle medium (DMEM). Cells were grown until they reached 70% confluency, then split into two batches and grown for two more days before transfection. IB4 is a latent EBV-infected lymphoblastoid cell line. The IB4 cells were maintained at 37°C in RPMI1640 plus 10% FBS. Body cavity-based lymphoma (BCBL-1) cells are primary effusion lymphoma-derived B-cell lines that were maintained at 37°C in RPMI1640 plus 10% FBS.

BCBL1 and IB4 cells were treated with varying concentrations of the drug sodium butyrate (0mM, 0.5mM, 1.0mM, 3.0mM, and 5mM). After treatment, the cells were incubated at 37°C for 12-16 hours before being used in protein analysis.

2.3 Transfection.

The 293T cells were chemically transfected using the Effectene Transfection Reagent Kit purchased from Qiagen and by following the manufacturer's recommended procedures. The plasmid was mixed with the cells and they were incubated overnight at 37°C.

2.4 Western blot analysis:

Samples were prepared by pelleting the cells then resuspending them in PBS. The cells were pelleted again and in order to extract the protein from the cell, 2x sodium dodecyl sulfate (SDS) protein loading buffer was added to the cell pellets to lyse them. The suspension of cells was boiled to denature the protein and centrifuged to obtain the supernatant and separate the DNA. The supernatant was added to an 8% polyacrylamide gel by adding a protein ladder to the first well, followed by 10 µL of sample in the remaining wells. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular weight. The samples were electrophoresed at 100 volts for approximately 90 minutes.

After separation, the proteins were transferred to an Immobilon PVDF membrane using 1x transfer buffer at 4°C at a voltage of 200mA for 2 hours. The membrane was then blocked with 5% non-fat milk dissolved in TBST buffer at room temperature for 45 minutes. It was then rinsed with TBST buffer and kept at 4°C overnight with the primary antibody. Recommended dilutions for antibodies were used: 1:100 TRIF, 1:500 IRF-7, 1:500 ORF-50, and 1:1000 GAPDH. After three washes with TBST for 5 minutes each, the membrane was incubated with the secondary antibody at room temperature for 30 minutes. Anti-rabbit secondary antibody was used for IRF-7, TRIF, and ORF-50 at a

concentration of 1:2000 and anti-mouse secondary antibody was used for GAPDH at a concentration of 1:2000.

Lastly, the membrane was washed again three times with TBST and treated with Super-Signal West Pico Chemiluminescent reagents (Pierce) for 3-5 minutes. The protein bands were detected using chemiluminescence that specifically detected the presence of HRP. The membrane was visualized by exposure to Kodak XAR-5 film. GAPDH levels were examined and later used as a loading control.

CHAPTER 3: RESULTS

IFNs play a key role in protecting a host against viral infection by inducing an innate immune response. TRIF helps regulate the production of IFNs by interacting with other proteins and activating IRFs. Investigating how K-RTA interacts with the protein TRIF is critical to understanding how viruses can evade a host's defense mechanism. In order to examine the expression of TRIF, cells were transfected and examined through western blot analysis. Specific antibodies for K-RTA, TRIF, and IRF-7 were used and the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

3.1 K-RTA does not degrade IRF-7.

Previous studies have found that K-RTA degrades IRF-7, thus blocking IFN production (13). However, when 293T cells were cotransfected with K-RTA and IRF-7, IRF-7 was expressed, as illustrated in Figure 4. To ensure that K-RTA is present after transfection, another analysis was done using the ORF-50 antibody which detects K-RTA. Further analysis has determined that K-RTA still significantly inhibits the expression of IFN- β , as illustrated in Figure 3. This data suggests that K-RTA regulates another intermediate in the interferon regulatory pathway, and consequently blocks IFN production.

3.2 K-RTA down-regulates the expression of TRIF.

After screening several different intermediates, it was found that K-RTA targets TRIF protein for down-regulation in the interferon regulatory pathway. 293T cells cotransfected with TRIF and K-RTA showed a decrease in the expression of TRIF, as displayed in Figure 5.

