UNIVERSITY OF CALIFORNIA SAN DIEGO

The effect of TLR3, RIG-I, PKR, PRKRA, and Fas on the containment of VSV in HeLa cells

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Mohona Datta

Committee in charge:

Professor Nan Hao, Chair
Professor Matthew Daugherty
Professor Dong-er Zhang

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The Thesis of Mohona Datta is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

I would like to dedicate my thesis to my family without whom I could not have had this amazing opportunity. My parents have made many sacrifices in order to help me study abroad and fulfill my dreams. Thank you for always believing in me and supporting all my dreams.

EPIGRAPH

Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained.

Marie Curie

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ILLUSTRATIONS

LIST OF IMAGES AND TABLES

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ABSTRACT OF THE THESIS

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Mohona Datta

Master of Science in Biology

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Professor Nan Hao, Chair

Human cells are capable of containing a virus through several different mechanisms. Most of these antiviral responses are mediated by the cellular secretion of chemicals called interferons which are secreted in response to the presence of the virus in the host cell. In this study, we created an overexpression cell line of various cellular double stranded

RNA (dsRNA) viral sensors such as TLR3, RIG-I, Fas, PKR, and PRKRA and used time lapse microscopy to investigate the changes in the spread of the viral infection in the host cell. We found that an overexpression of TLR3, PKR, PRKRA, and Fas in HeLa cells show no significant changes in Vesicular Stomatitis Virus (VSV) containment upon infection. However, there is a significant decrease in the spread of VSV upon infection when RIG-I was overexpressed in HeLa cells. This reveals the importance of RIG-I in the interferon induced antiviral response against VSV and also demonstrates that not all dsRNA viral sensors are important in triggering the same response. Future research on these sensors can provide us greater insight on how we can manipulate the host induced interferon antiviral response for efficient containment of the virus upon infection.

CHAPTER 1: INTRODUCTION

Viruses can cause a fatal threat to our immune system. Pandemic infections have often left a lasting imprint on human history and have led to the incalculable loss of lives (Iverson, 2018) . In order to develop antiviral therapeutics to treat infectious diseases, it is crucial to understand the intricacies of human-virus interactions. The human host and the virus are constantly evolving and competing to establish dominance in this interaction. This thesis discusses the interaction of the double-stranded RNA (dsRNA)- sensing cellular receptors of the host's innate immune system with Vesicular Stomatitis Virus (VSV) in HeLa cells.

1.1: WHY STUDY HOST-VIRUS INTERACTIONS?

The study of host-virus interactions is a dynamic topic that is constantly evolving as more scientific discoveries are being made in the field of virology, immunology, and cell and molecular biology. Understanding the intricacies of this interaction is important in the development of effective vaccines and antiviral drugs. Virus-host cell interactions involve viruses exploiting the host machinery for their own purposes since they are obligate intracellular parasites, but also provides a means for the host cell to combat viral infection. (Hoenen T. et al., 2022).

The first stage of the viral life cycle involves viral entry. This is the earliest interaction that a virus protein has with a host cellular receptor in order to facilitate virus uptake.This interaction provides an interesting checkpoint where the host's immune system can intervene and inhibit further infection. An 'infection' is an invasion of the host cells by pathogens such as bacteria, viruses, fungi etc. which may or may not be followed by a reaction

from the host. Viral infections are often dependent on the virus that is infecting, the host it infects, and the functional state of the host cell (Dowling P., Youngner J., 1999). We hypothesized that on viral infection, the more the activation of the viral sensing cellular receptors, the better should be the containment of the virus. Therefore, an overexpression of different kinds of viral dsRNA recognizing host cell receptors such as TLR3, RIG-I, PKR, PRKRA, and FAS in VSV infected HeLa cells was performed to evaluate whether this is true and gain a better understanding of the antiviral pathway during a virus infection. By examining cellular responses to infection, we can gain insights regarding the mechanisms associated with the restriction of virus infection and pathogenesis (Katze et al., 2022). Such knowledge allows us for the successful manipulation of these responses to develop precise targeted therapeutics for the control of viral infections and create antiviral drugs which have a reduced likelihood to develop resistance towards the virus due to their reliance on host cell components.

1.2: WHAT ARE VIRUSES?

Viruses are obligate, intracellular parasites that primarily depend on their hosts for energy, macromolecular synthesis machinery, genome replication and particle assembly (Gelderblom,1996). By definition, they must have some genome in the form of DNA or RNA that is surrounded by a protective protein shell called a capsid. Viruses are acellular, meaning they are biological entities that lack most of the components of cells, such as organelles, ribosomes, and the plasma membrane(9.2A Viral Morphology, Biology LibreTexts). Since viruses are parasitic in nature, they have developed numerous ways by which they can manipulate host machinery to benefit viral replication and sustenance of viral population. A

complete virus particle is called a virion. The main function of the virion is to deliver its DNA or RNA genome into the host cell so that the genome can be transcribed and translated by the host cell (Gelderblom, 1996)

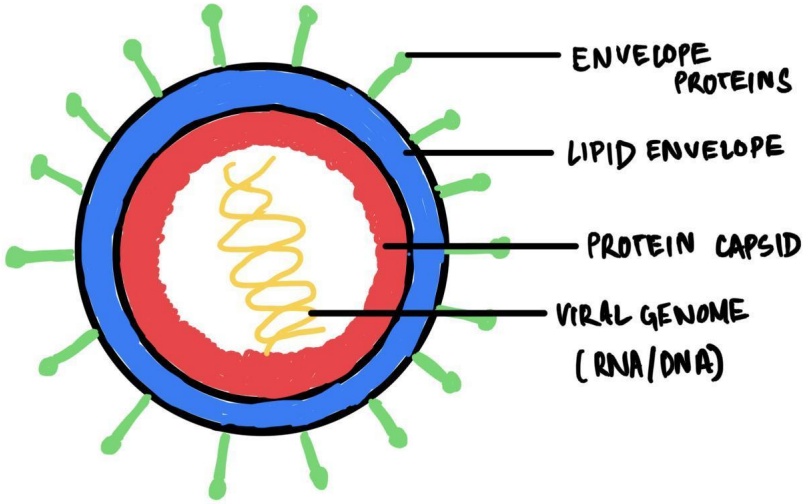

Illustration 1.1. A basic structure of a virus

1.3: VESICULAR STOMATITIS VIRUS (VSV)

VSV is a non-segmented, single-stranded, negative-sense RNA virus containing genes which encode five structural proteins: the glycoprotein (G), membrane (or matrix) protein (M), nucleoprotein (N), and two internal proteins (L and P). This virus is known to cause infection in livestock but has little to no disease causing tendencies in a human host. The only known cases of human infections are asymptomatic and caused by close contact to infected livestock (Whelan, 2008). Being one of the first negative-sense RNA viruses to be generated in labs, it has been widely researched upon and used as a prototype of a non-segmented -ssRNA virus.VSV is also known to show a high replication rate in a broad range of cultured cells. This

factor combined with the relative safety of using this virus, makes it a convenient virus model that is frequently used in research laboratories to study host-virus interactions. Studies with VSV have led to understanding of fundamental host cell processes, viral gene expression, and pathogenesis related to viral infection. In addition, VSV has also provided us with greater insight on fundamental principles in evolutionary biology and population genetics (Whelan, 2008).Thus, we chose to use VSV for studying properties of viral infections and host-virus interaction in different mammalian cell lines. Additionally,VSV is commonly used as an expression vector for foreign proteins due to its small genome and ease of genetic manipulation. It is also a promising vaccine platform due to its ability to express foreign antigens. It can be replicated in a wide variety of mammalian cells and grow to create a high viral load in these cells and elicit strong immune responses in vivo (Suder et al., 2018).

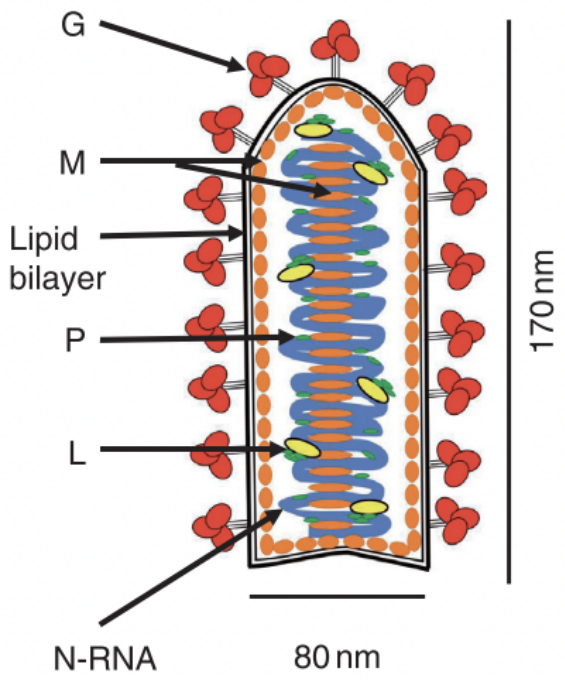

Illustration 1.2. Structure of Vesicular Stomatitis Virus. (Whelan, 2008)

1.4: INNATE IMMUNE SYSTEM

The main role of the host immune system is to attack 'non-self' molecules. The collective and coordinated response upon introduction of a foreign substance ('non-self') is called an immune response. There are different kinds of pathogens that the mammalian immune system recognizes such as parasites, fungi, bacteria, and viruses.

Most pathogens are selected to maximize replication and transmission. These goals of the virus have limited success in the host because of its ability to "sense" the virus. The immune system can be divided into two broad categories: Innate Immunity and Adaptive Immunity. This thesis primarily covers an understanding of the innate immune system.

Innate immunity is present in all animals and in all cells of the body, and becomes activated when a pathogen crosses the epithelial surface of cells. It is particularly important in the first few hours of exposure to a new pathogen. Innate immunity elicits an immediate response against a pathogen but has limited specificity towards different pathogens as it recognizes conserved features of pathogens to get activated and destroy invaders. The specificities of the innate pathogen-receptors are encoded in the germline and evolved over evolutionary time. These particular pathogen-associated molecules are called pathogen associated molecular patterns (PAMPs), and are common to a certain class of pathogens but absent in the host. PAMPs are recognized by different types of Pathogen Recognition Receptors (PRRs) in the host that induce a signaling pathway that leads to a response against the pathogen (Albert B. et al., 2002).

The signaling pathways that are activated by the PAMP-PRR interactions often lead to the secretion of chemicals called interferons (IFN). Interferons are known to elicit an antiviral state and are important regulators of innate and adaptive immune responses. The antiviral response is carefully controlled by the crosstalk between cellular regulatory pathways involved in IFN signaling, cellular apoptosis, pro-inflammatory pathways, and the stress response. There are two main types of IFN— Type I and Type II. In this project, we mainly focus on Type I IFNs which are secreted in response to a viral infection. IFNs bind to their type specific receptors and ultimately result in the transcription of IFN-stimulated genes (ISGs). The transcription of ISGs is strictly modulated by IFNs through different positive and negative feedback mechanisms. This ensures the perfect immune response to the virus. In turn, viruses encode many mechanisms to counteract the host antiviral response and minimize secretion of IFNs in order to maximize viral replication. In fact, viruses have adapted to nearly evade all aspects of IFN regulatory pathways including blocking receptors downstream of the signaling pathway (Katze et al., 2002).

In this project, we are investigating how overexpression of PRRs on HeLa cells affects the containment of VSV infection in these cells. Specifically, we are looking at the cluster size and the time it takes to contain the virus when we overexpress TLR3, FAS, PKR, PRKRA, and RIG-I.

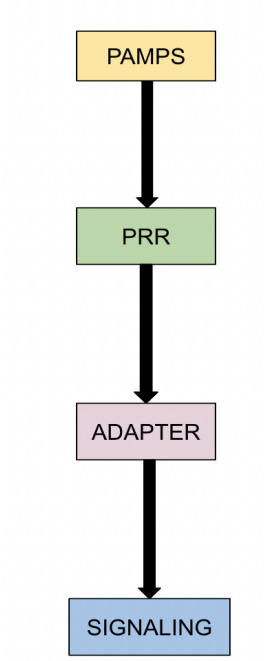

Illustration 1.3: A basic signal transduction pathway of innate immunity cellular receptors against a viral infection

1.5 : RIG-I RECEPTOR

Retinoic acid inducible gene I (RIG-I) is a key sensor of viral infection that mediates the type-I interferon induced antiviral host response. RIG-I is specifically a dsRNA sensor that is localized in the cytosol, although traces of RIG-I have also been seen in the nucleus. Once RIG-1 binds RNA and oligomerizes, the ligand-receptor complex interacts with the mitochondrial antiviral-signaling protein (MAVS), which then activates TBK1 and IKK-E which induce the IRF3/7 and NF-kB pathway. This results in the transcription of the genes that encode for Type-I interferons.

There are several key features that are utilized by RIG-I to 'sense' the viral RNAs. RIG-I monitors the 5'ends of RNA molecules for several biochemical features such as RNA molecules with a 5' triphosphate group or base pairing of one RNA molecule to a complementary strand of RNA (thus, a dsRNA) can trigger RIG-I activation. RNAs with uncapped diphosphate (PP) groups at the 5'end also activate RIG-I. Lastly, the 5' terminal nucleotide needs to be unmethylated at its 2'O position to allow RNA recognition of RIG-I. These important molecular features are found in several RNA viruses. The genomes of many negative sense RNA viruses contain partially self-complementary 5' and 3' ends that base pair to form an intermediate double stranded RNA structure that can be recognized by RIG-I. Often, viral polymerases are responsible for forming the unmethylated 5' PPP end during genome replication which then triggers RIG-I antiviral pathway.

