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Suppression of Pulmonary Innate Immunity by Pneumoviruses

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SUPPRESSION OF PULMONARY INNATE IMMUNITY BY PNEUMOVIRUSES

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DEDICATION

I want to dedicate this thesis to the most important people in my life whose support and blessings empowered me to finish the journey.

To my husband Debojyoti whose dream of seeing me achieve a doctorate degree has become a reality. Your love, support and guidance during my difficult times helped me to embark on this journey and to finish it. You have been my pillar of strength making me believe in myself and bringing me this far. I want to thank you for always being at the side of your most annoying wife.

To my Maa and Baba, there is not a single day when I don't miss you. The feelings that I belong to you and that you are always there for me make me feel safe. Your unconditional love and believe in me directed my life this way. I know no matter how badly the situation is, for you I am always the winner. Thank you for being there.

To my in-laws, for your caring nature, kindness, and support towards me. I have long admired your commitment to my education. I am so grateful to you always.

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SUPPRESSION OF PULMONARY INNATE IMMUNITY BY PNEUMOVIRUSES

JAYEETA DHAR

ABSTRACT

Pneumonia Virus of Mice (PVM) and Respiratory Syncytial Virus (RSV) are negative sense, single-stranded, enveloped RNA viruses from *Pneumovirus* genus, *Paramyxoviridae* family. RSV is the leading cause of respiratory diseases in infants. PVM causes similar respiratory illness in mice. PVM is used as an animal model to study RSV pathogenesis because of its similarity with RSV infection. Viral infection induces type I interferon (IFN) response as an antiviral strategy. PVM and RSV both have two non-structural (NS) proteins that are known to be IFN antagonists. While RSV can target different signaling components of IFN pathway, the mechanism of IFN suppression for PVM was unknown. We have identified that PVM can also target different signaling components of IFN pathway to circumvent the host immune system. Our observations showed that PVM NS proteins facilitate proteasome-mediated degradation of RIG-I, IRF3, STAT2 in IFN pathway by direct interactions with them. Production of several Interferon Stimulated Genes (ISGs) is the distal part of the IFN pathway. We have identified that NS proteins of PVM can also target a few of them such as TRAFD1, IFITM1, ISG20, and IDO for complete suppression of the host immune system.

RSV NS proteins play a similar role to suppress IFN pathway by targeting TBK1, RIG-I, IRF3, IRF7, and STAT2. Our study has identified one ISG, OASL, that has antiviral properties against RSV and documented that to counteract this antiviral property of OASL, RSV NS proteins can degrade OASL in a proteasome-dependent way.

These above observations help us to delineate the complete suppression mechanism for the whole *Pneumovirus* genus, both for PVM and RSV by providing the first experimental evidence of signaling components from the IFN pathway targeted by PVM to suppress the IFN response. PVM is a clinically relevant animal model that will help us to find new therapeutic strategies against *Pneumovirus* infection. RSV study with one of those important ISGs, OASL, is also important to uncover the target substrates of the entire IFN pathway. Together these findings help us to delineate new immune modulatory strategies for the whole *Pneumovirus* genus.

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LIST OF ABBREVIATIONS

Ala – Alanine

AP-1 – Activation Protein 1

BMDM – Bone Marrow Derived Macrophage

CD225 – Cluster of Differentiation 225

CBP – CREB Binding Protein

Cys – Cysteine

DAMPs – Damage-Associated Molecular Patterns

ds – Double Stranded

hPIV2 – Human Parainfluenza Virus 2

IFTM1 – Interferon Induced Transmembrane Protein 1

IKKε – Inhibitor of κB Kinase Epsilon

IB – Immunoblot

IDO – Indoleamine 2,3-Dioxygenase

IF – Immunofluorescence

IFNs – Interferons

IP – Immunoprecipitation

IRF3 – Interferon Regulatory Factor 3

IRF7 – Interferon Regulatory Factor 7

IRF9 – Interferon Regulatory Factor 9

ISGs – Interferon Stimulated Genes

ISG20 – Interferon Stimulated Exonuclease Gene 20

ISGF3 complex – Interferon Stimulated Gene Factor 3

ISRE – Interferon Stimulated Response Element

JAK-STAT – Janus Kinase - Signal Transducer and Activator of Transcription

LC – Lactacystin

LGP2 – Laboratory of Genetics and Physiology 2

Luc – Luciferase

MAVS – Mitochondrial Antiviral Signaling

MDA-5 – Melanoma Differentiation-Associated protein 5

MEF – Mouse Embryonic Fibroblast

MHC – Major Histocompatibility Complex

M.O.I. – Multiplicity of Infection

NF-kB – Nuclear Factor-Kappa B

NS proteins – Non-Structural proteins

NS1 – Non-Structural protein 1

NS2 – Non-Structural protein 2

OASL – 2′-5′ Oligoadenylate Synthetase-Like

PAMPs – Pathogen-Associated Molecular Patterns

PAS – Protein-A-Sepharose

PFU – Plaque Forming Unit

PKR – Protein Kinase R

PRRs – Pattern Recognition Receptors

PVM – Pneumonia Virus of Mice

RIG-I – Retinoic acid Inducible Gene-I

RSV – Respiratory Syncytial Virus

SeV – Sendai Virus

SH2 – Src homology 2

STAT2 – Signal Transducer and Activator of Transcription 2

SV5 – Simian Virus 5

TBK1 – TANK Binding Kinase 1

TLR – Toll Like Receptor

TRAFD1 – Type Zinc Finger Domain Containing 1

TRAF3 – TNF Receptor Associated Factor 3

TRIF – TIR-Domain-Containing Adapter-Inducing Interferon-β

TYK2 – Tyrosine Kinase 2

Ub – Ubiquitin

UBL – Ubiquitin Like

U – Uninfected

VSV – Vesicular Stomatitis Virus

WB – Western Blot

WT – Wild Type

CHAPTER I

INTRODUCTION

1.1. Pneumoviruses' overview

Pneumoviruses belong to the virus Group V according to David Baltimore's classification (1971). The classification is based on the virus's genome: precisely, the nucleic acid content (DNA and RNA), positive and negative strand, and sense-antisense mode of replication. Virus Group V comprises of one order- *Mononegavirales* and eight families (*Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Nyamiviridae*, *Arenaviridae*, *Bunyaviridae*, and *Ophioviridae*) of negative sense single stranded RNA viruses. The *Pneumovirus* genus belongs to family *Paramyxoviridae*, sub family *Pneumovirinae*. It is comprised of enveloped virus pathogens that are causative organisms for respiratory tract infections, pneumonia, and bronchiolitis. The best characterized Pneumoviruses are human Respiratory Syncytial Virus (hRSV) and Pneumonia Virus of Mice (PVM). There are also other three Pneumoviruses: Bovine Respiratory Syncytial Virus (cow-bRSV), ovine Respiratory Syncytial Virus (sheep) and caprine Respiratory

Syncytial Virus (goat) [Easton A. J. et al. 2004].

1.1.1. Human Respiratory Syncytial Virus (hRSV)

Human RSV is a leading cause of pediatric illness. RSV was first discovered in 1956 by Robert Chanock at National Institute of Allergy and Infectious Diseases. Every year more than 85,000 people get hospitalized only in USA with a severe lower respiratory tract infections caused by RSV [Wyde, P. R, 1998]. It is a seasonal infection, observed mainly in winter and spring. The transmission of the disease occurs through direct contact with respiratory secretions and the incubation period is 2-8 days. There are two strains of RSV: A and B. The main difference between these two groups are in the glycoprotein (attachment protein). RSV A is mainly responsible for the severe respiratory infections. There is still no effective antiviral therapy available for RSV [Easton, A. J. et al., 2004].

1.1.2. Pneumonia Virus of Mice (PVM)

PVM is a mouse pathogen from the same *Pneumovirus* genus which causes robust respiratory tract infections in bronchial epithelial cells of mice. The discovery of PVM was done by researchers F. L. Horsfall and R. G. Hahn at The Rockefeller University in 1939. PVM is the only other mouse virus in the *Pneumovirus* genus which closely resembles hRSV, although there are a very few sequence homologies between the virus encoded proteins of hRSV and PVM [Kimberly, D. D. et al. 2012]. There are two strains of PVM: strain 15 and strain J3666. PVM can replicate many of the clinical and pathological features of RSV infection, so it is largely used as a workable animal model to study the infection mechanism of hRSV. The replication rate of PVM is a period of 24-30 hrs. The comparison

and contrast studies between RSV and PVM provide a basic idea of the infection mechanism of the whole *Pneumovirus* genus. Notably that will further help in the evaluation of effective vaccines with new immunomodulatory and therapeutic strategies against respiratory viruses' infections [Rosenberg H. F. et al. 2008].

1.1.3. Genome structure of hRSV and PVM

Human RSV and PVM both are enveloped viruses. They have negative sense, single stranded non-segmented RNA as their genetic material. The virions [Figure 1] of both viruses are structurally similar. They are spherical and the average diameter is 100 to 200 nm. The outer lipid envelop is made up of three proteins, attachment/ glycoprotein (G), fusion protein (F) and small hydrophobic (SH) protein. F and G proteins resemble 14 nm spikes on the virion surface. The internal surface of the virion structure has matrix (M) protein layer. The inner nucleocapsid region is made up of nucleoprotein (N), phosphoprotein (P) and large polymerase protein (L). These are all structural proteins of viruses.

Non-structural proteins (NS)

There are also two other proteins which are not a part of the virion structure of hRSV and PVM. They are known as non-structural (NS) proteins of virus [Easton A.J. et al., 2004]. Both hRSV and PVM have two NS proteins. The transcription products of these two NS proteins, Non-Structural protein 1 (NS1), Non-Structural protein 2 (NS2) that are not structurally incorporated into progeny virion. Still the NS genes are located at the promoter proximal 3' most region of the genome, thus they are the most abundantly