3.3 TRIF degradation is specific to K-RTA.

E-RTA is the protein responsible for the switch from latency to lytic replication in the Epstein-Barr Virus, a gamma herpesvirus similar to KSHV. To determine if TRIF-degradation is unique to gamma herpesviruses, the relationship between TRIF and E-RTA was examined. In 293T cells cotransfected with E-RTA and TRIF, there was no effect on the expression of TRIF. Contrastingly, when cotransfected with K-RTA, the expression of TRIF was significantly reduced, as shown in Figure 6. This data suggests that the degradation of TRIF is specific to K-RTA.

3.4 K-RTA mutant does not significantly inhibit the expression of TRIF.

To determine if K-RTA is the specific protein in KSHV responsible for degrading TRIF, a K-RTA mutant (KRTA K152E) which had a mutation that converted a lysine to glutamic acid at position 152 in K-RTA was prepared. This mutant lacked the full viral capacity of wild-type K-RTA. The expression of TRIF was significantly inhibited in cells transfected with wild-type K-RTA; however, TRIF was expressed in cells transfected with KRTA K152E, as illustrated in Figure 7. This confirms that wild-type K-RTA specifically functions in degrading TRIF.

3.5 Expression of K-RTA and TRIF are inversely correlated at physiological concentrations.

When KSHV positive body cavity-based lymphoma cells at physiological concentrations were treated with sodium butyrate at concentrations of 0mM, 0.5mM, and 1.0mM, the expression of K-RTA was significantly increased. Western blot analysis showed the simultaneous down-regulation of TRIF as expression of K-RTA increased, as depicted in Figure 8. The same membrane was treated with IRF-7 antibody and the

expression of IRF-7 increased with increasing amounts of sodium butyrate. This data ensured that TRIF degradation was not due to cell necrosis, and that the sodium butyrate was working properly in inducing K-RTA.

3.6 The expression of TRIF is constant in IB4 cells treated with sodium butyrate.

IB4 cells from the Epstein-Barr Virus which do not contain KSHV were used as a control to determine the effect of just sodium butyrate on the expression of TRIF. TRIF expression was not affected when cells were treated with increasing concentrations of sodium butyrate as shown in Figure 9. These results eliminate sodium butyrate as being a factor in the degradation of TRIF.

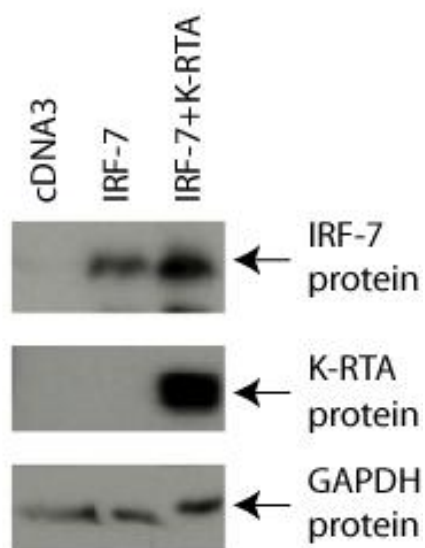
FIGURE 4: K-RTA DOES NOT DEGRADE IRF-7

Figure 4. IRF-7 is expressed in samples of 293T cells cotransfected with K-RTA and IRF-7. The presence of K-RTA after cotransfection is also verified. GAPDH was used as a loading control.

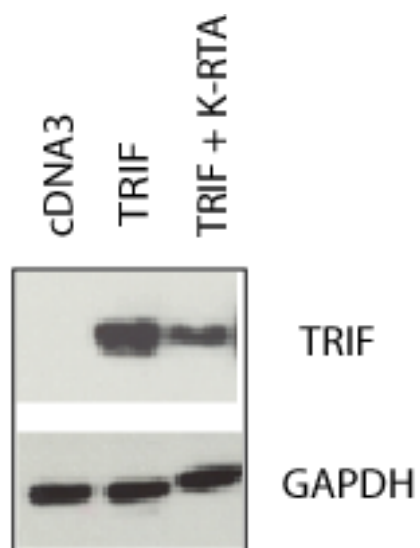
FIGURE 5: K-RTA DOWN-REGULATES THE EXPRESSION OF TRIF

Figure 5. 293T Cells were transfected with cDNA3, TRIF, and cotransfected with TRIF and K-RTA. Cells transfected with K-RTA show a decreased expression of TRIF. GAPDH was used as a loading control.