It is important to note that RIG-I can also be activated by host cellular RNAs instead of viral RNAs. Thus, it is crucial for our innate immune system to mediate and control the antiviral response that is initiated by the rapid signal activation of RIG-I. This is done by the host by using mechanisms such as post-translational modifications mediated by regulatory enzymes, regulation by interaction proteins, post-transcriptional mechanisms, and autophagy(Rehwinkel J. et al., 2020) . In this thesis, an overexpression study of RIG-I on HeLa cells was performed to analyze the changes in the antiviral response initiated on viral infection. To counter the efficient antiviral pathway that is initiated by RIG-I, different viruses have developed several molecules that can interact with and antagonize its function. This leads to the inhibition of the interferon activation pathway and it occurs via RNA dependent and RNA independent mechanisms. Influenza A virus (a negative sense single strand RNA virus) has a non-structural protein (NS-1) that is a multifunctional dsRNA binding protein that directly

interacts with RIG-1 and prevents its activation, thus inhibiting RIG-1 activated IFN-β production and facilitating viral evasion of the host immune response. The dsRNA binding activity of NS1 is extensive and can inhibit various other sensors such as PKR as well. Vaccinia Virus is a DNA virus that has a protein called E3 that binds to dsRNA and has an antagonistic effect on RLRs such as RIG-I. VP-35 in Ebola Virus is another multifunctional virulence factor that interacts with RIG-1 to prevent activation of the IFN-β promoter and other IFN stimulated response element (ISRE)-containing promoters induced by dsRNA or viral infection (Leung D. et al., 2012) .

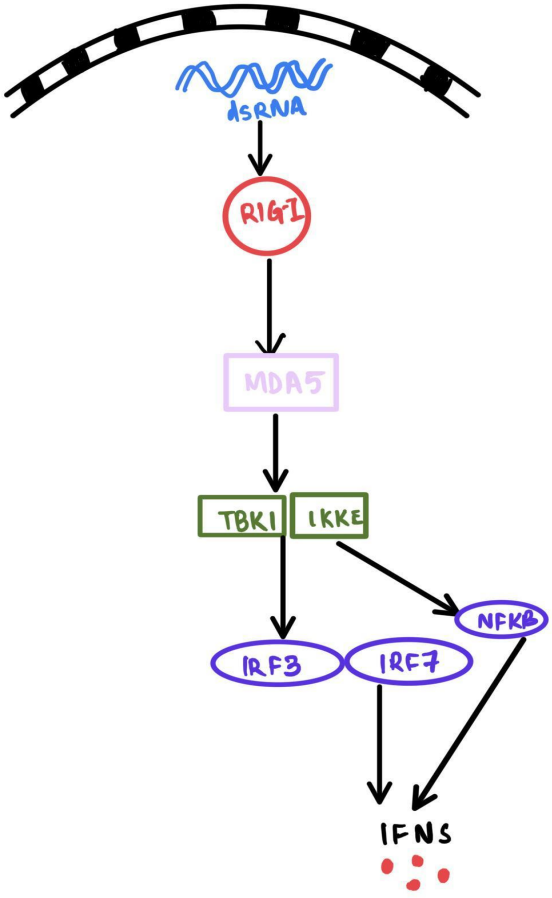

Illustration 1.4. RIG-I signaling pathway

1.6: TOLL LIKE RECEPTOR 3

The Toll-like Receptors are PRRs that comprise a family of type-I transmembrane receptors. All mammals have at least ten TLRs and each recognizes a specific PAMP during an infection. TLRs are localized either on the cell surface or the endosome. All TLRs recognize structurally unrelated ligands. It is often the case that a pathogen expresses multiple PAMPs that can be recognized by different TLRs. Activation of a TLR leads to the activation of an adapter protein that will then initiate a signaling cascade, thereby activating transcription of several immune response genes (Medzhitov, 2001).

TLR3 is a TLR that is localized in the endosome and recognizes double stranded RNA as its PAMP. TLR3 is found in different kinds of cells in the body including neurocytes, immune cells, fibroblasts, and various epithelial cells. TLR3 completely relies on TRIF as its adaptor protein to induce interferon production. TRIF interacts with tumor necrosis factor (TNF) receptor associated factor (TRAF3) and TRAF6 to trigger a series of signaling cascade reactions. This TLR3-TRIF pathway can be further classified as the TRIF dependent nuclear transcription factor-kB (nf KB) pathway and the TRIF dependent IFN regulatory factor 3/7 (IRF3/IRF7) pathway. Ultimately both pathways result in the production of type I interferon, proinflammatory cytokines and other chemokines (Chen Y et al., 2021) .

The double-stranded RNA that TLR3 specifically recognizes is often a viral replication intermediate, and so TLR3 can recognize viral replication and initiate downstream signaling, and expression of different interferons, and antiviral protein synthesis. VSV is a -ssRNA virus which forms a dsRNA intermediate for viral replication. We expect that during the replication stage of VSV, TLR3 will recognize the dsRNA and initiate an antiviral response.

Previous studies of viral infections by -ssRNA viruses such as Influenza A Virus have shown that TLR3 is essential for the antiviral response and the production of IFN I (Lim H. et al. 2019). TLR3 has also been presumed to be an important regulator of innate and adaptive immunity against SARS- Cov-2 (Mabrey, 2021). Based on what we know about this in the literature, we expect that levels of TLR3 expression may affect infection outcome. We hypothesized that an overexpression of TLR3 will lead to a better containment of VSV in HeLa cells.

As with most signaling mechanisms in immunology, the TLR3 signaling pathway is based on a careful modulation between its role in generating antiviral response while also making sure that the response does not negatively impact the host's immune system. TLR3 signaling is a well known response against viral infections that prevents disease in the host by diminishing the high viral load on infection and triggering th IFN mediated signaling cascade.However, an overactivation of TLR3, can lead to overstimulation of the host's immune system and ultimately lead to pathogenesis. Therefore, it is crucial that the immune response elicited by TLR3 is controlled. In case of chronic viral infections, the TLR3-TRIF signaling pathway becomes a key determiner of the balance established by the anti and pro-inflammatory pathways that lead to an IFN-induced antiviral response and other regulated immune responses (Chen Y et al., 2021).

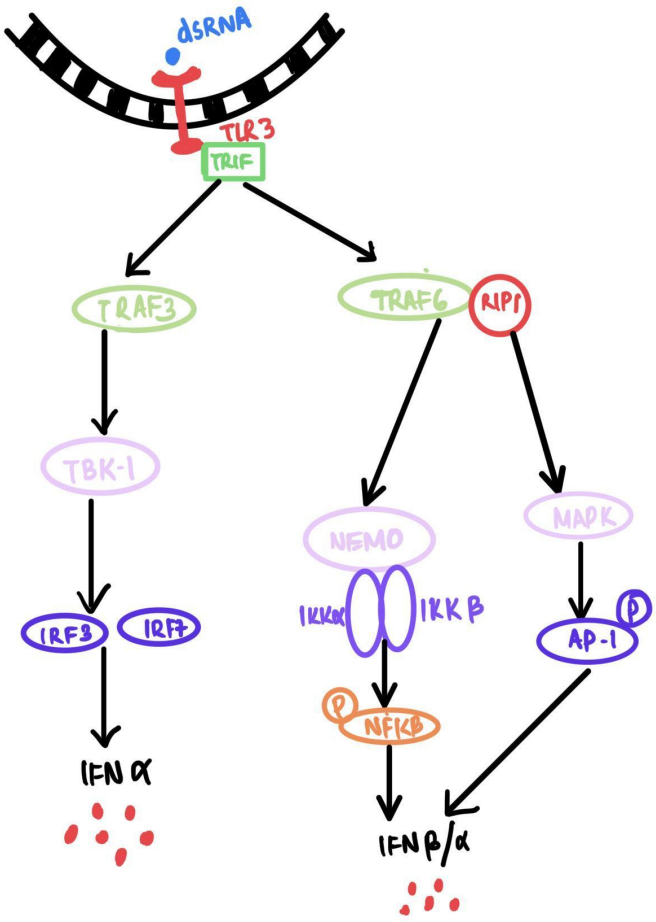

Illustration 1.5. TLR3 signaling pathway on recognition of dsRNA

1.7: FAS (CD95/APO-1)

Fas (also called CD95 or APO-1 or TNFRSF6) is a type I transmembrane protein that contains a death domain (DD) in its cytoplasmic region which plays a crucial role in the induction of apoptosis. Fas-mediated apoptosis is important for immune homeostasis. The induction of apoptosis is triggered by the interaction of FAS with its ligand FASL which is a 40kDa membrane protein. This interaction leads to the recruitment of the adaptor protein Fas-associated death domain (FADD) and facilitates the binding of procaspase-8, resulting in the

formation of the death-inducing signaling complex (DISC). Finally, this leads to the activation of effector caspase 3- by activated Caspase-8. Once the caspase cascade is activated, it leads to cellular apoptosis. Fas-mediated apoptotic signaling is one of the major cytotoxic mechanisms used by the immune system to kill endogenous infected host cells and exogenous pathogens (Yolku E. et al., 2017). The activation of the Fas-FasL pathway is a tightly regulated mechanism that is controlled by the formation of Fas microclusters, actin reorganization, inducible or constitutive association with membrane rafts and acid sphingomyelinase-mediated ceramide production. (Nainu F et al., 2017).

Cellular apoptosis followed by phagocytosis of virus-infected cells is an antiviral response that is conserved across several multicellular organisms. When a viral infection occurs, there is a higher transcriptional gene expression level of Fas and its ligand, FasL. Due to the higher expression level, more ligand-receptor interaction occurs on the surface of the virus infected cells, resulting in the high induction of cellular apoptosis (Nainu et al., 2017). In this project, the Fas receptor was overexpressed in the HeLa cells to study the induction of the apoptotic signaling pathway that can limit the spread of the viral infection by VSV.

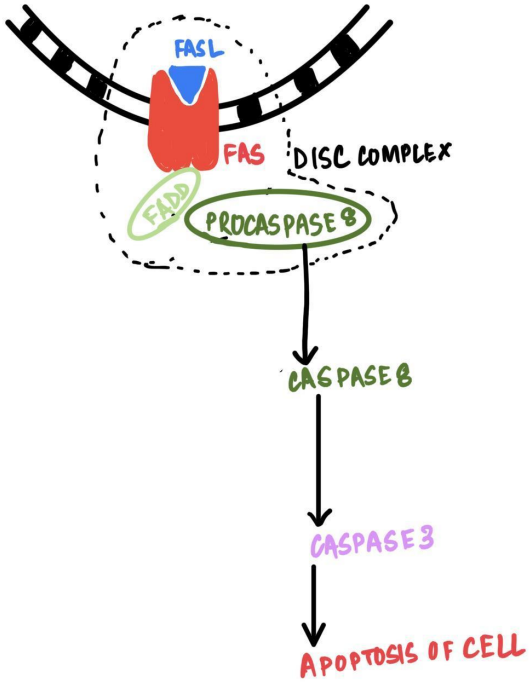

Illustration 1.6. Fas-Fas-L Apoptotic Signaling Pathway

1.8: PROTEIN KINASE R (PKR)

Protein Kinase R is a kinase that plays a key role in the interferon-induced antiviral response upon viral infection. While usually PKR is in its inactive state, once it binds to its substrate— double stranded RNA(dsRNA), it undergoes instant activation and autophosphorylation. Ultimately it limits viral replication by inhibition of cellular protein synthesis. The α-subunit of eukaryotic initiation factor eIF2 is the key substrate of PKR in the host cell.Phosphorylation of eIF2α interfere with the translation of the proteins and thereby leads to the inhibition of both viral and host protein synthesis (Lemaire P. et al., 2008). In this project, we overexpressed PKR in HeLa cells to investigate how PKR levels affect host-virus interaction on viral infection with VSV.

PKR activation by dsRNA has been extensively studied and there have been different proposed molecular models. Most models have agreed on the idea that PKR is capable

of dimerizing in the absence and the presence of dsRNA. PKR has a bell shaped activation curve that indicates that low concentrations of dsRNA activate but higher concentrations inhibit PKR activation. The researchers speculated that this activation curve indicated that at a lower concentration, PKR dimerizes on a single dsRNA whereas a higher concentration of dsRNA dilutes the PKR to form only monomers on the dsRNA (Lemaire P. et al., 2008) . In this experiment, while we hypothesize that an overexpression of PKR will lead to a better containment of VSV, an exposure to a high viral load to these cells containing a high quantity of dsRNA can induce a different effect to the VSV infection based on the balance of their stoichiometric interaction.

While the main activator of PKR is known to be dsRNA, previous studies have demonstrated that sometimes single stranded regions in the RNAs (ssRNAs) can also activate PKR and these ssRNAs bind at the basic region of the PKR structure as well as the dsRBD (double stranded RNA Binding Domain). The activation of PKR by the ssRNA requires the presence of the basic region. PKR activation is therefore regulated both by the interaction of the double and the single stranded regions(Mayo C., Cole J., 2017) . This is important because VSV is a –ssRNA virus and it is possible that PKR not only senses the replication intermediate of VSV, but also the genome itself.