transcribed genes [Figure 2]. Both the NS proteins are small in size. The length of NS proteins of hRSV and PVM are 139 and 113 amino acids for NS1 and 124 and 156 amino acids for NS2, respectively. Research showed that these NS proteins are only abundantly expressed in host cells during infection and are responsible for the viral pathogenesis. NS proteins play a significant role to increase the replication efficiency of progeny virions [Valarcher J. F. et al. 2003, Buchholz U. J. et al. 1999, Teng M. N. et al. 1999].
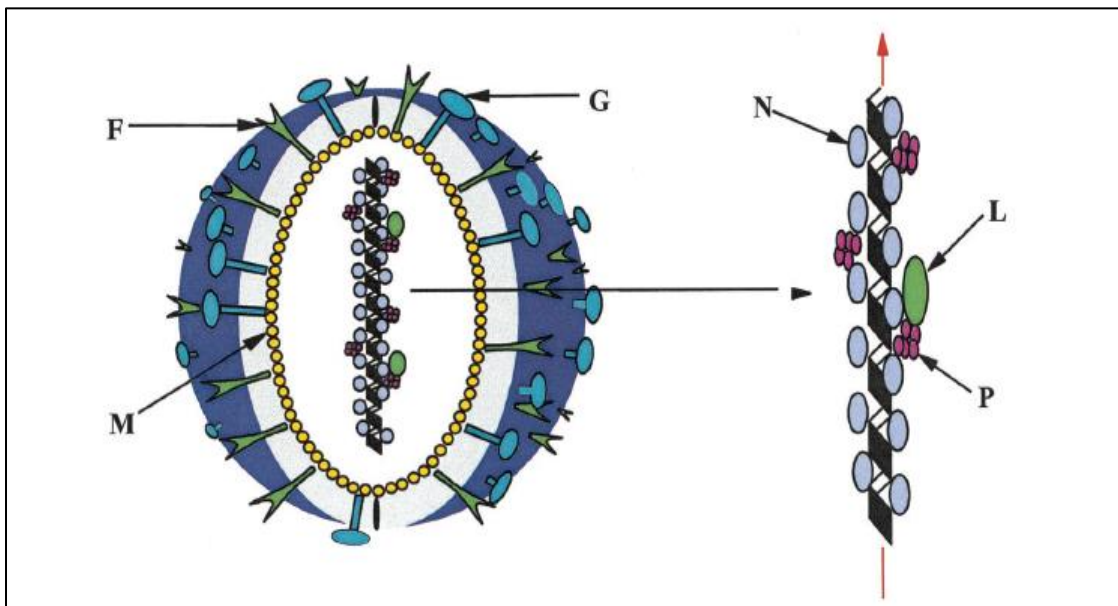
Figure 1: Schematic representation of virion structure of Pneumoviruses (hRSV and PVM). Outer lipid envelope consists of fusion (F), glycoprotein (G), and small hydrophobic protein (SH) (not shown). Inner nucleocapsid region is made up of nucleoprotein (N), phosphoprotein (P) and polymerase protein (L). Nucleocapsid region is encircled by matrix (M) protein. [Easton A.J. et al. (2004)].
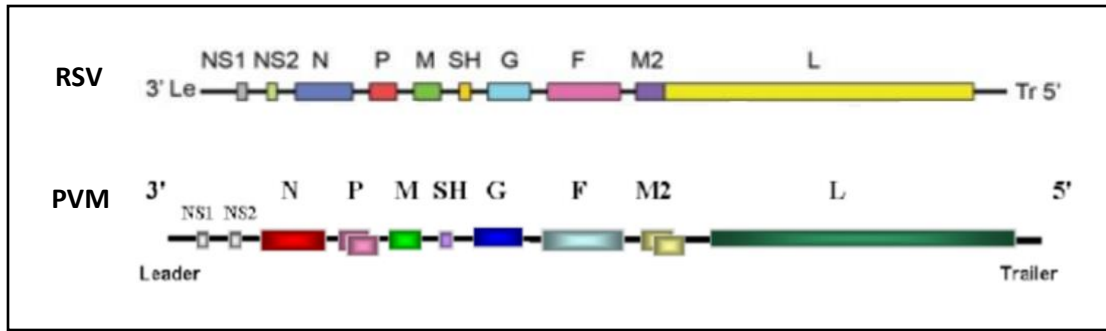
Figure 2: Genome organization of hRSV and PVM (from 3' to 5'): NS proteins (1 and 2): N, nucleoprotein gene; P, phosphoprotein gene; M, matrix protein gene; SH, small hydrophobic protein gene; G, glycoprotein gene; F, fusion protein gene; M2, encodes matrix proteins M2-1 and M2-2; L, RNA dependent RNA polymerase gene. [Kimberly D. D. (2012), Cox R. et al. (2015), Warwick School of Life Sciences].

1.2. Innate immune system

The immune system is the host's defense mechanism that protects against foreign pathogens and diseases. The immune system has many branches that can help in proper functioning by recognizing the foreign particles inside the host. The immune system is divided into two subgroups, innate immune system and adaptive immune system. The innate immune system, also known as non-specific immune system, is the first line of defense mechanism of host's immune system after any pathogen infection. The adaptive immune system is known as specific or acquired immune system. This adaptive system generates enormous, robust response to pathogens by creating an immunological memory from the previous initial response.

Invading pathogens encounter the innate immune system after entering through the body's anatomical (physical, chemical, and biological) barriers. The innate immune system is activated when special proteins pattern recognition receptors (PRRs) identify and bind

to the specific pathogen-associated molecules. There are two types of these pathogen associated molecules; pathogen-associated molecular patterns (PAMPs) associated with microbial pathogens, and damage-associated molecular patterns (DAMPs) associated with cell components responsible for cell damage. Signaling PRRs include membrane-bound Toll-Like Receptors (TLR) and cytoplasmic Nod-Like Receptors. There is also another PRR, cytoplasmic RIG-I receptors responsible for viral double-stranded and single-stranded RNA recognition [Medzhitov, R et al. 2001, Kumar H et al. 2009, Takahasi K. et al. 2008, Delbridge L.M. et al. 2007]. PRRs activation leads to the release of several inflammatory mediators. Interferons are one of them.

1.3. Interferons

Interferons (IFN) are the major part of the innate immune system. These are signaling proteins and a group of cytokines secreted by the host cells in response to foreign pathogens as a protective defense mechanism. The main function of IFN is to "interfere" in viral replication, that is why they are so named. IFNs are also involved in a variety of functions, they activate natural killer cells and macrophages for destruction of infected cells and upregulate antigen presentation by increasing the expression of Major Histocompatibility Complex (MHC) antigens, eventually increasing the host's defense mechanism. There are three classes of interferons: type I, II, and III. Viral infections mainly induce type I IFNs, which are the focus of this project. Type I IFNs are represented by IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. Type I IFNs are released by fibroblasts and monocytes cells. They bind to cell surface receptors which consist of IFNαR1 and IFNαR2. Type II is represented by IFN-γ and released by T helper cells and vascular smooth muscle cells and bind to IFNγR1

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and IFNγR2 receptors [Leon M.L et al. 2005]. Lastly, type III IFNs consist of three IFN-λ molecules (IFN-λ1, IFN-λ2 and IFN-λ3) and signal through IL10R2 and IFNLR1. The primary focus of this manuscript is effective and intrinsic antiviral activity of type I IFN signaling in response to Pneomoviral infection. Type I IFN signaling is mainly responsible for viral recognition and production of IFNα and IFNβ by establishing an antiviral state inside the host cells.

1.4. Type I interferon signaling

The production of type I IFNs occurs in response to viral infection. The pathway is bimodal, activated in two parts: (1) First, viral infection leads to the production of IFN in the IFN induction pathway and (2) the secretion of multiple ISGs in an autocrine and paracrine manner in IFN response pathway in order to establish antiviral state.

1.4.1. IFN induction pathway (type I)

The schematic diagram on Figure 3 illustrates the IFN induction upon viral infection. Viral RNA is recognized by two different receptors, (1) Toll Like Receptor 3 (TLR3) in the endosome, and (2) Retinoic Acid Inducible Gene-I (RIG-I) that is the cytoplasmic RNA sensors from the RNA helicase family. Another member of this family is Melanoma Differentiation-Associated protein 5 (MDA-5) that functions in a fashion in viral recognition with RIG-I. Another member of this family is Laboratory of Genetics and Physiology 2 (LGP-2) that serves as a negative regulator by inhibiting viral RNA recognition by RIG-I and MDA-5. LGP-2 has a natural capacity to cover the viral RNA from recognition. RIG-I and MDA5 have two N-terminal Caspase-Recruiting Domain

(CARD) and a C-terminal DExD/H RNA helicase domain [Kato H. et al., 2005; Yoneyama M. et al., 2004]. RIG-I recognizes non-segmented negative-strand RNA, while the positive strand viral RNA genome is recognized by MDA-5 [Takahasi et al. 2008, Myong S. et al 2009, Pippig D. A. et al. 2009, Gitlin L. et al 2010]. Viral RNA recognition induces the conformational changes between the CARD domain of RNA helicases and CARD containing downstream adaptor protein Mitochondrial Antiviral Signaling (MAVS). MAVS is attached to the outer mitochondrial membrane with its C-terminal domain. In parallel, the viral recognition by TLR3 in the endocytic compartments induces the activation of adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF). These two pathways then converge and activate TNF Receptor Associated Factor 3 (TRAF3) and TNF Receptor Associated Factor 6 (TRAF6) complex. This process leads to the recruitment, phosphorylation, and activation of two kinases, Inhibitor of kB Kinase Epsilon (IKKε) and TANK binding kinase 1 (TBK1). These two serine/threonine kinases then phosphorylate and activate C-terminal domain of transcription factors of interferon regulatory factor family members, Interferon Regulatory Factor 3 (IRF3) and Interferon Regulatory Factor 7 (IRF7). The activation leads to the homo or heterodimerization of IRF3 and IRF7 and they translocate to nucleus. With the help of other transcription co-activators in the nucleus [such as p-300, CREB binding protein (CBP)] the transcription of type I IFN genes initiates. IRF7 alone can activate IFNα. While IFNβ induction requires four transcription factors IRF3, IRF7 and Nuclear Factor-Kappa B (NF-kB) and Activation Protein 1 (AP-1). Type I IFN induction induces the production of mainly two IFNs: IFNα1 and predominantly expressed IFNβ. The transcription of IFN genes are followed by the translation and secretion of IFNs outside the host cells in response to pathogen recognition;