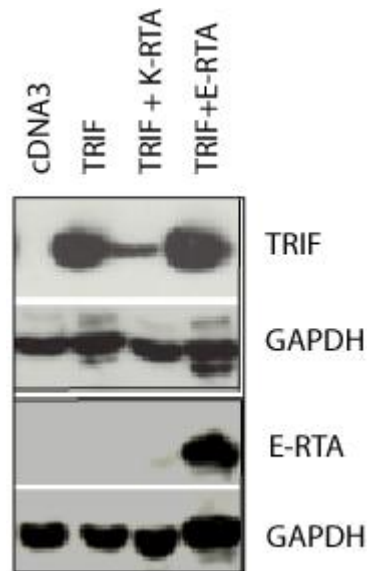
FIGURE 6: TRIF DEGRADATION IS SPECIFIC TO K-RTA

Figure 6. 293T cells cotransfected with TRIF and E-RTA show no effect on the expression of TRIF, whereas cells cotransfected with TRIF and K-RTA show significant inhibition of the expression of TRIF. GAPDH was used as a loading control.

**FIGURE 7: K-RTA MUTANT DOES NOT SIGNIFICANTLY INHIBIT THE
EXPRESSION OF TRIF**

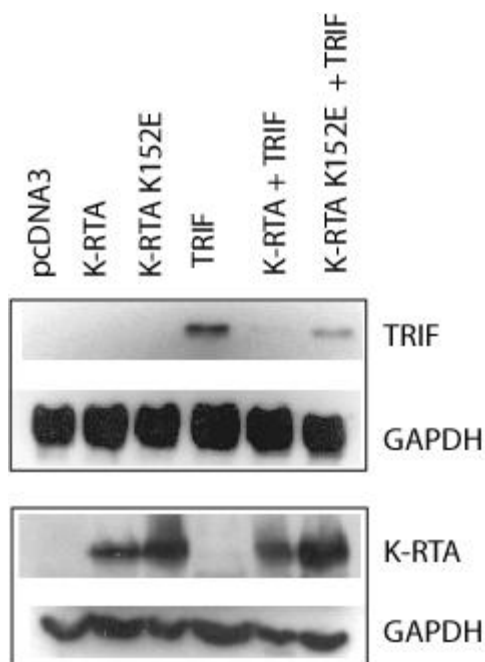


Figure 7. 293T cells transfected with wild-type K-RTA and K-RTA mutant K152E. Down-regulation of TRIF expression is seen only in cells transfected with wild-type K-RTA protein and not in cells transfected with K-RTA K152E mutant. GAPDH was used as a loading control.