PKR is a well known broad range antagonist since it is capable of inducing an IFN response through multiple pathways including inhibition of mRNA translation, apoptosis, and IFN mediated response. Thus, viruses have also evolved different methods to evade PKR. Middle East Respiratory Coronavirus (MERS-CoV) has developed a mechanism where it uses antiviral protein called p4a to “hide” or sequester the dsRNA molecules, thereby preventing the activation of the PKR induced antiviral response (Rabouw et al., 2016). Some viruses degrade

excess dsRNA molecules in order to restrict PKR activation. This is seen in Infectious Bronchitis Virus (IBV) which uses a protein called nsp15 endonuclease to perform this function(gao et al., 2021, Zhao et al., 2021). There are still other viruses, such as members of the Bunyavirales family, that choose to degrade PKR instead to dsRNA molecules in order to inhibit the antiviral response(Ikegami et al., 2009; Kalveram et al., 2013). Another virus called murine cytomegalovirus uses genes, m142 and m143, to affect the downstream signaling cascade of PKR, by dysregulating its activation and autophosphorylation (Valchanova R. et al., 2006). Viruses such as HIV-I contain proteins such as Tat that directly bind to PKR and prevent the PKR autophosphorylation and dimerization (Heinicke et al., 2009). A similar approach is used by the N protein of Respiratory Syncytial Virus (RSV) (Groskreutz et al., 2010). In RSV, its nucleoprotein can also recruit a protein called PP2 that binds to eIF2a which leads to its dephosphorylation and therefore, activates viral protein synthesis and viral spread (Groskreutz et al., 2010). There are many other viruses that have evolved different ways to inhibit the PKR mechanism by deactivating different parts of the signaling cascade. It is fascinating that there are so many diverse mechanisms against PKR induced antiviral response . Researchers have proposed that it could be possible that viruses try to not broadly inhibit the innate immune system because an excessive viral load in the host cell might backfire and lead to the host cell apoptosis and prevent further host to host viral transmission(Cesaro T, Michiel T., 2021).

1.9: PRKRA

The *PRKRA* gene encodes a protein called PACT, which performs several cellular pathways in which it functions as a PKR activator during an integrated stress response (ISR) (Vaughn L. et al., 2022). PACT plays a role in the cell's response to stress, such as exposure to viruses, damaging molecules called free radicals, or other toxic substances (Medline Plus).

When PACT is expressed, it activates and heterodimerizes with PKR in a dsRNA dependent fashion. This results in an elevated IFN induced antiviral response upon viral infection. As with most of the dsRNA binding host cellular receptors, the signaling pathway ultimately leads to the activation of NF-kB and IRF3 and IRF7. This shows that not only does PACT turn on the protein synthesis inhibition mediated by PKR but also interacts with other cellular pathways involved in the antiviral response (Iwamura et al., 2001). PKR's kinase activity remains latent until it is bound to dsRNA during viral infection or PACT during non-viral cellular stress signals. Once activated it follows its usual mechanism of phosphorylation of eIF2a (Vaughn L. et al., 2022).

PACT is essential for IFN production in response to some viral infections and many viruses have targeted PACT inactivation mechanisms to counteract the host defense mechanisms(Vaughn L. et al., 2022). Infection with viruses such as Herpes Simplex Virus (HSV) result in an interferon induced antiviral response that is mediated through by the different PRRs such as TLRs and RIG-I receptors. Optimal function of dsRNA sensors such as RIG-I requires PACT, which interacts with, and potentially activates RIG-I in a PKC-independent manner. HSV-I has a protein called Us11 that associates with PACT tightly to prevent it from binding with and activating RIG-I. This results in reduced RIG-I dependent IFN-I production and allows the virus to evade the host's innate immune response(Kew C. et al., 2013). Mouse Hepatitis Virus and severe acute respiratory syndrome (SARS)-CoV have also evolved new strategies to evade the immune response in a PACT dependent fashion. MHV N protein counteracts the IFN-β production by inhibiting the PACT-RIG-I/MDA5 interactions and RIG-I/MDA5 activation. The same strategy has also been utilized by SARS-CoV N protein (Zhen D. et al.,2017)

However, the role of PACT in viral infection seems to be fairly debated. *In vitro,* an overexpression of PACT has shown no effect on gene induction by poly(I:C) treatment which results in an activation of the antiviral pathways mediated by IRF-3, IRF-7, and NF-kB (Iwanmura T. et al., 2001). In NewCastle Disease Virus infection, the overexpression of PACT did not affect type I IFN gene activation. Another factor to consider would be that the role of PACT in viral infection might be virus dependent since different viruses exhibit a different host tropism (Iwamura T. et al., 2001).

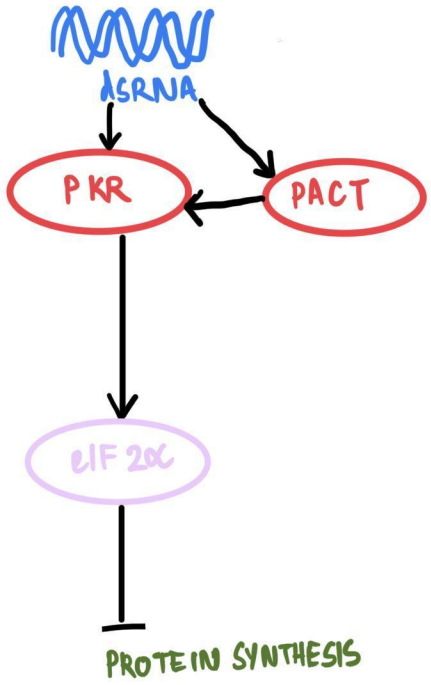

Illustration 1.7. PKR and PRKRA induced protein (PACT) signaling pathways on recognizing dsRNA

1.10: SUMMARY

In conclusion, this project aims to study whether an overexpression of different viral sensors can lead to a better containment of a viral infection. VSV which is a –ssRNA virus was used as the model virus for infection on HeLa cells. During viral replication, VSV momentarily forms a double stranded RNA (dsRNA). TLR3, RIG-I, and PKR are cellular receptors that directly bind to dsRNA and activate a Type-I IFN induced antiviral response that leads to the containment of the virus. Similarly, PRKRA is a gene that encodes for protein PACT which also directly binds to dsRNA or can activate the PKR antiviral response pathway by binding directly to PKR. Thus, we overexpressed these genes to demonstrate if more activation of TLR3, RIG-I, PKR, and PRKRA in HeLa cells could lead to a better and faster containment of the virus. Fas is a gene that encodes for its receptor that binds to its ligand, FasL and initiates an apoptotic pathway in cells. During a viral infection, the expression of the Fas ligand and receptor is often enhanced to lead to greater cellular apoptosis to prevent the virus from spreading. In this project, we overexpressed Fas to see whether an overactivation of Fas in the host cell would lead to a faster and better containment of the virus.

To evaluate the containment of the virus, we analyzed clusters of dead cells which form when the cells are killed by the VSV virus and undergo apoptosis to prevent the virus from spreading. The lesser the time it takes to form the cluster and the smaller these clusters are, the better is the containment of the virus by the host cell. On overexpressing these viral sensors, we expected to see a smaller cluster of cells within a shorter time period on viral infection.

CHAPTER 2: MATERIALS AND METHODS

2.1 Development of the desired plasmids

We developed plasmids to overexpress TLR3, PKR, PRKRA, FAS, and RIG-I. CMV promoter was used in the backbone of the plasmid since it is a strong promoter that is well known to provide strong expression activity in mammalian cells. A nuclear localization sequence followed by a P2A spacer was used in between the donor plasmid DNA and a CFP protein tag. This helped us detect a CFP signal whenever the donor DNA was expressed in the HeLa mutant cell lines. We ordered plasmids from Addgene to obtain the receptor sequences to use for downstream cloning. These plasmids are #13641: pcDNA3-TLR3-CFP,# 20030pSB819-PKR-hum, Plasmid# 106113pLenti_C_Myc_DDK_IRES_Puro_PRKRA, # 98334FAS_pLX307, #167288pLenti-RIG-I-Apex-P respectively. The backbone template for the overexpression plasmids was pLenti-puro-pCMV-USP18-P2A-NLS-CFP. A Gibson Assembly was first performed using this backbone to form the final RIG-I plasmid called pCMV-RIG1-P2A-NLS-CFP in pLenti-Puro. We used the previously constructed plasmid, pCMV-RIG1-P2A-NLS-CFP in pLenti-Puro, as the backbone for the final plasmids of the other receptors. This plasmid was digested using Restriction Enzymes SpeI + NheI and we extracted and purified the band at 8131 bp to use as the template. Once we obtained the template for the final plasmid, we designed primers for the inserts. The final primers used are listed below. We used PCR to amplify each of the inserts using the designed primers. Finally, we used Gibson Assembly to create the final plasmids consisting of the overexpression template and the respective inserts using NEBUILDER 2X HiFi DNA Assembly Master Mix. After the Gibson Assembly, we transformed the product into *E.Coli* and performed minipreps on the resulting colonies. We then sent the plasmids from the minipreps for Sanger sequencing to confirm

whether the desired plasmid was obtained. Once we confirmed the final plasmids, we performed midipreps to obtain the optimal amount of desired donor DNA to be used for transfection.