the transcription and production of type I IFN activates the IFN response pathway to establish the antiviral state inside the host cells [Haller O. et al. 2006].
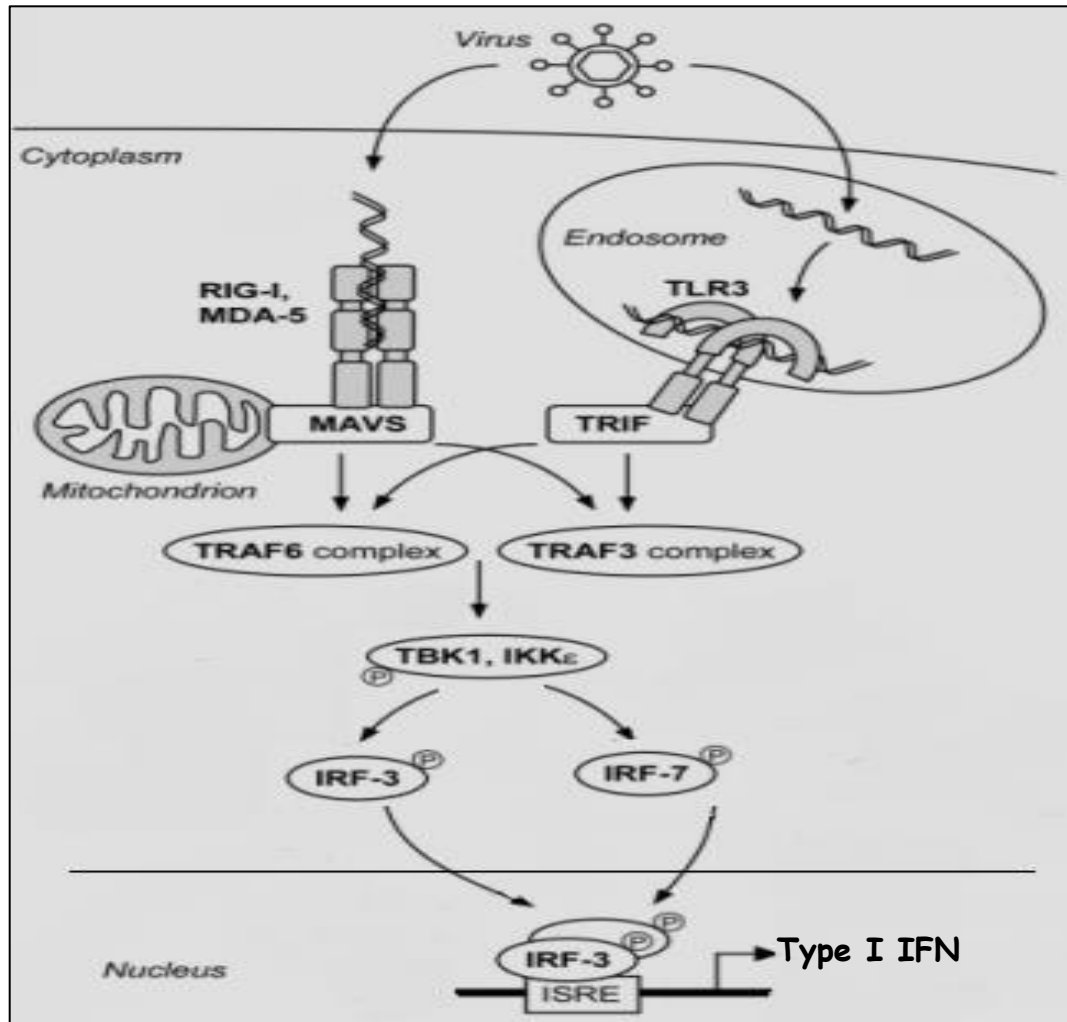
Figure 3: Interferon induction pathway (type I). Viral RNA is recognized by cytoplasmic RIG-I and MDA-5 and the endocytic TLR3 receptor. These two pathways merge together and phosphorylate and activate TRAF proteins, which later phosphorylates TBK1 and IKKε and phosphorylation and homo and heterodimerization of transcription factors IRF3 and IRF7. Phosphorylated IRF3 and IRF7 translocate to the nucleus and induce IFN production with the help of other transcription factors [Fensterl V. et al 2009].

1.4.2. IFN response pathway (type I)

IFNs bind to specific cell surface IFN receptors (IFNAR) and activate numerous downstream signaling pathways. Among those, the canonical Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway induces the production of a large number of Interferon Stimulated Genes (ISGs) [Figure 4 and Figure 5]. Binding of IFNs to the IFNAR phosphorylates and activates Janus Kinases: JAK-1 and Tyrosine Kinase 2 (TYK2). These set of processes activate the recruitment and phosphorylation of cytoplasmic transcription factors STAT1 and STAT2 proteins which induces the heterodimerization of two STAT proteins via Src homology 2 (SH2) domain. Phosphorylated STATs then recruit another factor Interferon Regulatory Factor 9 (IRF-9) and form a transcriptional complex known as Interferon Stimulated Gene Factor 3 (ISGF3 complex). The whole complex translocates to the nucleus and binds to Interferon Stimulated Response Element (ISRE) and transactivates several Interferon Stimulated Genes (ISGs) in an autocrine and paracrine manner. There a large number of ISGs available; the exact count is unknown, probably 500 to 1000. Some known ISGs are Viperin, 2′-5′ Oligoadenylate Synthetase-Like (OASL) group of proteins, Interferon Induced Transmembrane Protein (IFITM), Protein Kinase R (PKR), Indoleamine 2,3-Dioxygenase (IDO), ISG20, Type Zinc Finger Domain Containing 1 (TRAFD1), RIG-G etc. [Haller O. et al. 2006, Masumi A., 2013]. These ISGs have diverse functional features. Among those, some ISGs are known to also have antiviral properties that help to limit viral infections. We have discussed a few of them in our manuscript (Chapter II and Chapter III).
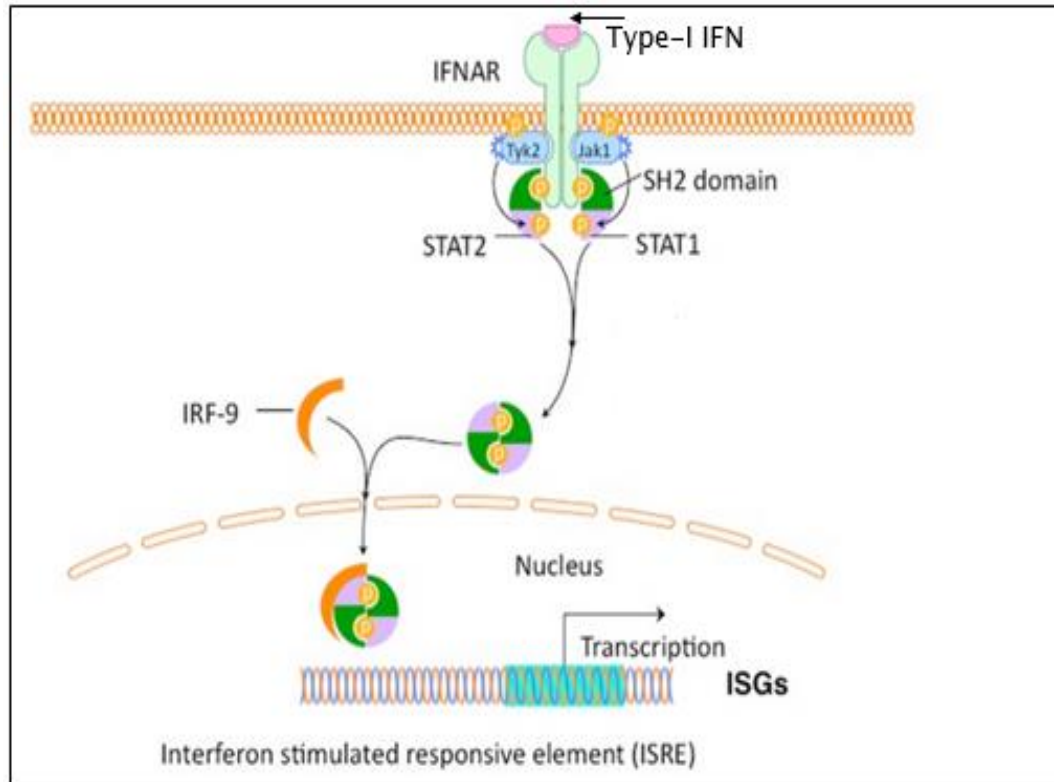
Figure 4: Interferon response pathway (type I). Type I IFN binds to cell surface receptors inducing the JAK-STAT pathway. JAK-1 and TYK2 phosphorylate and recruit cytoplasmic STAT proteins. STAT1, STAT2 and IRF9 together form a transcriptional complex ISGF3 and translocate to nucleus and induce the expressions of multiple ISGs. [Masumi. A., 2013].

1.4.3. Interferon stimulated genes – major antiviral proteins

In response to external stimuli mainly viral infection host cells produce IFNs. The IFN production involves multiple signaling pathways, and to counteract the viral state inside the host cells, hundreds of ISGs are transcribed. Recent studies show the antiviral properties of some of these ISGs. Apart from their antiviral activities, these ISGs have multiple other functions: apoptosis, regulation of cell growth differentiation, and in

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immune responses. The best characterized ISGs are PKR, OAS, IFITM family [Figure 5]. The second part of my project is based on OAS like protein (OASL) which also have antiviral activities and RSV can circumvent that. Recent studies also shown ISGs (such as, IFI6, IFI27, IRF1, IRF9, IFITM1, IFITM2, IFITM3, ISG20, OAS1, PKR, and Viperin) can inhibit specific virus replication [Itsui Y. et al, 2006, Jiang D. et al. 2008, Jiang D. et al. 2010]. These ISGs act as modest inhibitors of viral replication when acting alone. The combined effect of two or more ISGs have shown robust antiviral activities [Schoggins J. W. et al. 2011]. Viruses have different steps in life cycles: attachment (absorption), penetration (entry), uncoating (disassembly), gene expression, assembly (morphogenesis), budding, and release of progeny virions. Any of these steps can be targeted by ISGs that is also virus specific [Schoggins J. W. Charles M. R. 2011, Samuel E. C. et al. 2001].

There is also one important ISG which belongs to OAS family and known as OASL (2′-5′ oligoadenylate synthetase-like protein). It induces RNA degradation by activating RNase L. The third chapter of my Ph.D. dissertation research is based on that.
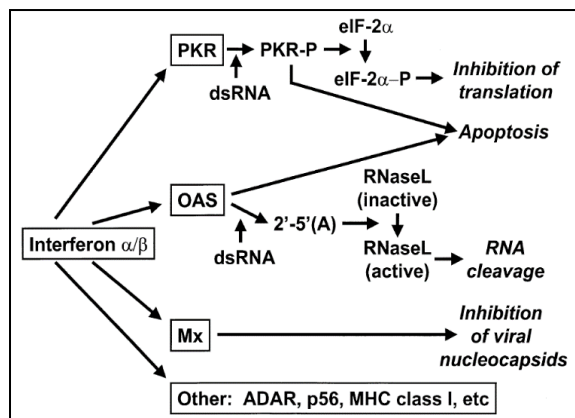
Figure 5: Functions of major antiviral proteins. (1) PKR (Protein Kinase R) – activated by dsRNA, inhibits viral protein translation; (2) OAS – activated by dsRNA, RNA cleavage activity; (3) Mx – viral nucleocapsid inhibition, (4) Other: ADAR, p56, MHC act on inhibition of viral replication [Schoggins J. W. Et al. 2011].

In this manuscript we have shown virus (PVM) has evolved inhibitory effects on some of these ISGs to counteract specific host antiviral functions. The ISGs are TRAFD1, IFITM1, ISG20 and IDO.

ISG20 – The full name is Interferon Stimulated Exonuclease Gene 20. ISG20 is a key player of adaptive immune response. It has 3'-5' exoribonuclease activity against single-stranded RNA viruses, such as Hepatitis C Virus (HCV), Hepatitis A Virus (HAV), Yellow Fever Virus (YFV). [Degols G. et al. 2007]. Recent reports show that ISG20 has antiviral activities against HIV-1 virus [Lucile E. et al. 2005]. Apart from its antiviral activities, ISG20 functions in ribosome biogenesis and maturations of snRNAs and rRNAs [Nguyen L. H. et al. 2001, Espert L. et al. 2003, Espert L. et al. 2005, Zhou Z. et al. 2011].

TRAFD1 – TRAFD1 is Type Zinc Finger Domain Containing 1 Gene. To control the viral pathogenesis inside the host cells, an inflammatory response is necessary, but excessive production of IFN is detrimental to host cells. TRAFD1 acts as negative regulator for uncontrolled inflammatory responses and has direct inhibitory effect on TRAF3 and TRAF6 of RIG-I and TLR3 mediated signaling pathways [Sanada T. et al., 2008]. Thus it can maintain the balance of the production of IFN after viral infection [Mashima, R. et al. 2005].