FIGURE 8: EXPRESSION OF TRIF AND K-RTA ARE INVERSELY CORRELATED AT PHYSIOLOGICAL CONCENTRATIONS

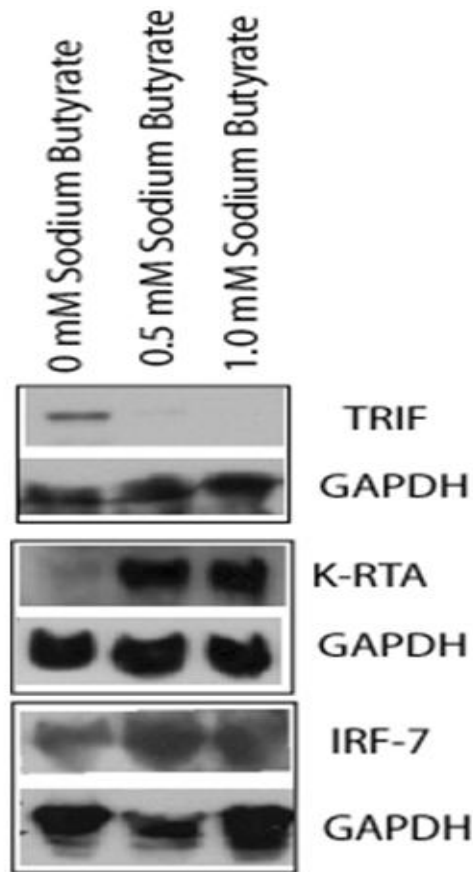


Figure 8. BCBL1 cells positive for KSHV at physiological concentrations are treated with different concentrations of sodium butyrate (0mM, 0.5mM, 1.0mM). With increasing levels of sodium butyrate, K-RTA is induced and TRIF is down-regulated. The same membrane is treated with IRF-7 antibody to verify that the degradation of TRIF was not due to cell death, and to ensure the sodium butyrate treatment was effective. The expression of IRF-7 is increased with increasing concentrations of sodium butyrate. GAPDH was used as a loading control.

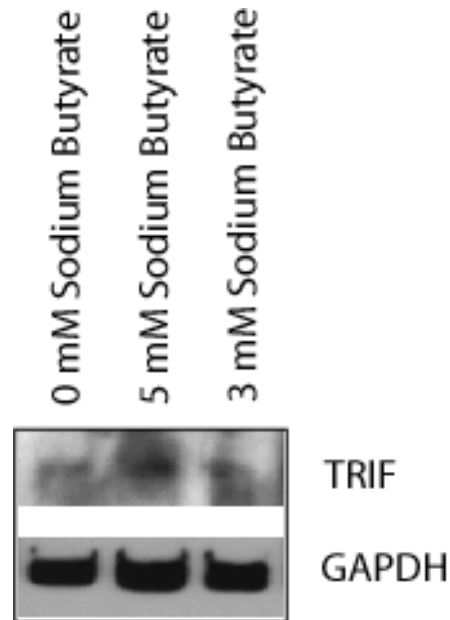
FIGURE 9: EXPRESSION OF TRIF IS CONSTANT IN IB4 CELLS

Figure 9. Epstein-Barr Virus IB4 cells that lack KSHV were treated with different concentrations of sodium butyrate (0mM, 5mM, 3mM). The expression of TRIF remains constant at each concentration of sodium butyrate. GAPDH was used as a loading control.

CHAPTER 4: DISCUSSION

KSHV has been implicated in the pathogenesis of Kaposi's sarcoma, body cavity-based primary effusion lymphoma and multicentric Castleman's disease (1,5,6). Numerous studies have indicated that KSHV is converted to its lytic stage of replication by the activity of the K-RTA protein (1,3,5,9). K-RTA acts to enhance the transcription of viral genes by binding to DNA or interacting with other cellular factors to activate target promoters (1,3,5). The present study analyzed the effect of K-RTA on the expression of proteins involved in an innate immune response soon after viral infection with KSHV.

IRF-7 is critical in the production of IFN- β through interaction with other cellular proteins and IRFs (2). Previous studies have shown that K-RTA can inhibit the expression of IFNs by targeting IRF-7 for proteasome-mediated degradation. A direct consequence of this reduction in IRF-7 levels is the inhibition of the transcription of IFN- β , which is critical to the cell's antiviral defense response. IFN- β is a marker for various subtypes of type I IFNs, therefore a decrease in IFN- β correlates to a decrease in the production of numerous IFNs. (13). Current results have shown that K-RTA does not degrade IRF-7 (Fig. 4). When cells were cotransfected with IRF-7 and K-RTA, IRF-7 was still expressed. However, K-RTA did inhibit the expression of IFN- β ; thus, it was hypothesized that K-RTA may regulate a different protein in the interferon regulatory pathway in order to inhibit the transcription of IFN- β .

A series of proteins in the interferon regulatory pathway were screened in order to detect the mechanism by which K-RTA blocks the transcription of IFN- β . It was determined that the expression of the protein TRIF was down-regulated by K-RTA.