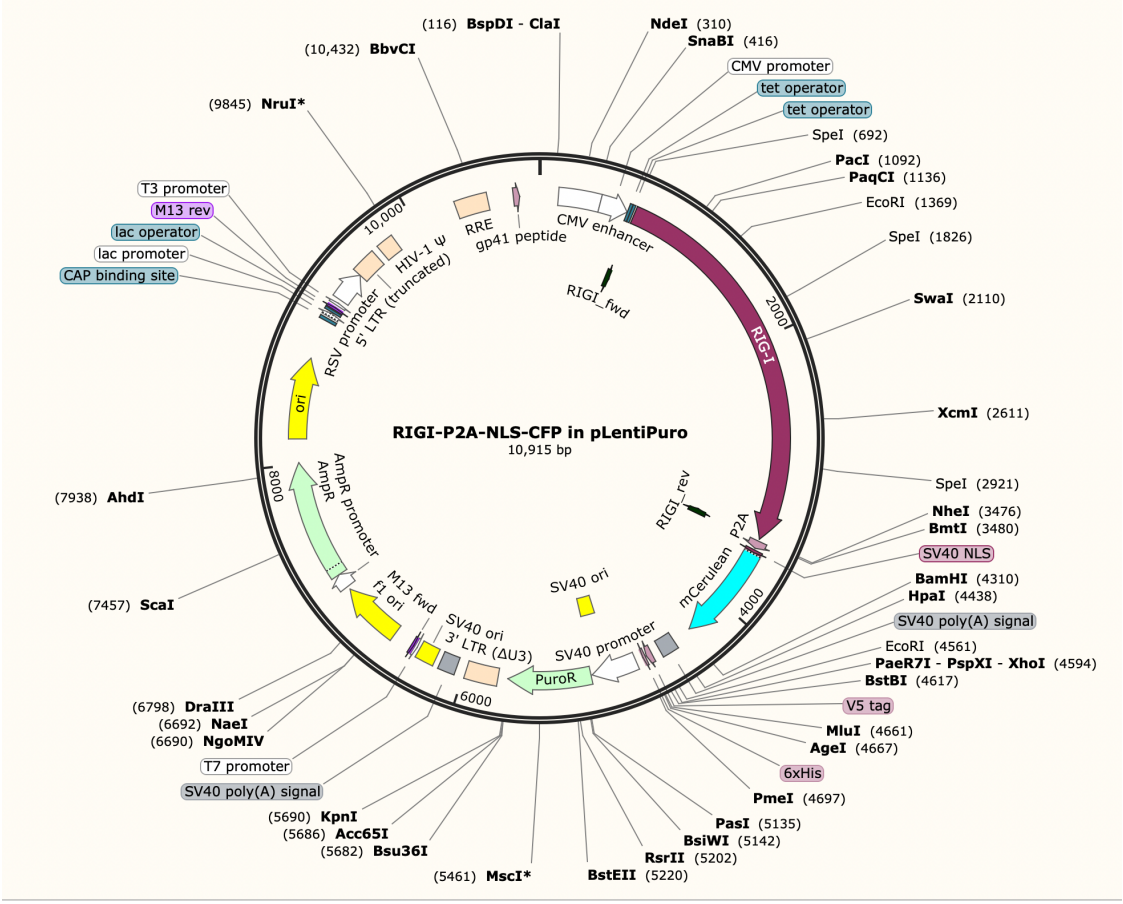

Image 2.1. RIG-I final plasmid

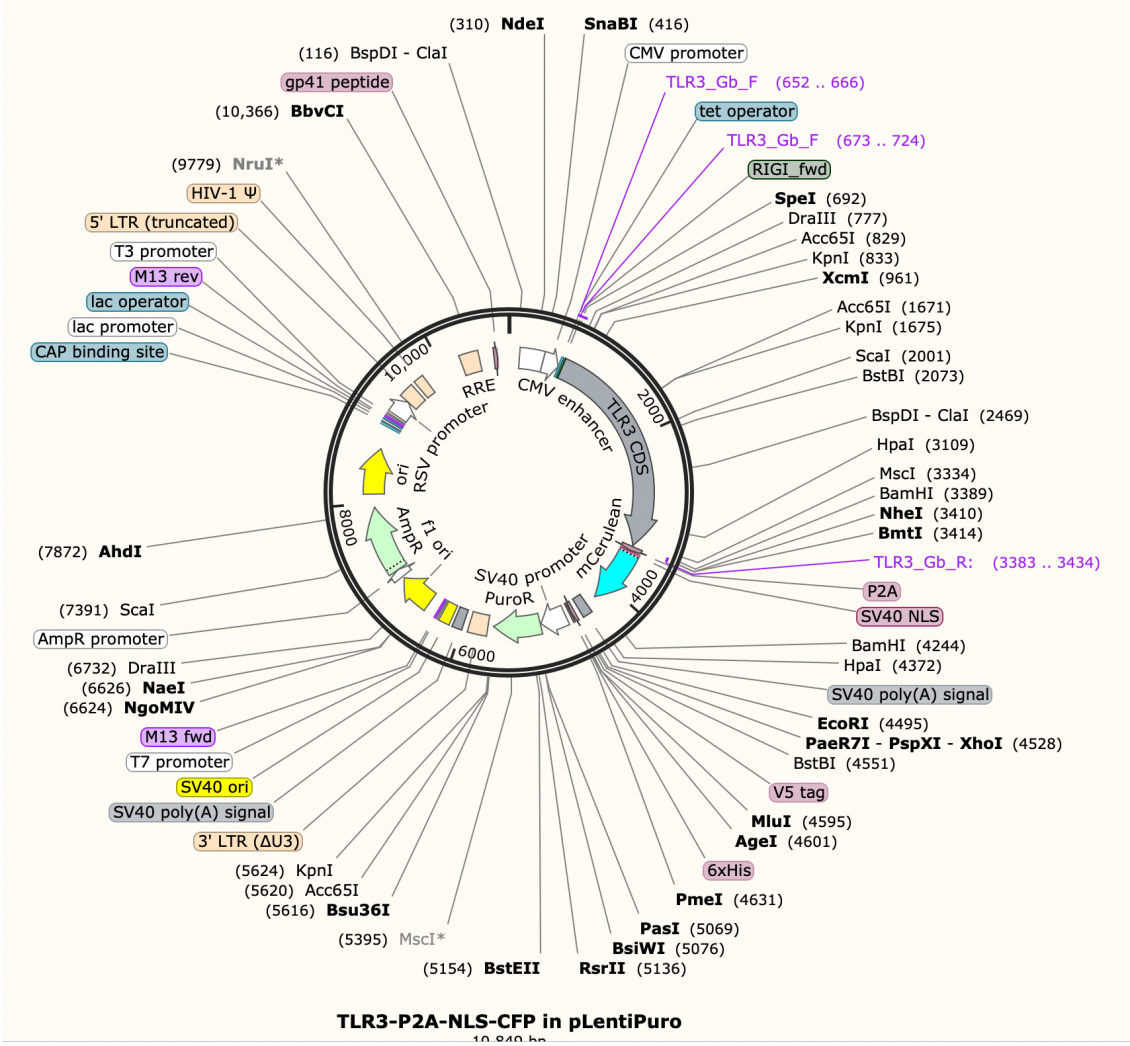

Image 2.2. TLR3 Final Plasmid

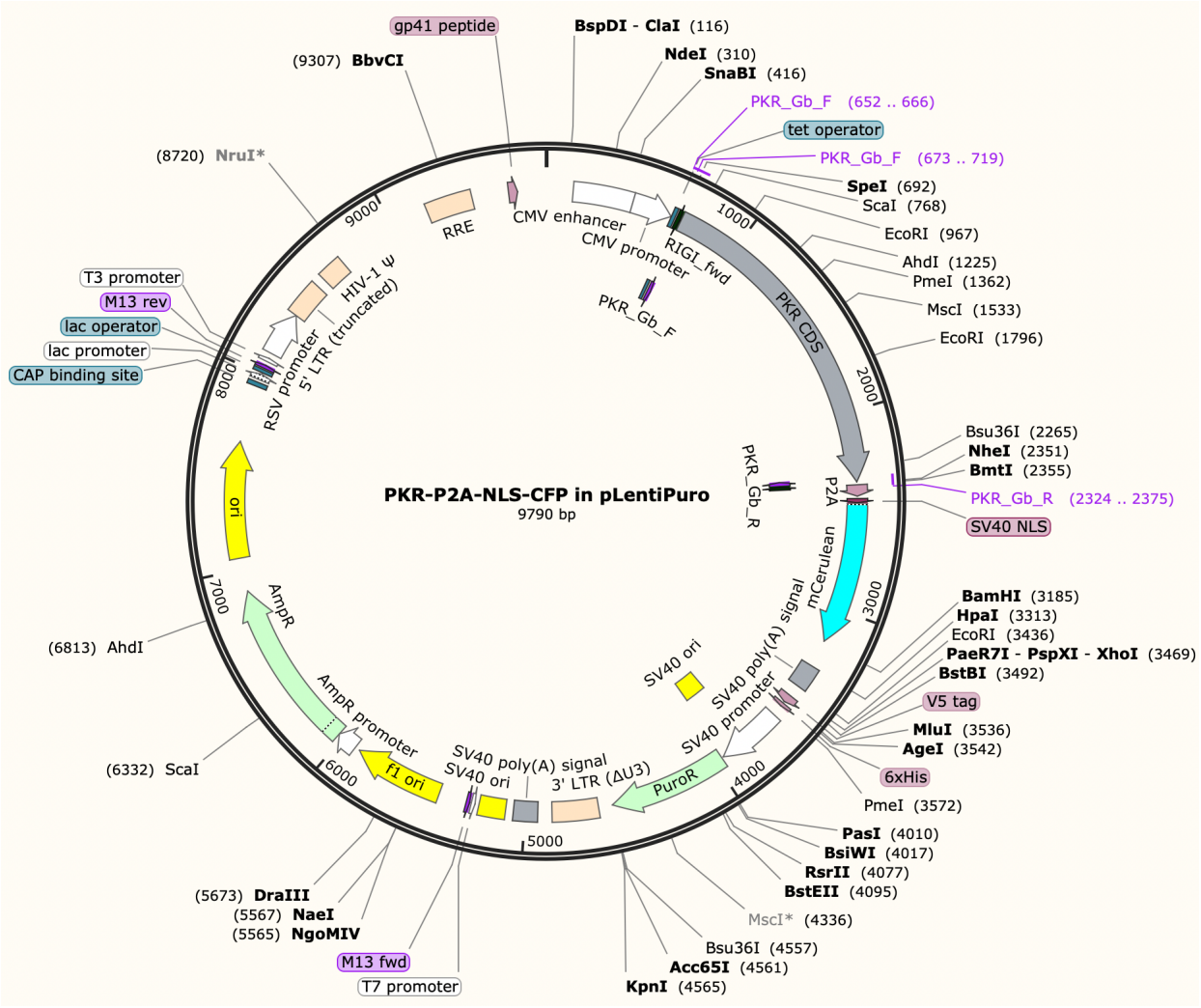

Image 2.3.PKR Final Plasmid

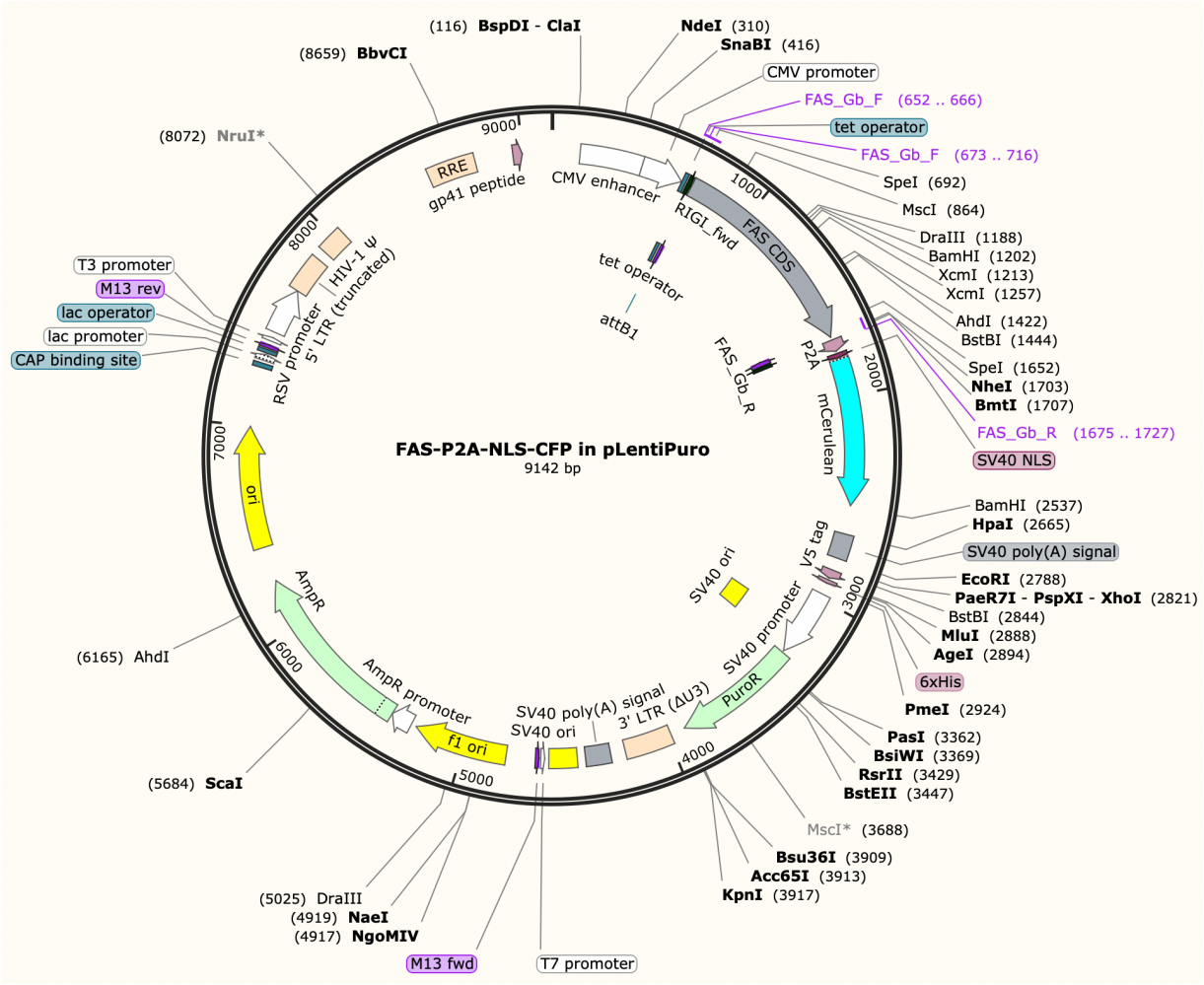

Image 2.4. Fas Final Plasmid

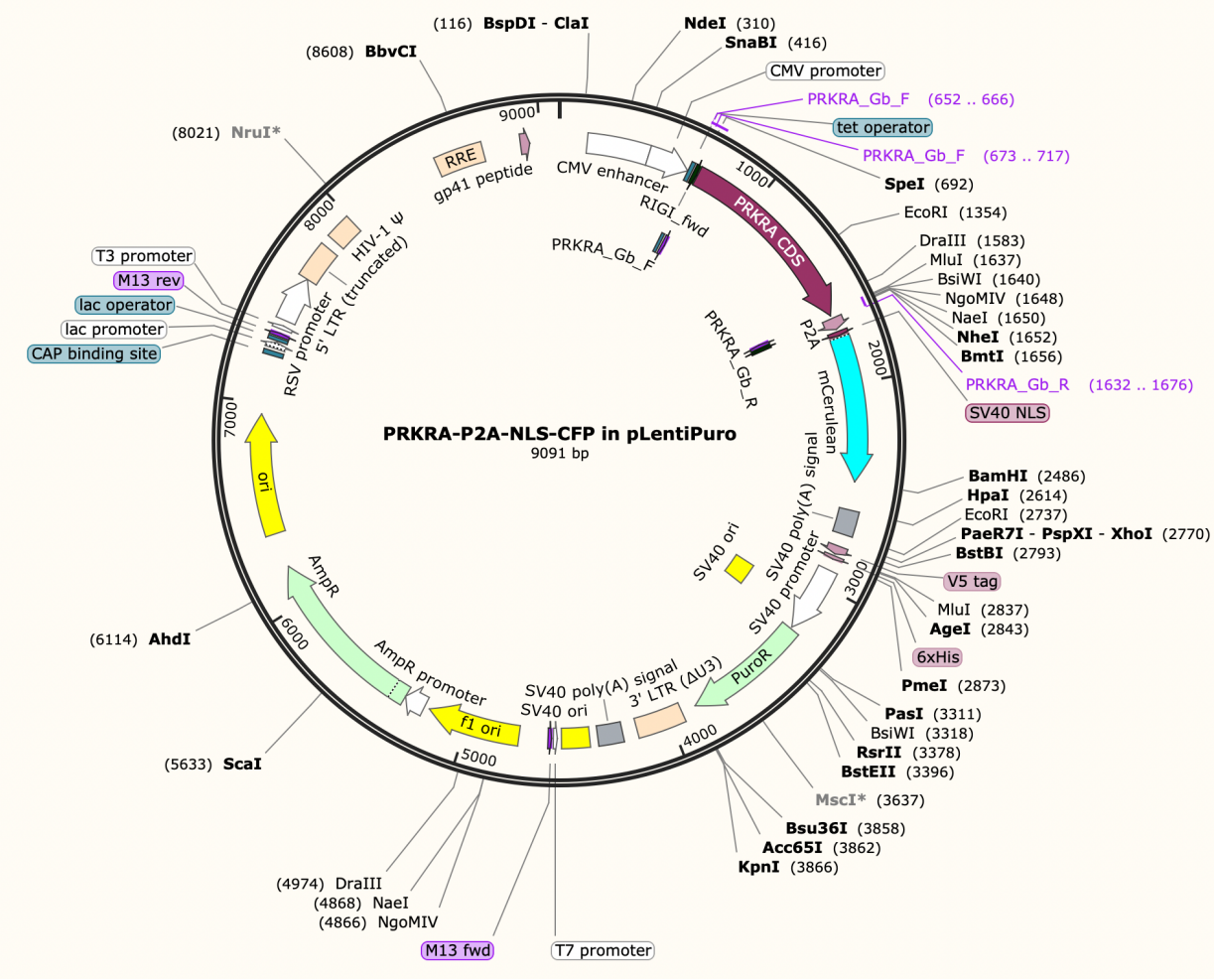

Image 2.5. PRKRA Final Plasmid

Table 2.1. Primers for TLR3, PKR, Fas, and RIG-I

TLR3_forward primer	TCAGTGATAGAGATCGTCGACTAGTatgagacagactttgccttgtatctac
TLR3_reverse primer	AGTTAGTAGCTCCGCTGCCGCTAGCatgtacagagtttttggatccaagtgc
PKR_forward primer	TCAGTGATAGAGATCGTCGACTAGTatggctggtgatctttcagcag
PKR_reverse primer	AGTTAGTAGCTCCGCTGCCGCTAGCacatgtgtgtcgttcatttttctctgg
FAS_forward primer	TCAGTGATAGAGATCGTCGACTAGTatgctgggcatctggaccc
FAS_reverse primer	AGTTAGTAGCTCCGCTGCCGCTAGCgaccaagctttggatttcatttctgaag
PRKRA_forwardprimer	TCAGTGATAGAGATCGTCGACTAGTatgtccccagagcaggcaccg
PRKRA_reverseprimer	AGTTAGTAGCTCCGCTGCCGCTAGCcggccgcgtacgcgtctttc
RIG-I_forward primer	tcagtgatagagatcgtcgactagtATGACCACCGAGCAGCGAC
RIG-I Reverse Primer	agttagtagctccgctgccg*ctagc*AGGTTTGGACATTTCTGCTGGATC

2.2: Lentivirus Transfection and Development of Stable Cell Lines

We performed a standard lentivirus transfection using the 1 ug Donor DNA (the midiprep DNA of the final plasmids), psPAX2, pMD2.G, and FuGeneHD transfection reagent. We performed the transfection with the 1ug DNA:2ul Fugene (Promega E2311) ratio. We

incubated this mixture at 37 C for fifteen minutes and added it to a 10 cm plate of HEK293T cells. Two six-well plates of HeLa cells seeded at 400,000 cells/well were seeded on the same day. The next day, we collected the supernatant containing the virus from the HEK293T cells into a 15 ml centrifuge tube and spinned it down at 1800 rpm for 5 minutes to remove any cells that may have been present. We recollected the supernatant and added polybrene at 1:1000 dilution and inverted the tube to mix it well. We then added 4 ml of this mixture to each well of the two 6-well plates containing HeLa cells. Thus, lentivirus transfection was used to develop the different viral sensor overexpression cell lines. The Hela and 293T cells were grown in Dulbecco's Modified Eagle Media (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate, Fetal Bovine Serum (FBS) (5%), Penicillin/Streptomycin (1%). We added Puromycin (1ug/ml) to the media for four days to select for cells. Survival cells were grown for 14 days. To ensure clonal populations of cells, we used fluorescence activated cell sorting (FACS) to sort single cells and grow clones from these cells. The clones were then selected based on their homogeneous overexpression of the desired viral sensor which was determined by measuring CFP levels as each overexpressed receptor has a NLS-CFP transcriptional reporter tagged to it.

2.3:Plating and Infection Experiment

On the day before the infection, we plated the overexpression cell lines (TLR3, PKR, FAS, PRKRA, RIG-I) at a cell density of 200,000 cells per well in a 24 well plate. Wild-type HeLa cells were used as a negative control. For infection, we replaced the media of the plated cells and added the diluted virus to this media. We infected the cells by adding normal medium containing 2500 plaque forming units (PFU) of VSV- mcherry, which corresponds to a multiplicity of infection (MOI) of ~ 0.01. The virus aliquot was diluted at a

ratio of 1:80. 1ul of diluted virus was used per well of the 24 well plate. After adding the virus containing media, the cells were left in the incubator at 37 C, 5% CO2 and 90% humidity for one hour. The virus containing media was then replaced by 0.8% Carboxymethyl Cellulose (CMC). The infected cells were placed back into the incubator for 6-10 hours to allow for the infection to occur.

2.4: Image Acquisition