IDO – The full name is Indoleamine 2, 3-Dioxygenase. This is a heme-containing rate limiting enzyme in tryptophan catabolism. IDO plays a pivotal role in antimicrobial and antitumor defense, inhibits cell proliferation, maintains immunoregulation, and induction

of immune tolerance during infection [Curti, A. et al. 2009, Schmidt, S.V. et al. 2014]. Recent reports revealed that there is increased IDO expression after viral infection (HIV, HBV, HCV, or influenza). IDO plays a role against the pathogen and simultaneously in parallel maintains the outcome of immune response [Schmidt, S. V. et al 2014].

IFITM1 – Interferon Induced Transmembrane Protein 1 is also known as CD225. It is a member of IFITM family. It acts as antiviral restriction factor and inhibits enveloped virus infection. It mainly prevents the fusion of cellular and viral membrane and thus inhibits the viral entry inside the cell cytoplasm and in endocytosis [Feeley E.M. et al. 2011, Olyaee A.B. S. et al. 2013]. Reports show that it can act against different viruses, starting from common Influenza A Virus, SARS Coronavirus, Dengue Virus (DNV), Human Immunodeficiency Virus Type 1 (HIV-1), Hepatitis C Virus (HCV), and even the deadliest Ebola Virus (EBOV), and Zika Virus [Savidis, G. et al. 2016 (41)]. HCV can induce expression of one microRNA that can inhibit IFITM1 expression [Choudhuri J. B. et al. 2012]. It can also control cell growth and migration, has antiproliferative effect and is a positive regulator of osteoblast differentiation [Olyaee, S. A. B. et al. 2013].

1.5. Viral evasion strategies to circumvent the type I IFN signaling

Induction of IFN signaling and production of multiple ISGs restrict viral replication inside host cells. Consequently, viruses have different evasion strategies to circumvent these host immune responses and counteract the antiviral state. Each stage of the defense mechanism can be targeted by the virus, from initial virus detection, disruption of cell surface receptors, targeting of individual transcription factors, and nuclear factors, to

impeding the entire response pathway. In addition, the ISGs are also targeted to generate a productive infection and that is specific for each virus [Taylor, K. E. et al. 2012]. Kaposi's Sarcoma-Associated Herpesvirus (KSHV) decreases TRIF levels in a proteasome-mediated degradation [Ahmed h. et al. 2011 (45)]. Human Cytomegalovirus (HCMV) down-regulates IFN-inducible Sp100 protein levels [Kim, Y. et al. 2011]. In several Paramyxoviruses V proteins can down regulate RIG-I and IRF7 levels [Kitagawa, Y. et al. 2011]. Hepatitis C Virus (HCV) can also down-regulate the mRNA levels of TLR3 and RIG-I [Eksioglu, E. A. et al. 2011 (47)]. HIV can interfere with IRF3 activation. Simian Virus 5 (SV5) targets STAT1 in a proteasome-dependent manner in IFN response pathway to inhibit type I and type II IFN signaling. From the same *Paramyxoviridae* family Sendai Virus (SeV) and Human Parainfluenza Virus 2 (hPIV2) can block type I IFN signaling by acting on ISGF3 complex [Young, D. F. et al. 2000]. RSV, which is a human *Pneumovirus*, can also suppress type I IFN signaling to establish pathogenesis inside the host cells.

1.5.1. Suppression of type I IFN signaling by human Respiratory Syncytial Virus (hRSV)

RSV is the leading cause of respiratory illness in children and also in elderly and in immunocompromised patients. Ribavirin, an aerosol drug and Palvizumab, a monoclonal antibody against RSV cannot reduce the risk of hospitalization [Chen, C.H. et al. 2008, Ohmit, S.E. et al. 1996, Nokes, J. D. et al. 2008, Forbes, M. et al. 2008]. Thus still there is no clinically approved effective vaccines are available for RSV infection. RSV can evade the host's IFN response and replicate efficiently inside the host cells. RSV encodes 11 proteins, in which the two non-structural proteins, NS1 and NS2, are responsible for

blocking of the IFN signaling resulting RSV pathogenesis. NS proteins are present at 3'

most region and made in abundant amount only after viral infection inside the host cell

cytoplasm [Tran, K. C. et al. 2004]. NS proteins can target multiple components of the

induction and response pathways to inhibit the IFN signaling. In the induction pathway,

both NS1 and NS2 individually and together can prevent the nuclear localization of

transcription factor IRF3 [Spann, K. M. et al. 2005]. The RIG-I molecule is also targeted

by NS2 protein of RSV and prevent the interaction to MAVS and inhibits the IFN signaling

[Ling, Z. et al. 2009]. Both NS proteins of RSV can also decrease the steady state level of

two key signaling molecules of the induction pathway, TRAF3 and IKKε [Sweden S. et al.

2009]. Regarding the response pathway, NS1 and NS2 proteins of RSV can block IFN

signaling by 26S mediated proteasome-mediated degradation of STAT2 protein. It can also

decrease the phospho-STAT2 and phospho-STAT1 level result in disruption of

transcription of ISGs [Lo, M. S. et al. 2005, Spann, K.M. et al. 2004]. The C-terminal tetra-

peptide DLNP domain, which is the only common sequence for both NS proteins of RSV,

play essential role in IFN suppression, specifically STAT2 degradation, while IKKε

degradation is dependent on N-terminal domain of NS1 [Sweden S. et al. 2011]. A recent

report also suggests that NS1 protein of RSV has a structural similarity with the ubiquitin

enzyme E3 ligase which has a consensus binding sequence of Elongin C and Cullin 2. So

NS1 has the potential to act as ubiquitin E3 ligase and decrease the STAT2 level [Elliot J.

et al. 2007]. Apart from the NS proteins, the F and G proteins of RSV can also interfere

with the IFN signaling by targeting on JAK/STAT signaling [Hashimoto, K et al. 2009,

Oshansky, C. M. et al. 2009].

The last part of the type I IFN signaling pathway is the production of several ISGs. The effect of RSV on ISGs and their antiviral effect on RSV are not well documented. There are a large number of ISGs available. We have decided to test one specific ISG – OASL family proteins in reference with RSV.

1.5.2. Effect of Pneumonia Virus of Mice (PVM) on type I IFN signaling

PVM causes respiratory diseases in mice lung tissues. PVM is the only other virus in the same *Pneumovirus* genus which is related to hRSV. PVM is a representative of acute respiratory infection in a natural host for RSV infection [Kimberly, D. D. et al. 2012]. Though it is a mouse virus, scientific reports show that growth and replication of PVM in in-vitro human cultured cell lines are efficient and as well suppress type I IFN response. That suggests that PVM has a potentiality to be a human pathogen [Brock, L. G. et al. 2012]. PVM also has two small, non-structural proteins, NS1 and NS2. Studies showed there is a reduced rate of replication in NS1 and NS2 deleted mutant PVM viruses [Buchholz U. J. et al. 2009]. A recombinant PVM virus study with single or both deleted NS1 and NS2 proteins showed that NS proteins of PVM are also highly IFN antagonists [Heinze B. et al. 2011]. However the definite molecular mechanism of this suppression is obscure.

Based on the foregoing, we have decided to test the effect of PVM on type I IFN signaling pathway. The study is mainly focused on the two NS proteins of PVM and their interactions with the signaling molecules of both IFN induction and response pathways. The main aim of this study is to identify specific host proteins that are affected by PVM.

As PVM is used as a model to study severe respiratory illness, any knowledge available from this study will help to deliniate several immunomodulatory and therapeutc strategies for RSV infections and this work also help to understand the infection mechanism of other respiratory viruses. The results discussed in this manuscript are the direct outcome of our initiative to identify the molecular mechanism of suppression of type I IFN by PVM.

This thesis covers two major studies. Chapter II is on suppression of type I IFN response by NS proteins of PVM. The effect of NS proteins of RSV on a specific ISG family protein OASL and inhibition of RSV replication by OASL studies are discussed in chapter III.

CHAPTER II

UNIQUE NONSTRUCTURAL PROTEINS OF PNEUMONIA VIRUS OF MICE (PVM) PROMOTE DEGRADATION OF INTERFERON (IFN) PATHWAY COMPONENTS AND IFN-STIMULATED GENE PROTEINS[1]

2.1. Abstract

Pneumonia Virus of Mice (PVM) is an enveloped, negative sense, single-stranded RNA virus. It belongs to family *Paramyxoviridae* and genus *Pneumovirus*. PVM is the only virus other than RSV which shares the same *Pneumovirus* genus. PVM causes respiratory tract infections in mice and reproduces similar clinical and pathological features of RSV infection. PVM is used as an animal model to study human RSV infection in a natural host, as RSV infection is not very effective and permissive in the mouse model. Two nonstructural proteins of RSV are required to circumvent the host immune system and suppress the type I IFN response. NS1 and NS2 alone or together can degrade many of those signaling components of induction and response pathways for optimal viral pathogenesis. PVM also encodes two nonstructural proteins, NS1 and NS2. But there is no

sequence homology between RSV and PVM NS proteins. PVM can also inhibit type I IFN signaling, but the mechanism is unknown. Our result shows that PVM NS proteins also play a pivotal role in the suppression of IFN signaling. NS proteins alone or together can target many components of the signaling pathway for degradation, RIG-I and IRF3 in induction pathway and STAT2 in response pathway. The degradation of RIG-I, IRF3 and STAT2 is done through proteasome-dependent pathway. Our observations also document that PVM virus can decrease the steady-state level of these three above mentioned IFN pathway proteins. Type I IFN signaling induces the production of several Interferon Stimulated Genes (ISG) with antiviral properties. To establish optimal infection inside the host cells and to evade the defense mechanism, PVM NS proteins can also target multiple ISGs, such as ISG20, TRAFD1, IDO and IFITM1. These observations enable us to document the basic molecular mechanism of IFN suppression by PVM in murine cells. In spite of the sequence dissimilarities between RSV and PVM nonstructural genes, our results show that the basic molecular mechanism of suppression of IFN signaling is relatively similar for the *Pneumovirus* genus. These findings could lead us to resolve new immunomodulatory strategies for respiratory diseases where PVM can serve as a surrogate animal model.