Numerous experiments were performed to verify this inverse relationship between K-RTA and TRIF. When cells were cotransfected with K-RTA and TRIF, the expression of TRIF was significantly reduced. EBV is another gamma herpesvirus that is the closest relative of KSHV, and has a protein with a similar function as K-RTA, known as E-RTA (3,7,9). To determine if TRIF degradation is unique to gamma herpesviruses, experiments were done to compare the expression of TRIF and E-RTA (Fig. 6). Results showed that E-RTA had no effect on the expression of TRIF; therefore, E-RTA does not inhibit IFN production by the same method as K-RTA does. This indicates that TRIF degradation is specific to K-RTA.

Furthermore, KSHV contains several viral proteins that can contribute to its effect on the innate immune system. To ensure that K-RTA is the specific protein that targets TRIF, analyses were done to determine if the same down-regulation in TRIF was experienced when K-RTA was mutated (Fig 7.). A K-RTA mutant was formed by mutating the amino acid lysine at position 152 to a glutamic acid. This mutant is not capable of DNA-binding, which is a key function of wild-type K-RTA (11). When this mutant was transfected into cells with TRIF, expression of TRIF was not significantly inhibited, unlike the effect of wild-type K-RTA on TRIF. This suggests that K-RTA must be one of the proteins in KSHV that function by specifically degrading TRIF. Additionally, KRTA's DNA-binding domain may play a role in K-RTA's interaction with TRIF and consequently contribute to TRIF's down-regulation.

Sodium butyrate is known to stimulate the transformation of KSHV from its latent to lytic cycle, thus inducing viral DNA replication and the expression of K-RTA (7,9,12). Cells containing KSHV at physiological concentrations were treated with sodium

butyrate and analyzed 12-16 hours later. K-RTA levels increased as the level of sodium butyrate in the cell increased. The effect on TRIF was analyzed in these cells and as the expression of K-RTA increased with increasing levels of sodium butyrate, the levels of TRIF simultaneously decreased. These findings agree with the previous results that K-RTA down-regulates TRIF. The expression of IRF-7 was also analyzed to verify that the sodium butyrate was effective and that cell death was not a factor in degrading TRIF. Previous studies have shown that sodium butyrate induces the expression of endogenous IRF-7 by altering the chromosomal structure of DNA, which is important in the regulation of IRF-7 expression (14). IRF-7 expression was marginally increased with increasing amounts of sodium butyrate, similar to the activating effect of sodium butyrate on K-RTA (Fig. 8). Therefore, the sodium butyrate was effective in inducing K-RTA expression. Additionally, because IRF-7 was not degraded, but instead induced, it is apparent that cell death did not occur after treatment with sodium butyrate, and as a result did not cause the degradation of TRIF.

The effects of sodium butyrate on the protein TRIF have not been well studied. To rule out sodium butyrate as one of the agents causing a down-regulation of TRIF, the interaction between just this drug and TRIF was examined. Cells from the Epstein-Barr Virus were used as a control because they contain no KSHV, therefore K-RTA is not present. When these cells were treated with increasing amounts of sodium butyrate, the expression of TRIF was not inhibited, and instead remained constant at all levels of sodium butyrate (Fig. 9). This suggests that K-RTA may be responsible for down-regulating the expression of TRIF at physiological concentrations, and sodium butyrate is not a factor in this down-regulation.

This inverse relationship between K-RTA and TRIF may help understand how KSHV can potentially cause oncogenesis. K-RTA specifically degrades TRIF which is an important signaling factor in one of the interferon regulatory pathways of the innate immune system. This degradation causes the transcription of IFNs to be inhibited and allows the virus to evade the host's innate immune defense response, thus facilitating the virus's survival. In addition, these results were obtained with transfected cells as well as with KSHV cells at physiological concentrations. Therefore, this phenomenon naturally occurs in individuals who are diagnosed with KSHV and this data can be used as a basis for further research concerning KSHV and its potential oncogenic transformation. Further investigations into different immunological pathways may lead to other mechanisms by which KSHV can down-regulate innate immunity, and increase the knowledge concerning its pathogenesis. Also, this research could lead to possible therapeutic treatments for KSHV patients by either inhibiting the function of K-RTA or increasing the expression of TRIF endogenously.