After the incubation period on the day of the infection (6-10 hours after washing out the virus), we brought the infected plate of cells to our microscope to set up for time-lapse imaging. Time lapse images were acquired using a Nikon Ti-E inverted microscope equipped with integrated Perfect-Focus (PFS), Nikon Plan Apochromat Lambda objective lens, and Evolve 512 EMCCD camera (Teledyne Photometrics). We imaged the cells with an on-stage incubator equipped with temperature control at 37 C and humidified 5% CO2. We acquired images for a time period of 72 hours with a 30 minute interval between each loop. The channels imaged were 10X phase (brightfield), Cy5 (nuclear marker) and mCherry (showing the virus infected cells). All channels were imaged at an interval of 30 minutes. We used an exposure time of 500 ms for the Cy5 and the mCherry channel and one frame for the phase channel. After 72 hours, the infected clusters of cells were analyzed and compared to the control.

2.5: Data Analysis

We used custom lab code in MATLAB to track the viral spread in cells. After 6-10 hours of washing out the virus from the cells, we located single infected cells in each well

of the 24 well plate by detecting the mCherry signal in the HeLa cells using the Nikon Ti-E inverted microscope. We used time lapse imaging to track the formation of the clusters of dead cells that formed around those infected cells. This helped us evaluate the containment of the virus in the cells. The codes used for the experiments allowed them to track the different clusters formed in the mutant and the wild-type cell lines and study the different features. The final data was then plotted using MATLAB. Statistical calculations were also performed using RStudio and MATLAB.

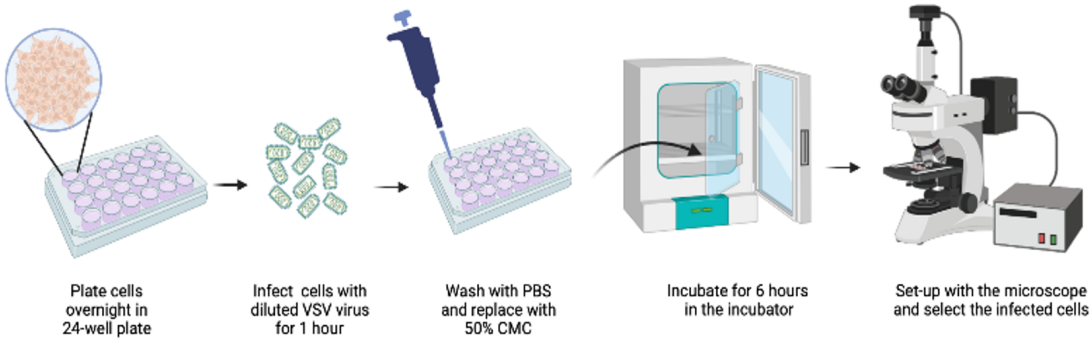

Illustration 2.1. The experimental set-up

2.6: Cell Tracking

Time lapse movies consisting of 433 frames of mutant infected cells of an overexpression RIG-I cell line and Wild Type HeLa cells were manually tracked using MATLAB. Cell death of the cells was defined by the loss of the Cy5 signal and a bright autofluorescence that was seen in the 10X phase channel. Cell death tracking was also performed for some time lapse movies of WT cells to get an accurate understanding of the death time of the cells on viral infection.

CHAPTER 3. RESULTS

3.1 Time lapse microscopy was used to record Brightfield channel, Cy5 channel, and mCherry Channel for the clusters of infected cells

We created HeLa cell lines which overexpress one of TLR3, FAS, PKR, PRKRA, and RIG-1, with the overexpressed receptor also expressing a NLS-CFP after a translational skip site to confirm expression level. These cell lines also have an iRFP nuclear marker (Figure 3.1 (top left)) for use in cell tracking and image analysis. We then infected each cell line with VSV-mCherry so that we could track the virus and identify virus-infected cells (Figure 3.1 (bottom)). We imaged these infected cells on the microscope to look at how receptor overexpression affected cell death and the size of the viral cluster and study the containment of the virus in the cell lines.

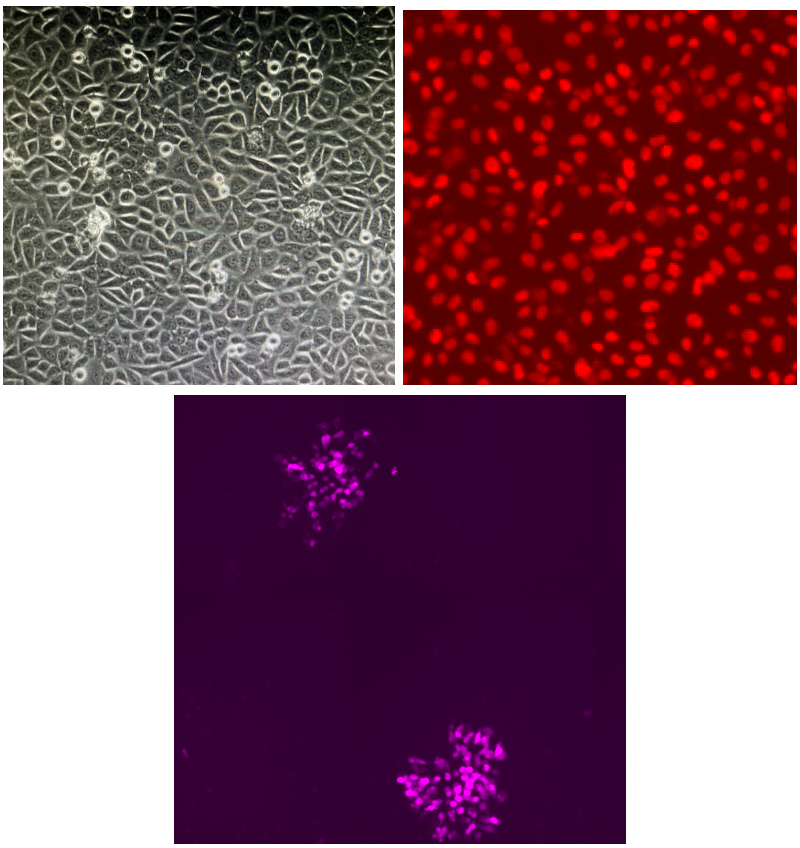

Figure 3.1: Channels used for the experiment. Brightfield channel (top left), Cy5 channel (top right), and the mcherry signal (bottom)

3.2: Different wells of wild-type cells show a similar time of cell death

We analyzed six movies of wild-type infected HeLa cells to determine the time at which cells in each movie die. These data show that in multiple wells and across multiple replicates, the wild-type cells die at approximately the same time and with the same distribution. While there is heterogeneity in the time of cell death, the median time of death is similar across wells. The median for the cell death time was calculated to be 150, 144, 145, 152, 154, and 169 frames respectively in different movies composed of 433 frames (Figure 3.2). On performing an Anova Test, the p-value obtained was more than 0.05. Thus, the differences in the time of death

of the cells in the six different clusters in different wells of wild type cells was not statistically significant. This suggests that wild type cells can be used as an effective control in this experiment.

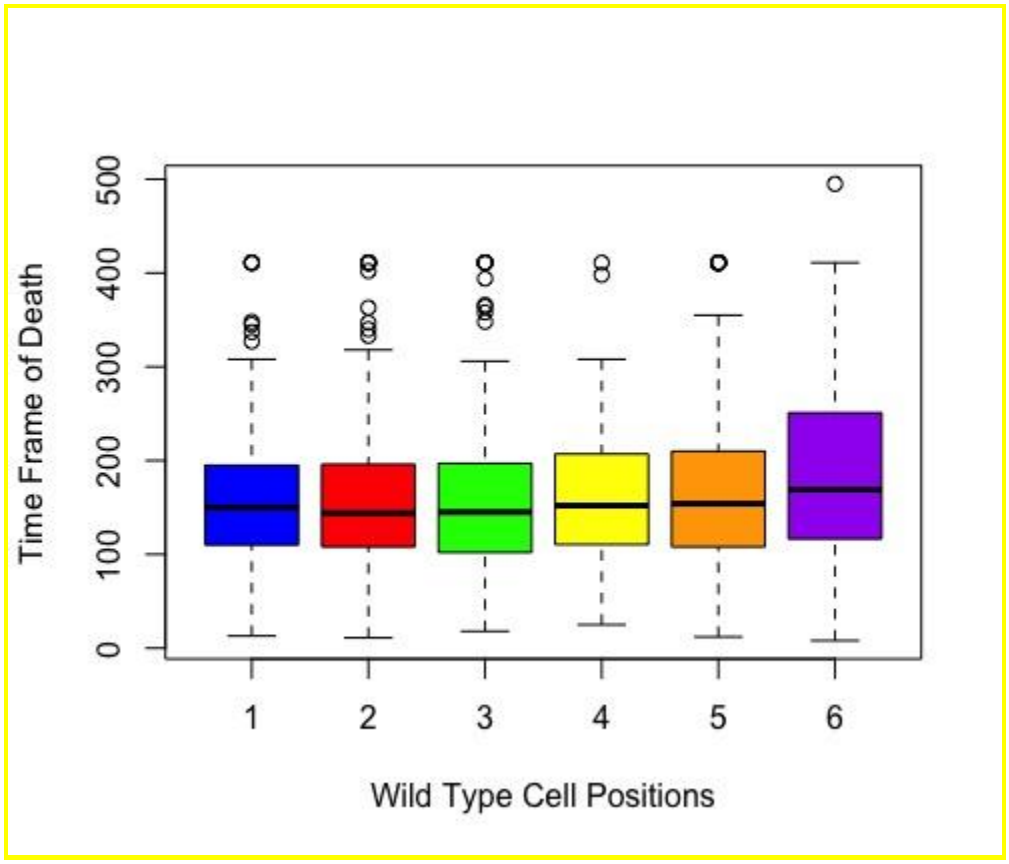

Figure 3.2. Wild type Hela cells can be used as the negative control for the experiment. Manual Cell Tracking of WT cells in six different wells indicates a similar mean time of cell death for wild type cells (**p>0.05).