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[1]As it appears in Scientific Reports, 2016

2.2. Introduction

The *Pneumovirus* genus is comprised of two viruses: RSV (human, cow, goat and sheep) and PVM (mouse). Both belong to family *Paramyxoviridae*. RSV causes lower respiratory tract infection in humans, mainly in infants and immunocompromised adults, where PVM is a deadly mouse pathogen causing high rates of morbidity and mortality. Both viruses have negative strand, single-stranded RNA as their genetic material. It encodes two nonstructural proteins (NS1 and NS2) in both the viruses [Easton, A. J. et al. 2004, Dyer, D. K. et al. 2012, Rosenberg, H. F. et al 2004, Horsfall, F. L., Hahn, R. G. et al 1939, Krempl, C. D. et al 2005]. NS proteins of RSV can evade the host's defense mechanism by preventing type I IFN response. They target the signaling molecules of the pathway and promote ubiquitin-mediated proteasomal degradation of them. Type I IFN pathway is bimodal (described before Chapter I). RSV NS proteins degrade multiple components of induction pathway, such as RIG-I, TRAF3, TBK1, IKKε, IRF3 and IRF7. In the response pathway, STAT2 is targeted for degradation [Barik, S. 2013, Ling, Z. et al. 2004, Lo MS et al. 2005, Ren J. et al. 2011, Spann, K. M. et al. 2005, Sweden S et al. 2009, Sweden S et al. 2011, Goswami, R. et. al 2013]. PVM replicates clinical and pathological features of RSV in the mouse model. Hence, PVM is used as a convenient surrogate model to study the entire *Pneumovirus* genus. Studies suggested that PVM can also suppress the host's immune response by inhibiting type I IFN signaling [Heinze B. et al. 2011, Buchholz U. J. 2009]. However, the mechanism of this suppression is not known. In an effort to understand the mechanism of the IFN suppression, we have compared the sequences of two NS proteins of PVM with the two NS proteins of RSV which have been established in

IFN suppression. By using Clustal Omega, the protein sequencing alignment tool [Sievers, F., 2011], we have aligned and compared the predicted primary structures of all four NS proteins to delineate any sequence homology with any combination of PVM and RSV NS proteins [Figure 6]. Both NS proteins of RSV and PVM do not have any significant sequence homology. RSV NS1 protein represents the hypothetical SOCS box with conserved Leu and Cys residues, which has been suggested has the E3 ligase activity only for RSV NS1 [Elliott J, 2007]. But PVM NS proteins do not have any discernible similarity with the SOCS box represented for RSV NS1. Both RSV NS1 and NS2 have a common DLNP tetra peptide sequence at C-terminal end which plays a major role in the suppression of IFN and in the degradation of the signaling molecules [Sweden S. et al. 2009, Sweden S. et al. 2011]. Sequence alignment explicates that PVM NS do not share the similar DLNP sequence as RSV. These observations led us to hypothesize that as there are no similarities between PVM and RSV NS proteins, the mechanism of action for suppression of IFN for PVM will be different from RSV.

A

```
PVM-NS1   MGCNVMMELDYG------------------G-------RAAWLAFHITNFDRSDLETILRGARVCNTWQDQRLSVYLVG   54
RSV-NS1   MGSNSLSMIKVRLQNLFDNDEVALLKITCYTDKLIHLTNALAKAVIHTIKLNGIVFVHVITSSDICPNNNI-------VV   73
          **.*.: . :.                               * ..:* ::: : :: .: :*.:         *

PVM-NS1   RDCNLLRPFVQAAKFIHNTRRGQTLTHWFTKNIVFSSTGQETEPPIDPTCELLVELISG--------------------   113
RSV-NS1   KSNFTTMPVLQNGGYI-----------WEMMELT---HCSQPNGLIDDNCEIKFSKKLSDSTMTNYMNQLSELLGFDLNP   139
          :.    *..:* . :*           *   ::.    . :: ** .**: ..   .
```

B

```
PVM-NS1   MGCNVMMELDYGGRAAWLAFHITNFDRSDLETILRGARVCNTWQDQRLSVYLVGRDCNLLRPFVQAAKFIHNTR------   74
RSV-NS2   ------MDTTHNDT-TPQRLMITDMRPLSLETIITSLTR--D-IITHRFIYLINHECIVRKLDERQATFTFLVNYEMKLL   70
              *:  :   :  : **::    ****: .        : :**: ::* : :   : *.* . ..

PVM-NS1   --RGQT----LTHWFT------KNIVFSSTGQETEPPIDPTCELLVELISG-----   113
RSV-NS2   HKVGSTKYKKYTEYNTKYGTFPMPIFINHDGFLECIGIKPTKY--TPIIYKYDLNP   124
            *.*     *.: *      *.:. *      * .**    . :*
```

C

```
PVM-NS2   MSTAMNKFTQTISKPATILNISDSEESGDEAGVGKVSRTTQSSERWLDLLIEKFQPS-----LQNITR-YINWNFIRICN   74
RSV-NS2   MDT------------------------------------THNDTTPQRLMITDMRPLSLETTITSLTRDIITHRFIYLIN   44
          *.*                                    *:..    *.* ::.*      : .:** *. .** : *

PVM-NS2   DRLKKEKMGYIEA-KQYVEDMAWMVIASEADSIEWKCIRRQEKVTGVKYPKFFFVQHKEDWIECTGCIPYPGHDLIYDED   153
RSV-NS2   HECIVRKLDERQATFTFLVNYE-MKLLHKVGSTKYKKYTEYNTK-YGTFPMPIF-INHDGFLECIGIKPTKHTPIIYKYD   121
          ..   .*:   :*   :: :   * : :. * ::*    . :.    .:* :*  .:: ::** * *      :**. *

PVM-NS2   DDD   156
RSV-NS2   LNP   124
           :
```

D

```
PVM-NS2   MSTAMNKFTQTISKPATILNISDSEESGDEAGVGKVSRTTQSSERWLDLLIEKFQPSLQNITRYIN--------------   66
RSV-NS1   -----------------------------MGSNSLSMIK----VRLQNLFDNDEVALLKITCYTDKLIHLTNALAKAVI   46
                                       *  .:*  .      *: *::: : :* : ** * :

PVM-NS2   -------WNF------IRICNDRLKKE-----KMGYIEAKQYVEDMAWMVIASEA-DSIEWKCIRRQ-EKVTGVKYPKFF   126
RSV-NS1   HTIKLNGIVFVHVITSSDICPNNNIVVKSNFTTMPVLQNGGYIWEMMELTHCSQPNGLIDDNCEIKFSKKLSDSTMTNYM   126
                  *        ** :.     .*  ::   *: :*   :. .*:     *: :*   :   :*:: .   :::

PVM-NS2   FVQHKEDWIECTGCIPYPGHDLIYDEDDDD   156
RSV-NS1   -----NQLSELLGFDLNP------------   139
               :: * *    *
```

Figure 6: PVM and RSV NS sequences alignment. A, B, C, and D represent all the possible combinations of sequence alignment of PVM and RSV NS proteins; **A:** PVM NS1-RSV NS1, **B:** PVM NS1-RSV NS2, **C:** PVM NS2-RSV NS2, **D:** PVM NS2-RSV NS1. Comparing the predicted primary sequences of PVM and RSV show that there are no sequence similarities between them. They are completely two random sequences.

PVM can circumvent the host defense mechanism. Studies showed that in the absence of NS proteins, the infection and replication of the virus is less efficient [Kimberly, D. D. et al. 2012, Rosenberg, H. F. et al. 2008, Heinze B. et al. 2011, Buchholz U. J. et al. 2009]. There is, however, no common sequence motif in RSV and PVM NS proteins; our studies delineate that NS proteins of these two viruses are almost functionally homologous. They use nearly similar strategies to circumvent the host immune system that is targeting the signaling molecules of the pathway. The initial immune response in the form of type I IFN can clear viral infection. This immune response further helps in the recruitment of the components of the adaptive immunity and develops immunological memory. As we discussed previously RSV is the leading cause of pediatric illness causing hundreds of thousands of mortalities per year because of the lack of effective antiviral drugs.

Our study aims to reveal the suppression mechanism of PVM through understanding the suppression mechanism of NS proteins with the signaling molecules of the IFN induction and response pathway. Both viruses cause similar pathogenesis having functional similarities but share no sequence resemblances. The knowledge from our study will allow us to find new immunomodulatory strategies for the whole *Pneumovirus* genus. That will help to limit the viral replication up to a certain threshold level, that the infection can induce long lasting immunity but cannot inhibit the complete shutdown of the immune response.

Thus, we hope to develop a novel antiviral approach against the whole *Pneumovirus* genus. It can also be used for those other viruses from different genus and families that also cause similar types of respiratory illnesses and interfere with IFN signaling.

2.3 Materials and methods

2.3.1. Cell lines

The immortalized mouse embryonic Fibroblast (MEF) cells were principally used for all the experiments showed here. The cell line was obtained from the Lerner Research Institute of the Cleveland Clinic from DR. Ganes Sen's laboratory. These cells possess the ability to induce type I IFN with viral infection. Phenotypically similar characteristics of these cell lines with the naturally infected mouse cell lines make the observations physiologically relevant.

Human lung alveolar epithelial (carcinoma) cells, A549 cells (ATCC CCL-185; American Type Culture Collection, Manassas, VA) were also used in a couple of experiments. Both of these cell lines were cultured in monolayers in an incubator at $37^{o}C$ with 5% CO_2. The cells were maintained in Dulbecco's Minimum Essential Media (DMEM) which was supplemented with L-Glutamine, Penicillin (100 IU/ml) and Streptomycin (100 µg/ml) and heat-inactivated Fetal Bovine Serum (FBS-10%).

FLIP-IN T Rex (Invitrogen) cell lines were used in some of the experiments that can express different ISGs. These cell lines were obtained from Dr. J. T. Gao laboratory. Cells were cultured and maintained in Tetracycline-free DMEM media, that was supplemented with 10% Tetracycline free FBS (Hyclone). For maximum accuracy in selectivity of ISG expressions, cells were treated with 250µg/ml Hygromycin and 5µg/ml Blasticidin. The specific ISG proteins were expressed in these cell lines with the addition of 0.2 µg/ml Tetracycline into the cell culture medium. The Tetracycline induced ISG cell lines are all

FLAG-tagged and the expression of these ISGs were tested and confirmed with Western Blot analysis [Jiang, D. et al. 2008].

In the majority of the experiments, cells were infected with viral strains or transfected with recombinant plasmid DNAs.

2.3.2. Virus strains

Pneumonia Virus of Mice (PVM) strain J3666 was used for all the experiments. The required amount of virus infections for the experiments was used at a multiplicity of infection (m.o.i.) 3. The strain J3666 was obtained from Dr. Helene Rosenberg's (NIH) laboratory. RAW mouse monocyte cell lines were used for the virus growth.

Human Respiratory Syncytial Virus (hRSV) strain A serotype was used for confocal experiment at m.o.i. 2. They were grown in HEp-2 cell line.

Sendai Virus type 1 strain was also used at m.o.i. 2 to for induction of IFN β promoter to measure luciferase activity.

All the cells were stored frozen in small aliquots in -80°C.

2.3.3. Antibodies

The large numbers of primary and secondary antibodies were used in all the experiments, starting from Immunoblot (IB) or Western Blot (WB) analysis, Immunoprecipitation (IP) and Immunofluorescence (IF).

The attached table gives the lists of the antibodies with their targets, dilution ranges, name of the hosts where the antibodies raised, and manufacturer details.