FIGURE 10: K-RTA DEGRADES TRIF AND BLOCKS INTERFERON PRODUCTION

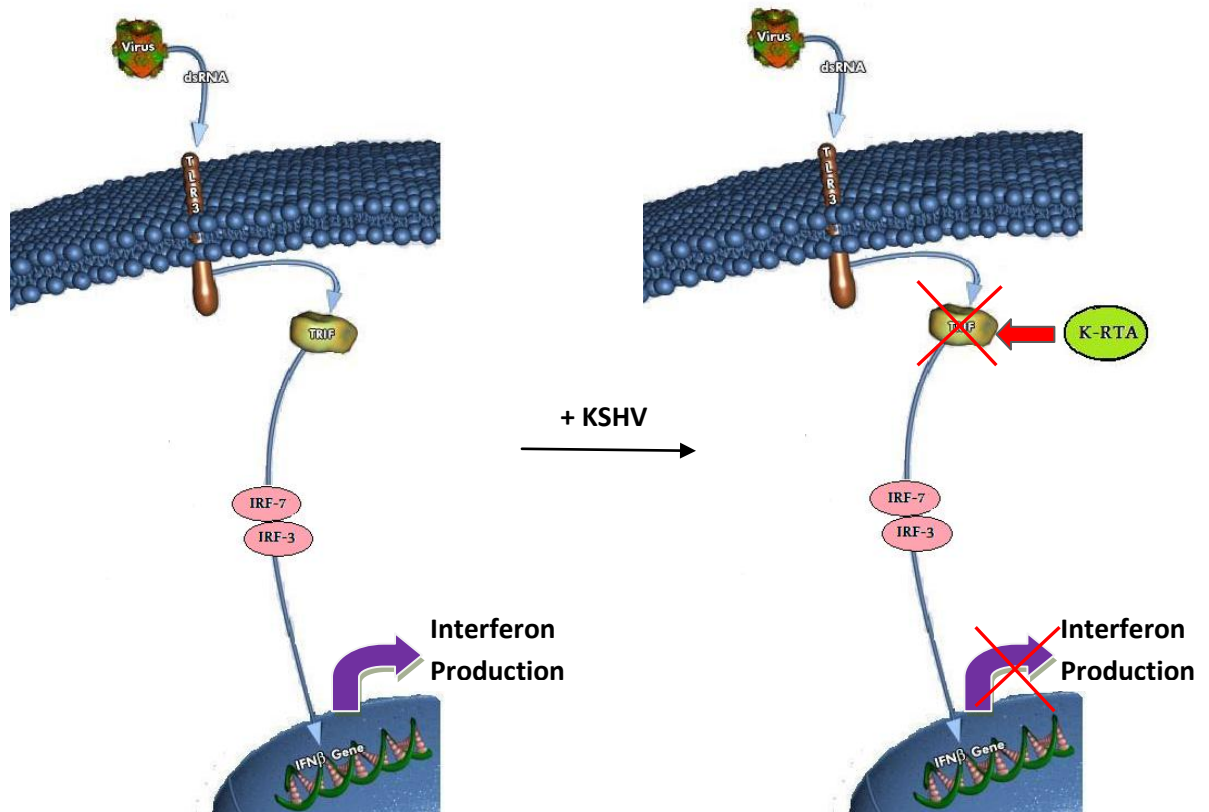


Figure 10. Depiction of KSHV infecting the cell and activating an innate immune response. Introduction of viral RNA activates TLRs which initiate a signal transduction pathway. K-RTA inhibits the expression of TRIF in this pathway, and the production of the cascade of interferon factors including IRF-7, IRF-3, and IFN- β is therefore inhibited. Figure is modified from Qiagen (<https://www1.qiagen.com/Geneglobe/PathwayView.aspx?pathwayID=436>).

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