3.3: RIG-I overexpression cells show a faster time of cell death

We performed an experiment where we manually tracked 200 infected RIG-I overexpression cells in one well to record the time frame in the movie at which the cells die. We compared these results to a control of infected wild-type cells at another position on the same 24 well plate. Our results show that RIG-I overexpression cells die faster than wild-type cells upon viral infection (Figure 3.3). Comparing the mean death times using a t-test gives a p-value of less than 0.05, showing a statistically significant difference in the mean time of cell death between the RIG-I overexpression cells and the wild-type cells after viral infection.

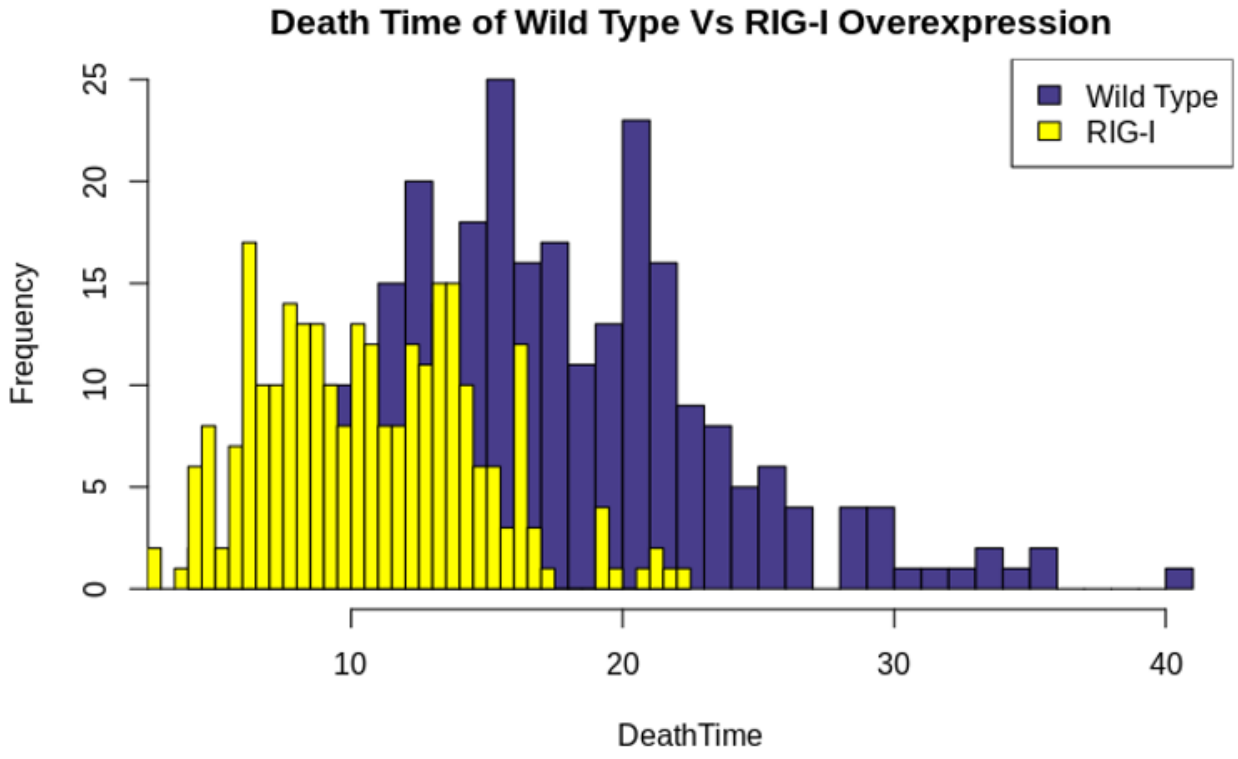

Figure 3.3. Comparing the death time of infected RIG-I overexpression cells with infected wild-type cells. On viral infection, cells with an overexpression of RIG-I (yellow) died at earlier than the wild type cells (blue).

3.4: Overexpression of RIG-I in Hela cells lead to a significantly smaller cluster size on viral infection

RIG-I overexpression cells and the wild-type cells were infected with Vesicular Stomatitis Virus (VSV) and imaged every 30 minutes after seeing the first infected cell for 72 hours. The clusters of Vesicular Stomatitis Virus (VSV) in the infected cells were shown as an mCherry signal tagged to the VSV. At the end of the time lapse imaging experiment, we had 17 VSV clusters in the RIG-I overexpression cells and 8 VSV clusters in the wild-type cells. These clusters were then tracked from 6-10 hours after infection till the end of the 72 hours and analyzed using custom lab code on MATLAB. The results showed that RIG-I overexpression cells have an overall smaller mean cluster size as compared to the wild-type cells starting from 20 hours after we started the time lapse experiment (Figure 3.4). On performing a two-sample t-test on the normal distribution of RIG-I overexpression cells and the wild-type cells, we got a p-value less than 0.05. Thus, we could conclude that there is a true significant difference in the mean cluster size of the RIG-I overexpression and the wild type cells. This confirms the hypothesis that an overexpression of RIG-I in Hela cells leads to a better containment of the virus.

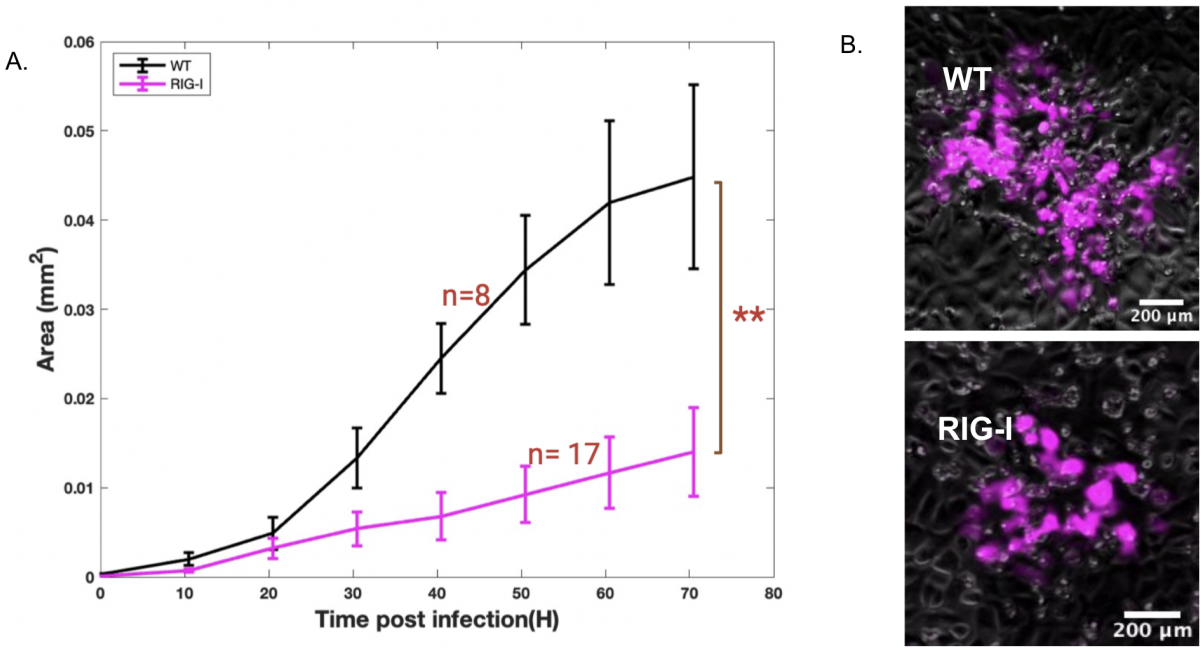

Figure 3.4. An overexpression of RIG-I in Hela cells leads to a decrease in the size of the cluster of Vesicular Stomatitis Virus (VSV) infected cells. A. 17 clusters of RIG-I-overexpressed VSV-mcherry infected cells were imaged for 72 hours and the mean area of the VSV cluster was plotted every 10 hours (magenta line). This was compared to the 8 clusters of the control wild-type cells (black line). A two sample t-test was performed between the normal distribution of the two groups at 72 h and a significant difference on the cluster sizes between the RIG1-overexpressed and the control group was recorded (** p-value<0.05). B. Time lapse images of a cluster of VSV-mcherry infected wild-type cells (top) and RIG-I overexpression cells (bottom). The images were obtained at the end of the experiment and the mCherry channel and phase 10X channel were merged using ImageJ.

3.5: Overexpression of TLR3 in HeLa cells lead to no significant change in cluster size on viral infection

TLR3 overexpression cells and the wild-type cells were infected with Vesicular Stomatitis Virus (VSV) and imaged every 30 minutes after seeing the first infected cell for 72 hours. The clusters of Vesicular Stomatitis Virus (VSV) in the infected cells were shown as an mCherry signal tagged to the VSV. At the end of the time lapse imaging experiment, we had 15 VSV clusters in the TLR3 overexpression cells and 10 VSV clusters in the wild-type cells. These

clusters were then tracked from 6-10 hours after infection till the end of the 72 hours and analyzed using custom lab code on MATLAB. The results showed that TLR3 overexpression cells have no overall difference in mean cluster size as compared to the wild-type cells even though it seems like TLR3 overexpression cells result in a slightly smaller cluster (Figure 3.5). On performing a two-sample t-test on the normal distribution of TLR3 overexpression cells and the wild-type cells, we got a p-value more than 0.05 at every time point. Thus, we could conclude that there is no true significant difference in the mean cluster size of the TLR3 overexpression and the wild type cells. This refutes our primary hypothesis that an overexpression of TLR3 in Hela cells leads to a better containment of the virus.

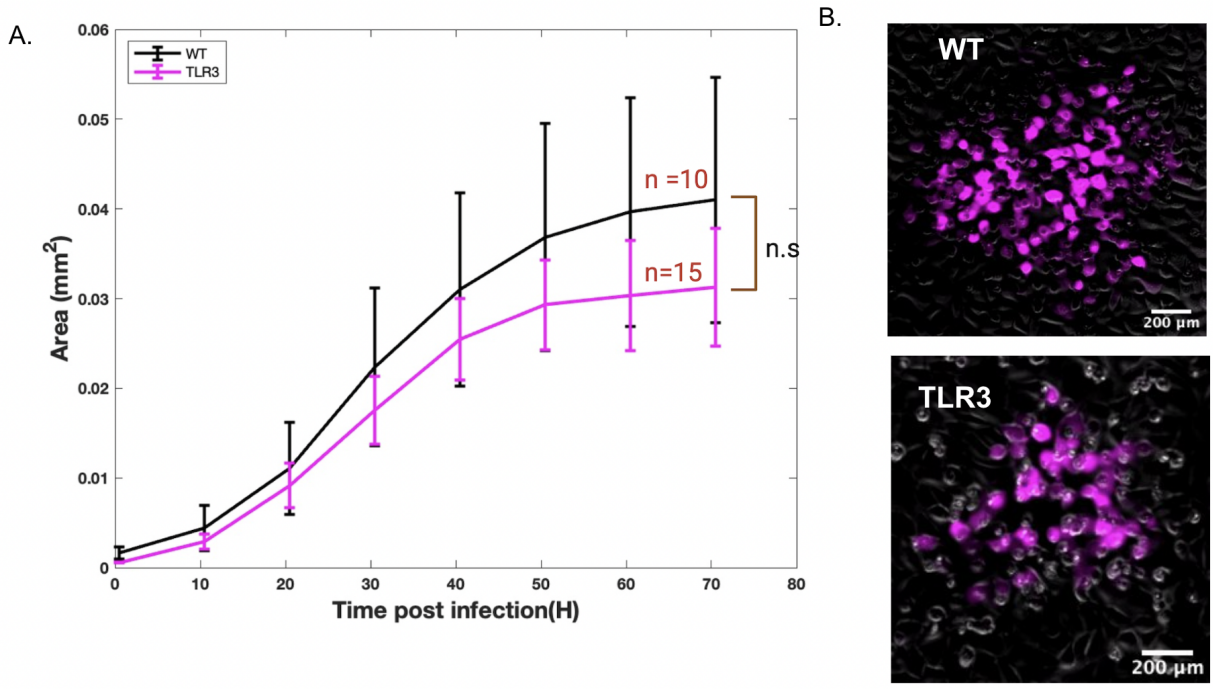

Figure 3.5. An overexpression of TLR3 in Hela cells leads to a no significant change in the size of the cluster of Vesicular Stomatitis Virus (VSV) infected cells. A. 15 clusters of TLR3-overexpressed VSV-mcherry infected cells were imaged for 72 hours and the mean area of the VSV cluster was plotted every 10 hours (magenta line). This was compared to the 10 clusters of the control wild-type cells (black line). A two sample t-test was performed between the normal distribution of the two groups at 72 h and no significant difference on the cluster sizes between the TLR3-overexpressed and the control group was recorded (p-value>0.05). B. Time lapse images of a cluster of VSV-mcherry infected wild-type cells (top) and TLR3 overexpression cells (bottom). The images were obtained at the end of the experiment and the mCherry channel and phase 10X channel were merged using ImageJ.