Table 1: Lists of primary antibodies

No.	Antibody target	Host	Dilution	Experiments	Manufacturer
1	PVM [Cys-conjugated peptide SQQLNIVDDTPDDDI of PVM N protein sequence (residue 379-393)]	Rat	1:500	WB	Bio-Synthesis, Lewisville, TX
2	FLAG	Mouse	1:1000 1:200	WB IF	Sigma, SLBF6631/F1804
3	FLAG	Rabbit	1:1000 1:200	WB IF	Sigma, F7425
4	V5	Mouse	1:5000 1:300	WB IP, IF	Thermo Scientific, MA5-15253
5	Myc (C-Myc)	Rabbit	1:1000 1:200	WB IP, IF	Thermo Scientific, PA1-981
6	Ub	Mouse	1:1000	WB	Santa Cruz, SC-8017
7	IkBα	Mouse	1:1000 1:200	WB IP	Santa Cruz, SC-56710
8	GAPDH	Mouse	1:5000	WB	Santa Cruz, SC-365062
9	β actin	Mouse	1:2000	WB	Santa Cruz, SC-81178
10	RIGI	Goat	1:1000	WB	Santa Cruz, SC- 48929

Table 2: Lists of secondary antibodies

No.	Antibody	Host	Dilution	Experiments	Manufacturer
1	IgG-HRP anti-mouse	Goat	1:2000	WB	Santa cruz, SC-2031
2	IgG-HRP anti-rabbit	Goat	1:5000	WB	Santa cruz, SC-2030
3	IgG-HRP anti-goat.	Mouse	1:3000	WB	Santa cruz, SC-2354

The secondary antibodies which are conjugated with fluorophores for microscopy experiments are as follows: anti-goat Alexa Fluor 488-conjugated (1:300 dilution) [Molecular probes: Invitrogen A11055]; anti-mouse Alexa Fluor 488-conjugated (1:300 dilution) [Molecular probes: Invitrogen A21202]; anti-rabbit Alexa Fluor 594-conjugated (1:300 dilution) [Molecular probes: Invitrogen A21442]. DAPI was used for nuclear staining [Molecular probes, D1306]. Prolong gold was used as a mounting media [Life Technology P36930].

2.3.3. Recombinant proteins and mutagenesis

PVM NS genes (NS1 and NS2) were commercially synthesized by GenScript. These codon-optimized NS genes were cloned in pCAGGS eukaryotic expression vectors. They have EcoRI-BglII restriction sites and an N-terminal FLAG-tag (DYKDDDDKP). The original length of the native NS1 protein is 113 amino acids and NS2 protein is 156 amino acids long. On SDS-PAGE the molecular weights of NS1 and NS2 are approximately 13 kD and 18 kD, respectively.

The six C-terminal mutants of both PVM NS1 and NS2 proteins were also commercially synthesized. Each NS protein lacks 5, 10, and 15 amino acids from their C-terminal end. These mutants were generated by introducing primers carrying upstream stop codons in PCR amplification reaction to produce pCAGGS-FLAG-NS1 or NS2 5, 10, and 15 amino acids deletions mutants. The expressions of each of these C-terminal deletions were tested by transient transfection in MEF cells. All deletions mutants were well expressed; the molecular weights are as follows: ΔC5-NS1 (11.8 kD), ΔC10-NS1 (11.2 kD), ΔC15-NS1 (10.6 kD) and ΔC5-NS2 (16.6 kD), ΔC10-NS2 (16 kD), ΔC15-NS2 (15.4 kD). The expression of ΔC10-NS1 was weak consistently.

mSTAT2-FLAG recombinant protein was obtained from Dr. Adolfo Garcia-Sastre from the Mount Sinai School of Medicine, NY; Myc-IKKε (pcDNA3.1-myc-IKKε), FLAG-mIRF7, FLAG-Myc-mRIG-I, and V5-mIRF3 recombinant plasmids were the generous gifts from Dr. Ganes C. Sen from the Lerner Research Institute of the Cleveland Clinic, OH. The firefly-Luciferase reporter plasmids were provided by Chris Basler of the Mount Sinai School of Medicine, NY. Dr. Ramesh Pillai EMBL, Grenoble, France, supported our project with the control Renilla luciferase plasmid.

2.3.4. Site directed mutagenesis

The sequence alignment of PVM and RSV NS proteins reveal that there is no significant sequence homology in PVM and RSV NS proteins. Therefore, to investigate whether there is any specific Cysteine residue that is important for the ubiquitin activity of PVM NS proteins, three Cys of PVM NS1 (Cys 40, Cys 57, and Cys 104) and one cysteine of PVM NS2 (Cys 137) were mutated to alanine by using Quick-change II Site-Directed

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Mutagenesis Kit (Agilent). By using specific forward and reverse primers in PCR amplification technique, the desired changes were introduced. Amplified products then subjected to *Dpn* 1 digestion. The digested products were transformed with XL1-Blue competent cells and were plated in Ampicillin-agar plate (100 µg/ml). Obtained colonies were tested for anticipated changes using the DNA sequencing method (Genescript).

All these point mutations have an N-terminal FLAG-tag.

Table 3: Lists of primers: All primers are written 5' to 3' (Site directed mutagenesis)

PVM NS1- Cys 40	Forward: GGGGAGCCCGCGTGGCCAATACTTGGCAGG Reverse: CCTGCCAAGTATTGGCCACGCGGGCTCCCC
PVM NS1- Cys 57	Forward: CTGGTCGGCCGAGATGCTAACCTGCTGAGACC Reverse: GGTCTCAGCAGGTTAGCATCTCGGCCGACCAG
PVM NS1- Cys 104	Forward: CTATCGACCCCACCGCCGAGCTGCTGGTCG Reverse: CGACCAGCAGCTCGGCGGTGGGGTCGATAG
PVM NS2- Cys 137	Forward: CACAAGGAGGACGACTGGATCGAAGCCACCG GATGTATTC Reverse: GAATACATCCGGTGGCTTCGATCCAGTCCTCC TTGTG

2.3.5. Transfection

All the recombinant plasmids were prepared using Qiagen Midi-prep kit according to the manufacturer's protocol. The concentration of the DNA was measured by Nano-Drop machine and plasmids were stored in -20°C. MEF, ISG, and A549 cell lines were used for transfections. Cells were transfected at approximately 80-90% of confluency. For mouse cell lines Lipofectamine LTX and PLUS reagent (Invitrogen/ Life Technologies) and for human A549 and ISG cell lines, Lipofectamine 2000 (Invitrogen/ Life Technologies) were

used. In all the transfections experiments, cells were transfected for 24 hrs, (unless otherwise indicated). In Lipofectamine 2000 method, the transfection reagent was replaced and in LTX method the reagent was diluted with DMEM media after 8-10 hrs according to the manufacturer's protocol. The amount of transfected plasmid is constant for all wells, ~1.6 µg for 12 well plate. After 24 hrs of transfection, cells were collected the next day with Laemmli buffer [Composition of 2X Laemmli buffer; 1M Tris pH 6.8, 20% SDS, 50% Glycerol, β-marcaptoethanol, Milli Q-water and pinch of Bromophenol blue].

To detect proteasomal activities in some of the experiments after 8-10 hrs of transfection cells were treated with one proteasomal inhibitor MG132 (Sigma). MG132 has toxic effects on cells, and therefore, we optimized the concentration of MG132. We also tested another proteasomal inhibitor, namely lactacystin, in a few of our experiments (Chapter 3). But we have found that the toxic effect of this chemical on cells is greater than that of MG132. Thus, we have used MG132 in all our experiments.

2.3.6. Western blotting (WB) or Immunoblotting (IB)

Monolayer of cells at 80-90% confluency were transfected with required and constant amounts of recombinant plasmids. After 24 hrs of post-transfection (unless otherwise indicated) cell lysates were harvested using 1X Laemmli Buffer. Lysates were then sonicated twice with 15 seconds of pulse (each time at amplitude 90% with 10 seconds of interval between each pulse). Samples were then heated at 95°C heat bath and centrifuged at 10000 rpm for 5 minutes, supernatant were collected then and stored at -80°C. These are the samples for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The standard protocol follows the application of the Tris-HCL polyacrylamide gel

and the Tris-Glycine-SDS running buffer. The samples were loaded in polyacrylamide gel and they were separated according to their electrophoretic mobility. PVDF membrane (0.45 µm, Immobilon-P, Millipore) was used to transfer the protein at constant current for 2 hrs. Tris-Glycine buffer with 20% methanol was used as a running buffer. Membranes with the transferred proteins were blocked for 2 hrs in blocking buffer at room temperature in shaking. The blocking buffer is composed of 6% non-fat dry milk solution in TBST with 0.1% Tween-20 in it. Membranes were then probed with specific primary antibodies overnight at 4°C, followed by corresponding secondary antibodies conjugated to Horseradish-Peroxidase the next day for 2 hrs in shaking. After incubation with primary antibodies, membranes were properly washed 6 times for 10 minutes each with TBST and after secondary antibodies with TBS. Bands were then developed by using ECL prime, Western Blotting detection reagent (GE Healthcare). Bands were detected in LI-COR Odyssey Fc Imaging System machine and densitometric analyses were conducted with the integrated LI-COR software. As a control for normalization, β-actin and GAPDH were used.

2.3.7. Immunoprecipitation (IP)

Immunoprecipitation is a protein pull down assay where a protein complex is precipitated with a specific antibody that can bind to a specific protein of that complex. In our experiment Protein A-Sepharose (PAS-Sigma) was used to pull down the protein of interest. MEF cells were transfected with FLAG-STAT2, Myc/FLAG-RIGI, V5-IRF3 and FLAG-NS recombinant plasmids. Cell lysates were collected after 24 hrs of post transfection with RIPA buffer. Lysates were then incubated with resuspended PAS beads

for 30 minutes in 4℃ in proper mixing. Anti-myc, anti-v5 and anti-FLAG antibodies were used with resuspended PAS for 3 hrs to pull down the protein of interest. After that the samples were washed with IP specific wash buffer and eluted with 2X sample buffer. The samples were then examined with denaturing SDS-PAGE followed by Western Blotting analysis.

2.3.8. Immunofluorescence (IF): Confocal microscopy

MEF cells were grown on square coverslips in 6-well plates. For RIG-I and NS colocalization study, to detect native RIG-I cells were induced with RSV at m.o.i. 2 for 8 hrs and then transfected with FLAG-NS recombinant plasmids. In IRF3-NS colocalization experiment, cells were transfected with V5-IRF3 and FLAG-NS recombinant plasmids. To prevent complete degradation of the protein of interest, proteasomal inhibitor MG132 (10μM for a 12-well plate) was added post-transfection. The next day, to stain the cells, media was removed and washed with PBS twice 5 minutes each followed by the cell fixation with ice-cold 4% paraformaldehyde for 20 minutes. Fixed cells were then permeabilized with PBS containing 0.1% Triton X-100. Blocking was done in the next step for 1 hr with PBS containing 1% BSA followed by incubation with the specific primary antibodies solutions made in PBS for 2 hrs. Cells were rinsed in PBS and incubated with Alexa Fluor-conjugated secondary antibodies solution in PBS in the dark for 1.5 hrs. For nucleus staining DAPI was used. Cells were washed three times and mounted with Prolong Gold (Invitrogen). Slides were kept in the dark at room temperature for overnight and then stored at -20℃. Images were captured in a Nikon A1RSI confocal microscope with NIS element 2.3 software at 60x magnification.

2.3.9. Dual luciferase assay

The Luciferase (Luc) assay is done to study the gene expression at the transcription level. The luciferase experiments were done according to the manufacturer protocol (Promega's Dual-Luciferase Reporter Assay System E1910). Cells were cotransfected with pCAGGS vector, NS expression recombinant plasmids, and firefly-luciferase expression vector, for induction assay IFNβ promoter firefly luciferase, and for response assay an ISGF54 promoter firefly luciferase. As internal control, pCMV Renilla luciferase vectors were used for normalization of firefly luciferase activity. Experiments were done in triplicates and graphs were generated accordingly.