3.6: Overexpression of Fas in Hela cells lead to no significant change in cluster size on viral infection

Fas overexpression cells and the wild-type cells were infected with Vesicular Stomatitis Virus (VSV) and imaged every 30 minutes after seeing the first infected cell for 72 hours. The clusters of Vesicular Stomatitis Virus (VSV) in the infected cells were shown as an mCherry signal tagged to the VSV. At the end of the time lapse imaging experiment, we had 25 VSV clusters in the Fas overexpression cells and 10 VSV clusters in the wild-type cells. These

clusters were then tracked from 6-10 hours after infection till the end of the 72 hours and analyzed using custom lab code on MATLAB. The results showed that Fas overexpression cells have no overall difference in mean cluster size as compared to the wild-type cells (Figure 3.6). On performing a two-sample t-test on the normal distribution of Fas overexpression cells and the wild-type cells, we got a p-value more than 0.05 at every time point. Thus, we could conclude that there is no true significant difference in the mean cluster size of the Fas overexpression and the wild type cells. This refutes our primary hypothesis that an overexpression of Fas in Hela cells leads to a better containment of the virus.

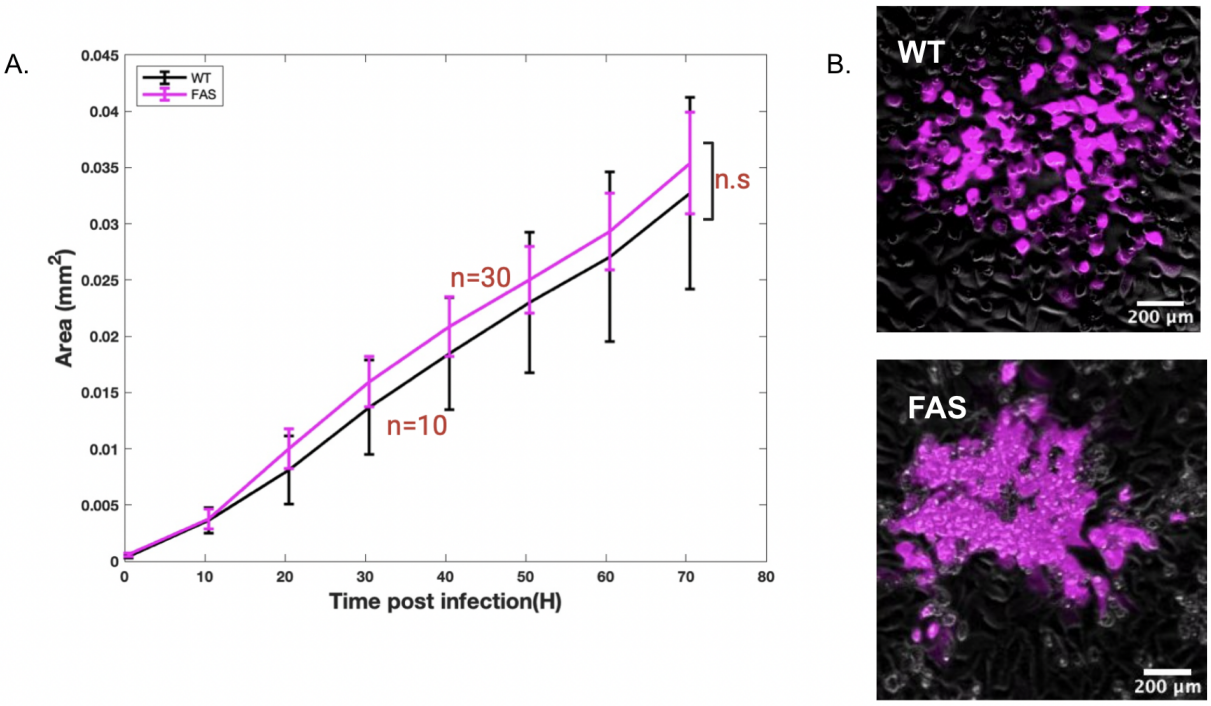

Figure 3.6. An overexpression of Fas in Hela cells leads to a no significant change in the size of the cluster of Vesicular Stomatitis Virus (VSV) infected cells. A. 30 clusters of Fas overexpressed VSV-mcherry infected cells were imaged for 72 hours and the mean area of the VSV cluster was plotted every 10 hours (magenta line). This was compared to the 10 clusters of the control wild-type cells (black line). A two sample t-test was performed between the normal distribution of the two groups at 72 h and no significant difference on the cluster sizes between the Fas overexpressed and the control group was recorded (p-value>0.05). B. Time lapse images of a cluster of VSV-mcherry infected wild-type cells (top) and Fas overexpression cells (bottom). The images were obtained at the end of the experiment and the mCherry channel and phase 10X channel were merged using ImageJ.

3.7: Overexpression of PKR in Hela cells lead to no significant change in cluster size on viral infection

PKR overexpression cells and the wild-type cells were infected with Vesicular Stomatitis Virus (VSV) and imaged every 30 minutes after seeing the first infected cell for 72 hours. The clusters of Vesicular Stomatitis Virus (VSV) in the infected cells were shown as an mCherry signal tagged to the VSV. At the end of the time lapse imaging experiment, we had 25 VSV clusters in the PKR overexpression cells and 10 VSV clusters in the wild-type cells. These clusters were then tracked from 6-10 hours after infection till the end of the 72 hours and analyzed using custom lab code on MATLAB. The results showed that PKR overexpression cells have no overall difference in mean cluster size as compared to the wild-type cells (Figure 3.7). On performing a two-sample t-test on the normal distribution of PKR overexpression cells and the wild-type cells, we got a p-value more than 0.05 at every time point. Thus, we could conclude that there is no true significant difference in the mean cluster size of the PKR overexpression and the wild type cells. This does not support our primary hypothesis that an overexpression of PKR in Hela cells leads to a better containment of the virus.

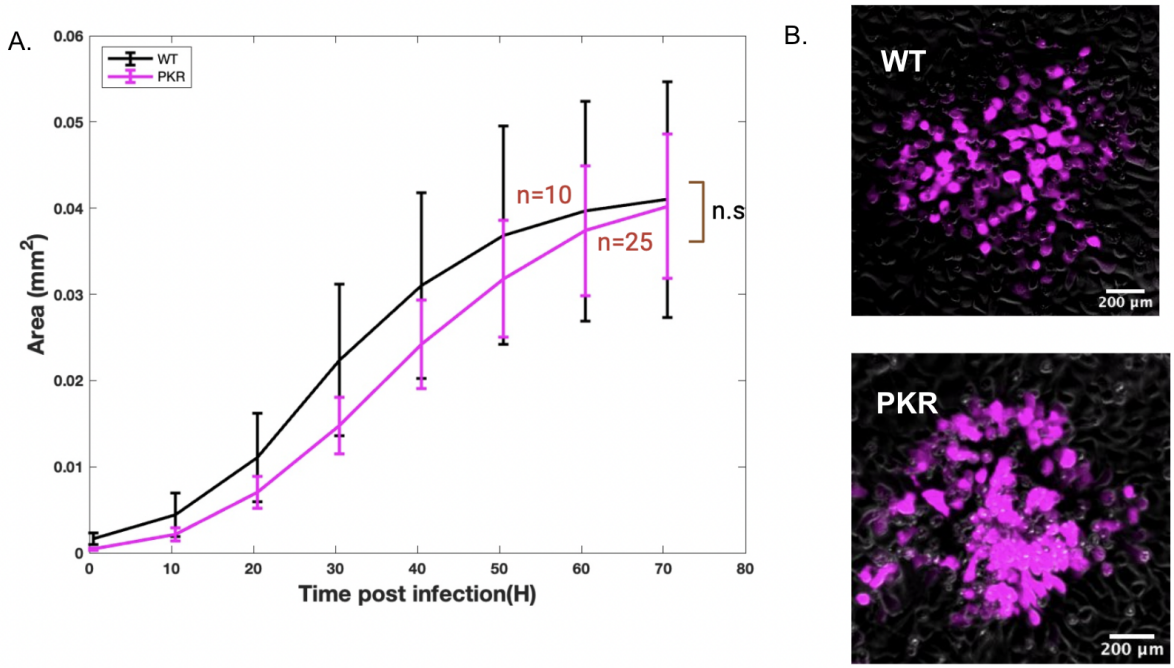

Figure 3.7. An overexpression of PKR in Hela cells leads to a no significant change in the size of the cluster of Vesicular Stomatitis Virus (VSV) infected cells. A. 25 clusters of PKR overexpressed VSV-mcherry infected cells were imaged for 72 hours and the mean area of the VSV cluster was plotted every 10 hours (magenta line). This was compared to the 10 clusters of the control wild-type cells (black line). A two sample t-test was performed between the normal distribution of the two groups at 72 h and no significant difference on the cluster sizes between the PKR overexpressed and the control group was recorded (p-value>0.05). B. Time lapse images of a cluster of VSV-mcherry infected wild-type cells (top) and PKR overexpression cells (bottom). The images were obtained at the end of the experiment and the mCherry channel and phase 10X channel were merged using ImageJ.

3.8: Overexpression of PRKRA in Hela cells lead to no significant change in cluster size on viral infection

We plated 8 wells of Fas overexpression cells in a 24 well plate. 4 wells of the wild-type cells were plated on the same plate to be used as the negative control. We took a 3X3 large image of each well every 30 minutes after seeing the first infected cell for 72 hours. The images were of the clusters of virus infected cells showing an mCherry signal in the wells. At the end of the time lapse imaging experiment, we had 35 clusters of PRKRA overexpression cells and 10 clusters of wild-type cells. These clusters were then tracked and analyzed using custom lab code on MATLAB. The results showed that PRKRA overexpression cells have an overall similar mean cluster size as compared to the wild-type cells (Figure 3.8). On performing a two-sample t-test on the normal distribution of PRKRA overexpression cells and the wild-type cells, we got a p-value greater than 0.05. Thus, we could conclude that there is no true difference in the mean cluster size of the PRKRA overexpression and the wild type cells.

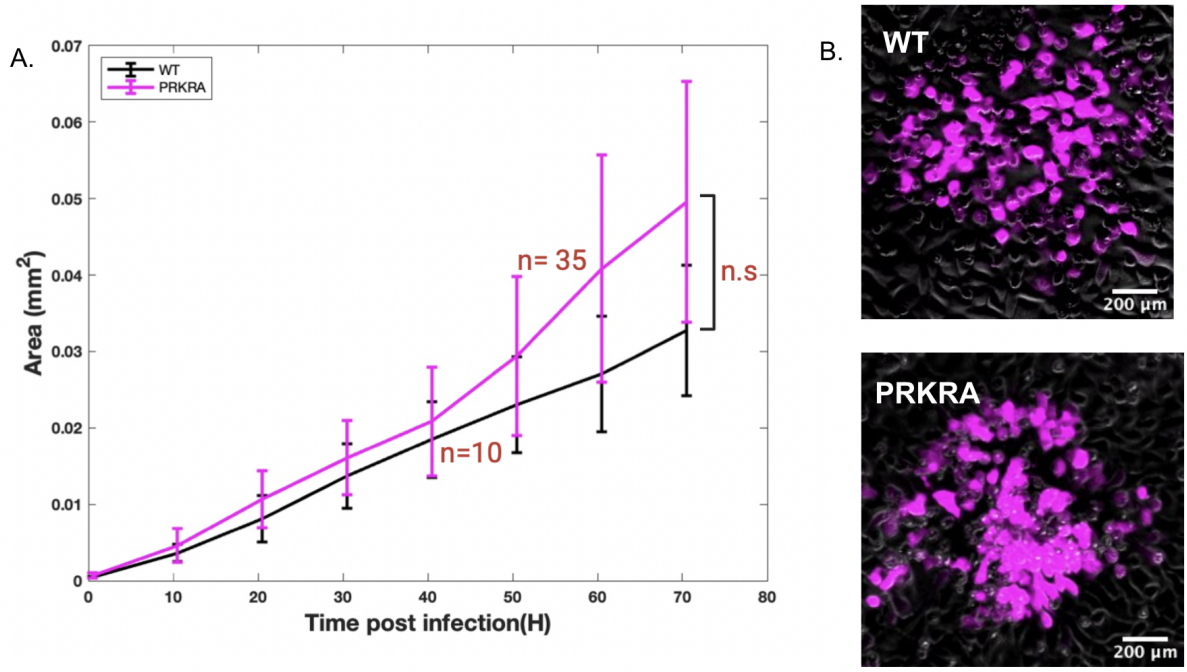

Figure 3.8. An overexpression of PRKRA in Hela cells leads to a no significant change in the size of the cluster of Vesicular Stomatitis Virus (VSV) infected cells. A. 35 clusters of PRKRA overexpressed VSV-mcherry infected cells were imaged for 72 hours and the mean area of the VSV cluster was plotted every 10 hours (magenta line). This was compared to the 10 clusters of the control wild-type cells (black line). A two sample t-test was performed between the normal distribution of the two groups at 72 h and no significant difference on the cluster sizes between the PRKRA overexpressed and the control group was recorded (p-value>0.05). B. Time lapse images of a cluster of VSV-mcherry infected wild-type cells (top) and PRKRA overexpression cells (bottom). The images were obtained at the end of the experiment and the mCherry channel and phase 10X channel were merged using ImageJ.