2.3.10. Quantitative RT-PCR

MEF cells were grown in 100mm cell-culture dishes and cotransfected with either of these four recombinant plasmids; mSTAT2, mRIGI, mIRF3, mIRF7, and NS recombinant plasmids. Total RNA were extracted after 24 hrs of post-transfection using Trizol. DNA free kit was used to purify the total RNA from any residual genomic DNA. Complementary cDNA was prepared and real time PCR reaction was performed using Real-Time Detection System and SYBR-GREEN Supermix (BioRad). Primers for the genes (mSTAT2, mRIGI, mIRF3, and mIRF7) used for the PCR are listed in Table 4. GAPDH was used as a loading control.

Table 4: Lists of primers: All primers are written 5' to 3' (qRT-PCR)

mRIG-I	Forward: TGGACAAAAAGGGAAAGTGG Reverse: TGCTGCACTGAGACGCTATC
mIRF3	Forward: ACGTGTCAACCTGGAAGAGG Reverse: GGCACCCAGATGTACGAAGT
mIRF7	Forward: CCAGTTGATCCGCATAAGGT Reverse: GAGCCCAGCATTTTCTCTTG
mSTAT2	Forward: AAACTTCTGAAGGGGGCATT Reverse: CTTCGGCAAGAACCTGGTAG
GAPDH	Forward: CTGGAAAACCCTGCCAAATA Reverse: TGCTCAGTTTAGCCCAGGAT

2.3.11. Statistical analysis

All results presented here include the mean $\pm$ the standard deviation from three separate experiments. Statistical analyses were performed using GraphPad Prism 5.0 software. All statistical analyses were calculated by using student's T-test and one-way Anova. The statistical significance was considered as $p < 0.05$.

2.4. Results

2.4.1. NS proteins of PVM are well expressed

Previous studies with recombinant ΔNS PVM viruses revealed that NS proteins are IFN antagonists and can suppress both type I and type III IFN responses. To establish the infection and pathogenesis effectively inside the host cells, NS proteins are necessary. ΔNS PVM virus has a lower rate of viral replication inside the host cells [Heinze B. et al. 2011, Buchholz U. J. et al. 2009]. Since we commercially made the FLAG-tagged recombinant NS1 and NS2 proteins, our first aim was to test their successful expressions in MEF cell lines. MEF cells were transfected with FLAG-tagged NS1 and NS2 expression vectors and subjected to immunoblot with the FLAG antibody. Results show that both of the recombinant NS1 and NS2 proteins are well expressed [Figure 7].

FLAG-tagged in MEF Cells

NS1   NS2
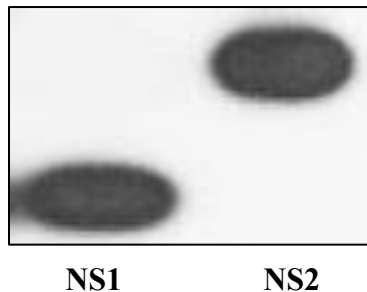
Figure 7: Recombinant NS proteins of PVM are well expressed. Immunoblot analysis detected with the FLAG antibody is showing NS1 (left panel) and NS2 (right panel) proteins. The molecular weight of NS1 is ~13 kD and NS2 is ~18 kD.

2.4.2. Recombinant NS proteins are multifunctional and IFN antagonists

Since we had the well expressed recombinant NS proteins for PVM, our next aim was to confirm that these NS proteins are functional. To test the IFN suppressor function of PVM NS proteins, we have decided to use the established luciferase reporter assay to study the gene expressions at the transcription level, as the primary regulation of type I IFN induction occurs at the transcriptional level. In our experiment, the luciferase reporter plasmid is attached to either IFN-β gene promoter for IFN induction pathway or the ISG54 promoter for IFN response pathway. Thus the transcriptional expression of Luc gene is under the influence of IFN-β and ISG 54 gene promoter. First, we determined the ability of PVM NS proteins to suppress the type I IFN induction. In this experiment, MEF cells were cotransfected with the empty pCAGGS vector along with luciferase reporter plasmid that is attached to IFN-β promoter, and also with FLAG-tagged NS1 and NS2 alone and together. As an internal control to normalize the firefly luciferase activity, we have used Renilla Luciferase gene under the control of the constitutively active CMV promoter. To induce the type I IFN response, SeV at m.o.i. 2 was added after 6 hrs of transfection. Cell extracts were collected after 24 hrs of transfection and the luciferase activity was measured. Results based on this luciferase assay experiment document that both PVM NS proteins can suppress the SeV induced IFN-β promoter induction. NS1 and NS2 can act individually or together to inhibit the transcription of the IFN-β promoter [Figure 8A].

Since both the NS proteins alone and together are able to interfere with IFN induction, next we have determined to test their ability on IFN response, specifically on transcription of the ISG54 promoter. The reason for using ISG54 is that it comprises the ISRE element which induces anti-viral genes or ISG production by binding with ISGF3 complex upon

IFNAR activation by type I IFN. Dual luciferase assay was performed where cells were transfected with Luc reporter plasmid which is attached to ISG54 promoter along with NS proteins and with CMV-Renilla Luc plasmid for luciferase transfection control. At 6 hrs of post transfection, cells were treated with recombinant universal type I IFN (1,000 U/ml) to induce the ISG54 promoter. After 24 hrs cells were assayed for luciferase activity. The observations from the experiment showed that both NS1 and NS2 are capable of suppressing ISG54 promoter activity alone. But the co-expression of both NS proteins leads to the robust suppression of the IFN response [Figure 8B].

Our observations indicate that both NS proteins cooperatively can suppress the transcription of IFN responsive genes. Hence, NS proteins of PVM are functional. But the mechanism behind this suppression remains unclear and needs to be determined.
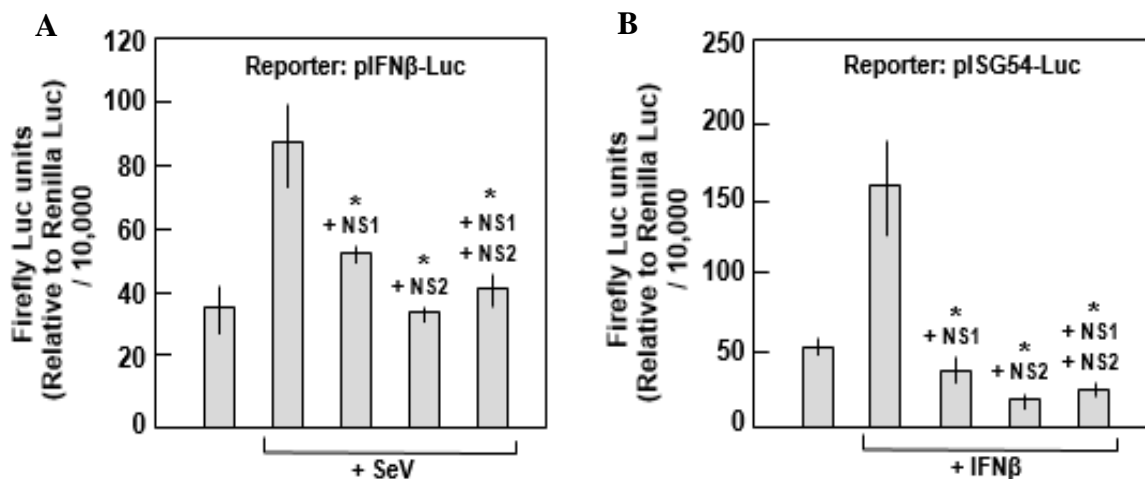
Figure 8: Functional IFN suppression by PVM NS proteins. A: Effect of NS proteins on IFN-β promoter activity. PVM NS proteins individually or together can suppress SeV induced IFN induction. Firefly luciferase gene under the control of IFN-β promoter measures the induction of the IFN-β promoter activity. Renilla luciferase was used as an internal control. NS proteins were cotransfected and luciferase activity was measured after

24 hrs of transfection. N = 3, asterix denotes p < 0.05, which is denoted as a significant inhibition of IFN suppression by PVM NS proteins. **B: Effect of NS proteins on ISG54 promoter activity.** The graphs represent the luciferase activity under ISG 54 promoter control with transfection of both PVM NS proteins individually and together. Renilla Luciferase as an internal control was used. N= 3, p < 0.05 is a significant inhibition of response pathway by PVM NS proteins.

2.4.3. NS proteins can decrease the steady state level of mouse RIG-I

Previous experiments showed that PVM NS proteins can suppress both of the IFN induction and response pathways. We then considered to test the effect of the PVM NS proteins on the components of both the IFN induction and response pathways to delineate the mechanism of suppression of type I IFN by PVM NS. The focus of our project is the TLR-independent pathway of type I IFN induction. The viral RNA is first recognized by cytoplasmic RNA sensor RIG-I. Previous reports also showed that NS proteins of other Pneumoviruses have the ability to interact with RIG-I and can decrease its level eventually preventing the IFN induction [Ling Z. et al. 2009].

We have performed the overexpression approach with recombinant PVM NS proteins to test the effect of each NS proteins on mouse RIG-I. MEF cells were transfected with FLAG-tagged recombinant NS1, NS2, and with FLAG/Myc tagged mRIG-I and for control empty pCAGGS vector. After 24 hrs of transfection, cell-lysates were processed for IB using the FLAG antibody. The results documented that steady-state level of RIG-I is affected. Both NS proteins can decrease the level of RIG-I significantly [Figure 9A, 9B]. The approximate molecular weight of mRIG-I is 101kD. Additionally, the dose-dependent

approach using different amounts of recombinant NS1 and NS2 proteins validates that both

NS proteins individually and together can decrease the mRIG-I level.