CHAPTER 4: DISCUSSION

One of the most important mechanisms by which our innate immune system fights against viral infections is the antiviral pathway regulated by Type I and Type II Interferons. There are many ways by which a host cell can recognize a virus. In this thesis, we mainly focused on the pattern recognition receptors such as TLR3, RIG-I, PKR, and PRKRA that recognize dsRNA as its PAMP. Once these receptors bind to the dsRNA, they trigger a series of signaling cascades that ultimately leads to the production of Type I interferon. A feedback loop is formed by which the Type I IFN leads to the transcription of more interferon induced genes (ISG) such as these PRRs and these PRRs further lead to the production of Type I IFN. This intertwined relationship between interferon production and ISG expression provides room for further investigation regarding the biological role of these ISGs in the host-virus interaction and the antiviral response against the virus(cite). We hypothesized that an overexpression of certain viral sensors such as TLR3, RIG-I, PKR, PRKRA, and Fas can better contain Vesicular Stomatitis Virus (VSV) in Hela cells.

A receptor that has shown to play a crucial role in the interferon induced antiviral response is Retinoic Acid Inducible Gene (RIG-I). RIG-I recognizes dsRNA and triggers interferon production in the host cell. A study revealed that RIG-I is capable of recognizing dsRNA as an antiviral response primarily by receptor binding to the viral dsRNA even when this signaling is not directly induced by interferon I (Thoresen et al, 2023). We hypothesized that an overexpression of RIG-I in Hela cells would result in an enhanced antiviral response against VSV and result in the better containment of the virus. Thus, we would see smaller clusters of

virus infected host cells using time lapse microscopy. Our results confirmed our hypothesis and showed that the RIG-I overexpression cells had a significantly smaller cluster of VSV infected cells when compared to the control wild-type cells. A previous study by Furr et al., had also shown that an intranasal administration of VSV to murine glial cells resulted in a rapid upregulation of RIG-I to induce cytokine production(Furr et al., 2013). Another paper confirmed these results and further proved that if RIG-I is knocked down in the glial cells infected with VSV, there is a significant reduction in the VSV induced cytokine production (Crill et. al., 2016) Our results second these findings and support the important role of RIG-I in host virus interactions. A recent study also found that RIG-I is capable of restricting SARS-CoV-2 in an interferon I/III independent manner. The researchers found that RIG-I interacts with the 3 untranslated regions of the SARS-CoV-2 RNA genome and an upregulation of this gene is correlated to the decreased activity of the virus (Yamada et al., 2021) . This further demonstrates the importance of studying RIG-I in the context of its antiviral responses to different viruses. In the future, we could investigate the role of RIG-I using a knockout model in Hela cells to see whether a complete abolishment of RIG-I will completely reverse or worsen the virus containment in these cells. It would also be interesting to test the accuracy of our experiment in an in-vivo model and examine whether we would be able to see a significant difference in virus containment if RIG-I was upregulated in this system.

Double stranded (dsRNA), as discussed before, is a very common danger signal for the host cell to trigger innate and adaptive immune responses. For a negative single stranded virus such as Vesicular stomatitis virus (VSV), dsRNA is formed as a replication intermediate. Thus, at this point, if the dsRNA binds to the host cell receptors, it can trigger an immune response initiated by different transcription factor activation such as NF-kB, IRF3, and IRF7

(Chattopadhay et al., 2014). Toll like Receptor 3 (TLR3) is a well known dsRNA sensor that recognizes both virus derived dsRNA and its synthetic analogue in the form of polyriboinosinic: polyribocytidylic acid (Poly(1:C)) . Upon viral infection, due to an increased production of dsRNA, TLR3 expression is also upregulated to bind to the dsRNA and activate an antiviral response. Our experiment showed that the cluster size of the VSV infected dead cells was not significantly different from that of the wild-type control cells. This indicates that an overexpression of TLR3 in the cells does not necessarily lead to a better containment of the virus. In previous studies, a pretreatment of IFN-I in addition to a viral infection leads to an upregulation of TLR3 in the host cell (Matsumoto et al., 2011). In the future, it might be interesting to see if a pretreatment of IFN-I causes any changes to the cluster size of the overexpression of TLR3 cells upon viral infection.

The role of TLR3 in a viral infection has been debated in the past. While previous studies have shown that TLR3 triggers an immune response, there is evidence that TLR3 can sometimes lead to further deterioration in health in the host (Matsumoto et al., 2011). A previous experiment performed by Le Goffic et al., demonstrated that mice that were TLR3 deficient actually showed a better survival rate than the WT mice on viral infection. The findings suggested that TLR3 is capable of triggering excessive pro-inflammatory responses which can have a fatal effect on the host cell (Le Goffic et al., 2006). Another study performed by Edelmann et. al, also showed that TLR3 is not universally needed for effective antiviral responses since it causes no change in viral pathogenesis and the host's generation of the antiviral response to VSV (Edelmann et al., 2004). Our experiment also showed no significant difference in the viral pathogenesis rate and the antiviral response elicited by the host in the TLR3 overexpression cells. According to Elisabeth Vercammen, the outcome of expressing

TLR3 during a viral infection can be dependent on several factors such as virus type, the viral load, the mode of infection (whether it is cytoplasmic or endoplasmic), the experimental model (in-vivo or in-vitro), the cell type infected, and the stage of infection (Vercammen et al., 2008). Another significant factor that could affect how TLR3 interacts with the virus could simply be the overwhelming viral load *in vitro* that may trigger a fast and strong TLR3 mediated Type I IFN response (Edelmann et al., 2004). This provide room for further experimentation where we can try testing for a change in virus containment in TLR3 overexpression cells in an *in vivo* model or try using different concentrations of the viral dsRNA to see whether there is a significant change in cluster size of the virus infected cell above a particular concentration of the virus.

Protein Kinase R (PKR) is another important dsRNA sensor that plays a role in the Type I IFN induced antiviral response. PKR recognizes viral dsRNA as its PAMP and undergoes autophosphorylation. This is followed by the phosphorylation of eIF2a which inhibits further protein synthesis in virus infected cells (Lemaire et al., 2008). Since VSV forms a dsRNA replication intermediate, we hypothesized that an overexpression of PKR in VSV infected cells would lead to a greater antiviral response due to higher recognition of the viral dsRNA. Quantifiably, we would see smaller clusters of virus infected dead cells indicating better virus containment. This was not experimentally true for PKR overexpression cells and the differences in cluster size between these cells and the control cells were not significant. PKR is also a heavily contested universal player in the antiviral response since studies have shown different outcomes in viral spread with respect to PKR. A previous finding in mouse embryonic fibroblasts from PKR knock-out mice, showed that PKR has no effect on VSV replication and does not show a better antiviral effect when compared to the control. The authors claimed that

the inhibitory effect of PKR on viral protein synthesis was counteracted later by a stimulatory effect in another part of the viral life cycle (Krishnamoorthy J. et al., 2008). However, another paper showed that PKR knockout mice had a greater susceptibility to VSV infection but this inhibitory effect of PKR in mice was mouse strain dependent. It was concluded that PKR plays a significant role in the protection against VSV but there is a greater interplay of multiple factors in the host-immune system that regulate its function in the antiviral response (Durbin et al., 2002). Another factor to consider in this specific PKR-virus interaction is the co-evolution of viruses in response to host cell machinery. For example, several viruses have evolved ways to inhibit PKR response. Sometimes the virus can mimic eIF2a and trick PKR to bind with a redundant viral protein such as K3L. On the contrary, the constant arms race between the host and the virus also allows the host PKR to evolve newer interactions with other molecules in our body to strategically evade the virus mimicry mechanism (Elde N. et al., 2009). A recent study showed an interesting interaction between E3 ubiquitin protein ligase HECTD3 and PKR and demonstrated a faster virus clearance and reduced inflammatory response both *in vitro* and *in vivo* using a VSV infection model (Huang J. et al., 2023). Such interaction further strengthens our PKR mediated immune response against viruses. Our PKR overexpression model showed no significant decrease in cluster size of virus infected dead when compared to the wild type cells. Since the virus containment was not different as expected, further experimentation in a PKR knock-out model in HeLa cells could be performed to test the accuracy of our results and see how crucial PKR is in the antiviral response in a VSV infection.

Another gene that we overexpressed in Hela cells was PRKRA. PRKRA encodes for a protein called PACT that activates the PKR induced inhibition of protein synthesis (Vaughn et al.,2022) to trigger an antiviral response. A previous study showed that a knockout model of

PACT in mice is fatal. They found that PACT can recognize dsRNA as its ligand and can also interact and activate RIG-I mediated cytokine production in the host. This interaction is also an essential component of the amplification of the innate immune system response and interferon production (Kok. K et al., 2011). Another study showed how PACT directly interacts with another protein called LGP2 which is also a RIG-I like receptor (RLR) to influence and mediate the IFN production between RIG-I and MDA-5 (another RLR). They concluded that this interaction between LGP2 and PACT allows to regulate the inflammatory responses mediated by the RLRs and enables a balance between the innate immune system response and the RNA silencing machinery (Sanchez et al., 2021). Thus, recognizing the importance of PACT in the antiviral response triggered by the innate immune system, we hypothesized that an overexpression of PRKRA gene in Hela cells would result in smaller clusters of infected cells, indicating a better containment of the virus. However, our results showed no significant difference in the cluster size of the VSV infected Hela cells on overexpression of PRKRA. A previous study conducted by Joao T. Marques et al., showed that PRKRA is not required in the antiviral response against VSV. They used a knockout model of PACT in mouse embryonic fibroblast (MEFs) cells and found that there was no significant change in the virus yield between the control wild type cells and the PACT knockout cells (Marques et al, 2007) . Our experiment which showed that an overexpression of PRKRA does not cause a significant difference in the containment of VSV aligns with this observation. Since the PACT-RIG-I interaction is so prominent in the interferon induced antiviral response, many viruses have shown to develop ways in which they can evade this interaction. VP-35 in Ebola virus is an interesting protein that can also inhibit RIG-I signaling by interacting with PACT and disrupting the PACT-RIG-I activity for inducing IFN responses (Luthra et al., 2013). SARS-CoV N protein also interacts

with PACT to directly disrupt the PACT-RIG-I interaction (Ding et al., 2017). Thus, it is unclear why PACT can sometimes play such an important role in the host-virus interaction and yet, not affect some viruses at all. We can speculate that an effect of an overexpression of PRKRA gene can be both cell type dependent and virus dependent. It is also possible that an overexpression of PRKRA gene does not necessarily produce an upregulated amount of PACT protein. In the future, we can perform a knockout experiment or explore more cell type and virus model combinations to investigate the differences in host-virus interaction due to the PRKRA gene.

After looking at different PRRs that recognize dsRNA, we also tried to investigate the extrinsic apoptotic mechanism used by the host cell to inhibit viral replication. Fas mediated apoptosis is regulated by the interaction of Fas with its ligand FasL. Once FasL binds to Fas, an adaptor protein, FADD is recruited. This further leads to the binding of procaspase-8 which results in the formation of the death-inducing signaling complex (DISC). Ultimately Caspase 3 is activated by Caspase 8 and this results in the apoptosis of the infected cell (Yolcu et al., 2017). Thus, we hypothesized that an overexpression of Fas would lead to a faster apoptosis of the host infected cell. This would result in a decrease in viral replication as the virus requires live host cells for replication, and this would ultimately lead to smaller clusters of virus infected cells, indicating a better containment of VSV. However, we did not notice any significant difference in the cluster size of infected cells when Fas was overexpressed upon viral infection. A previous study conducted by Fujimoto et al., found an interesting rise in the expression levels of both Fas and FasL after 6-12 hours of infection with Influenza A virus in Hela cells. However, they also showed that artificial dsRNA in the form of Poly I:C, showed no change in the expression level of FasL while showing a rise in Fas. Thus, there was no change in the induction of apoptosis in the infected cells in this experiment (Fujimoto et al., 1998). It might be possible that an

overexpression model of Fas did not necessarily increase the co-expression of FasL to induce apoptosis of the infected cells. Another study found that while apoptosis in virus infected cells can serve as an effective strategy for host cells to control viral replication, viruses can also manipulate the death receptor mediated apoptosis to their advantage for releasing their progeny and continuing the spread of infection (Zhou et al., 2017). Many viruses encode different proteins that can upregulate the death receptors or their ligands on the surface of the infected cells. This is seen in HIV-1 that uses proteins such as Env and Vpu to mediate Fas signaling (Casella et al.). Thus, in conclusion, while Fas seems to play an important role in the antiviral response against some viruses, we were unable to show a significant change in the host-virus interaction in VSV infected Hela cells on an overexpression of Fas.

In conclusion, we found that only RIG-I has a significant impact on the containment of VSV in HeLa cells. An overexpression of TLR3, Fas, PKR, and PRKRA did not result in a better containment of the virus. These results demonstrate that RIG-I might be the key viral sensor that causes a host mediated interferon induced antiviral response in VSV infected Hela cells.

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