We have also tested the effect of these NS proteins on native mouse RIG-I. The similar

experimental procedure was followed. RSV at m.o.i. 0.5 was used after 6 hrs of transfection

to induce the native RIG-I. PVM NS proteins also can decrease the level of native RIG-I

level [Figure 9C]. IB results from transfection experiments with recombinant NS proteins

consistently show that the production of NS2 is abundant than the NS1 protein, the reason

is unknown for that.
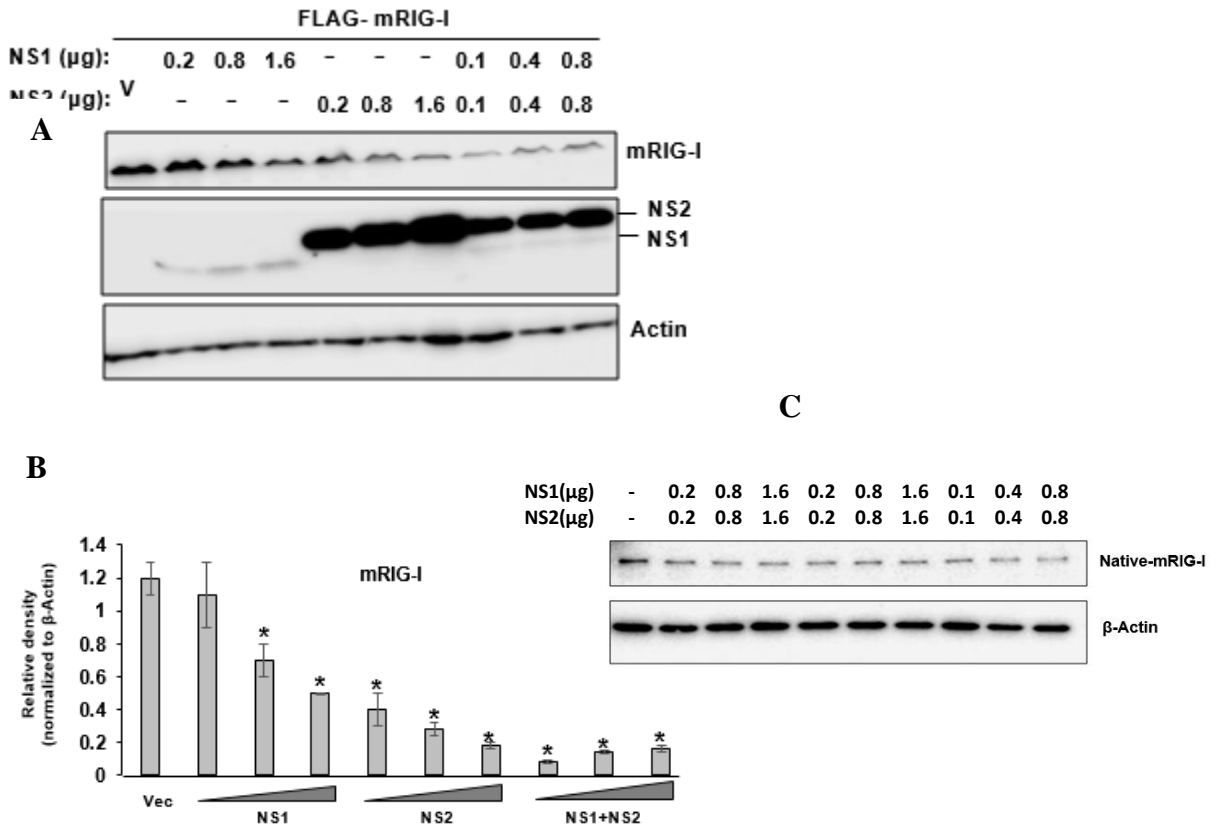
Figure 9: mRIG-I level is decreased by PVM NS1 and NS2. A: Dose dependent response of FLAG-NS on FLAG/Myc mRIG-I. Recombinant NS proteins transfection in MEF cell lines followed by immunoblot is showing decreased level of mRIG-I. **B:** Densitometric analysis of the previous blot represents FLAG-mRIG-I level after normalization with β-actin levels. N=3, p< 0.05 shows significant reduction.

2.4.4. NS proteins selectively decrease downstream signaling molecule mIRF3

IRF3 is the downstream signaling molecule of the IFN induction pathway. The inactivated cytoplasmic form of IRF3 phosphorylates to become activate and translocate to the nucleus. It is one of the key components to induce the transcription of type I IFN genes. Previous studies reported that RSV NS1 plays predominant role to prevent the activation and nuclear localization of IRF3 and thus suppresses type I IFN production [Spann, K. M. et al. 2005]. We took the similar overexpression approach and examined the effect of PVM NS proteins on mIRF3. MEF cells were transiently cotransfected with V5-tagged IRF3 and FLAG-NS recombinant plasmids and were subjected to IB. Normalization with GAPDH showed that NS proteins can also decrease the steady-state level of mIRF3. The molecular weight of mIRF3 is ~ 47 kD. This time the reduction level was not as efficient as RIG-I, though both NS proteins can significantly decrease the level of mIRF3 [Figure 10: A, B].

Native mIRF3 also showed similar results. NS transfected MEF cells showed reduced level of native mIRF3 [Figure 10C].
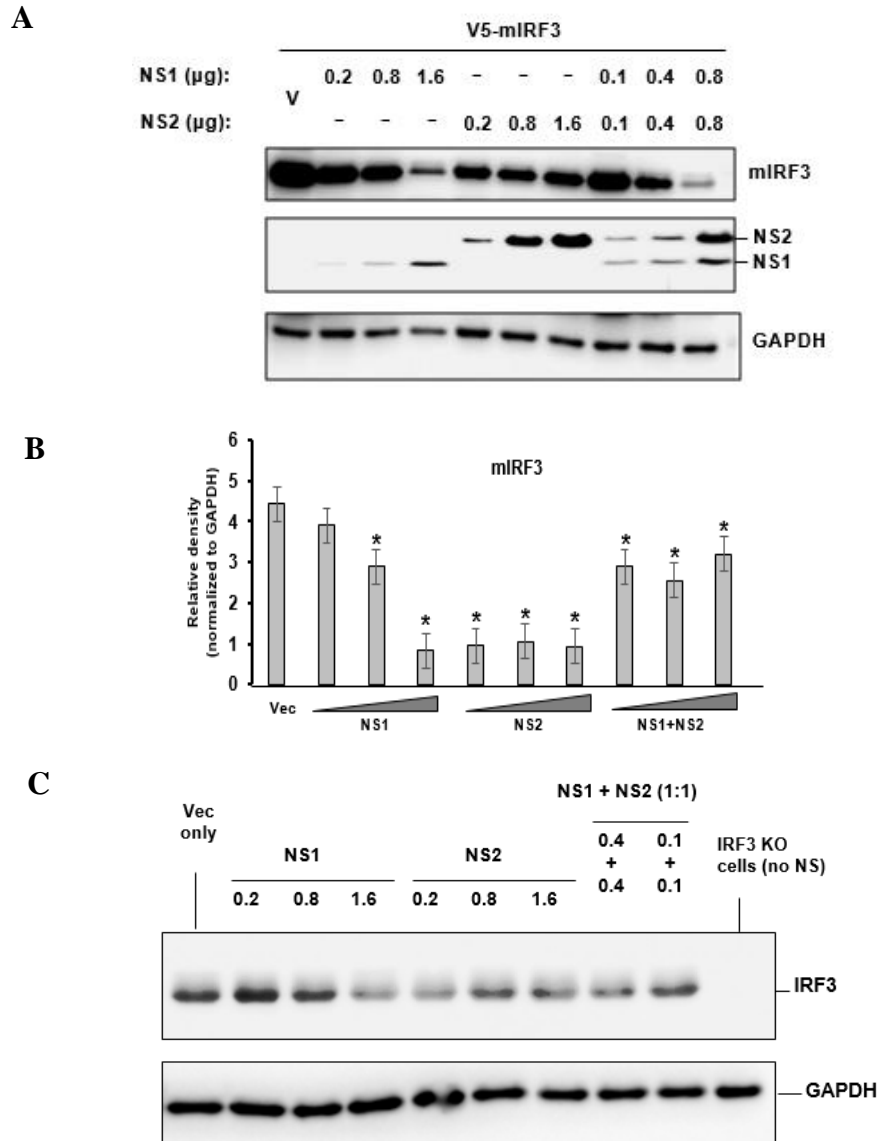
Figure 10: Decreased level of mIRF3 with PVM NS proteins. A: Dose dependent response of FLAG-NS on V5-mIRF3. Different amount of NS proteins were transfected in MEF cell lines with V5-mIRF3. Immunoblot probe with V5 antibody is showing decreased level of mIRF3. **B:** Densitometric analysis represents V5-mIRF3 level, normalized with GAPDH. N=3, $p< 0.05$ shows significant reduction. **C:** Reduction of steady state level of native mIRF3 with recombinant PVM NS proteins. IB represents the indicated protein levels.

2.4.5. NS proteins have no effect on mIRF7

The immediate signaling partner of IRF3 is IRF7, another member of interferon regulatory transcription factor. IRF7 phosphorylates and activates the transcription of virus-inducible cellular genes. It forms homo or heterodimer with activated IRF3 and then translocates to the nucleus to participate in the induction of type I IFN genes. To explore that PVM NS proteins can also reduce IRF7 expression as the proximal signaling components, such as RIG-I and IRF3, we have performed similar experimental procedures, where MEF cells were cotransfected with FLAG-IRF7 and FLAG-NS and subjected to IB analysis with the FLAG antibody. The molecular weight of mIRF7 is ~51 kD. Results show no change in the level of IRF7, thus it can be concluded that PVM NS proteins have no effect on mIRF7 level [Figure 11].
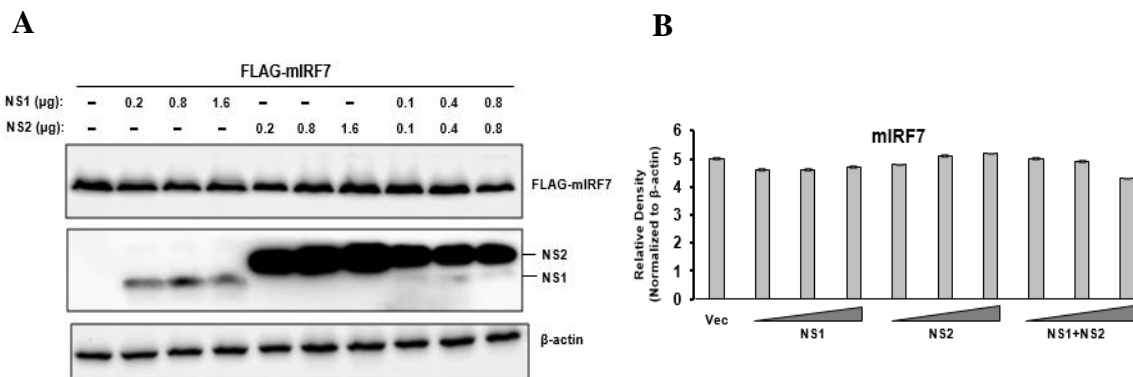
Figure 11: No effect on mIRF7 with PVM NS proteins. A. A dose dependent study with PVM NS proteins on recombinant mIRF7 shows no effect. A similar experimental procedure was followed as figure 7. **B.** The densitometric analysis also documents there is no significant reduction of mIRF7 with PVM NS proteins.

2.4.6. PVM NS proteins have no effect on the transcript level of mRIG-I, mIRF3 and mIRF7

Our previous observations demonstrate that PVM NS proteins can decrease the level of some components of the IFN induction pathway. Results show that the effect is on the protein level. To elucidate the effect of PVM NS on mRIG-I and mIRF3 transcription level, we have next measured the transcript level of these two genes with PVM NS proteins. We have also measured the transcript level of mIRF7 to determine if there is any change with recombinant NS proteins on it. MEF cells were transfected with FLAG-NS, FLAG/Myc mRIG-I, V5-mIRF3 and FLAG-mIRF7. RT-PCR analyses showed no significant changes in their mRNA level for mRIG-I, mIRF3, and mIRF7 [Figure 12A, B, C]. These observations also rule out the effect of PVM NS proteins at the RNA level of these different components of the induction pathway. Moreover, these observations also suggest different mechanisms of action for PVM NS proteins activity.
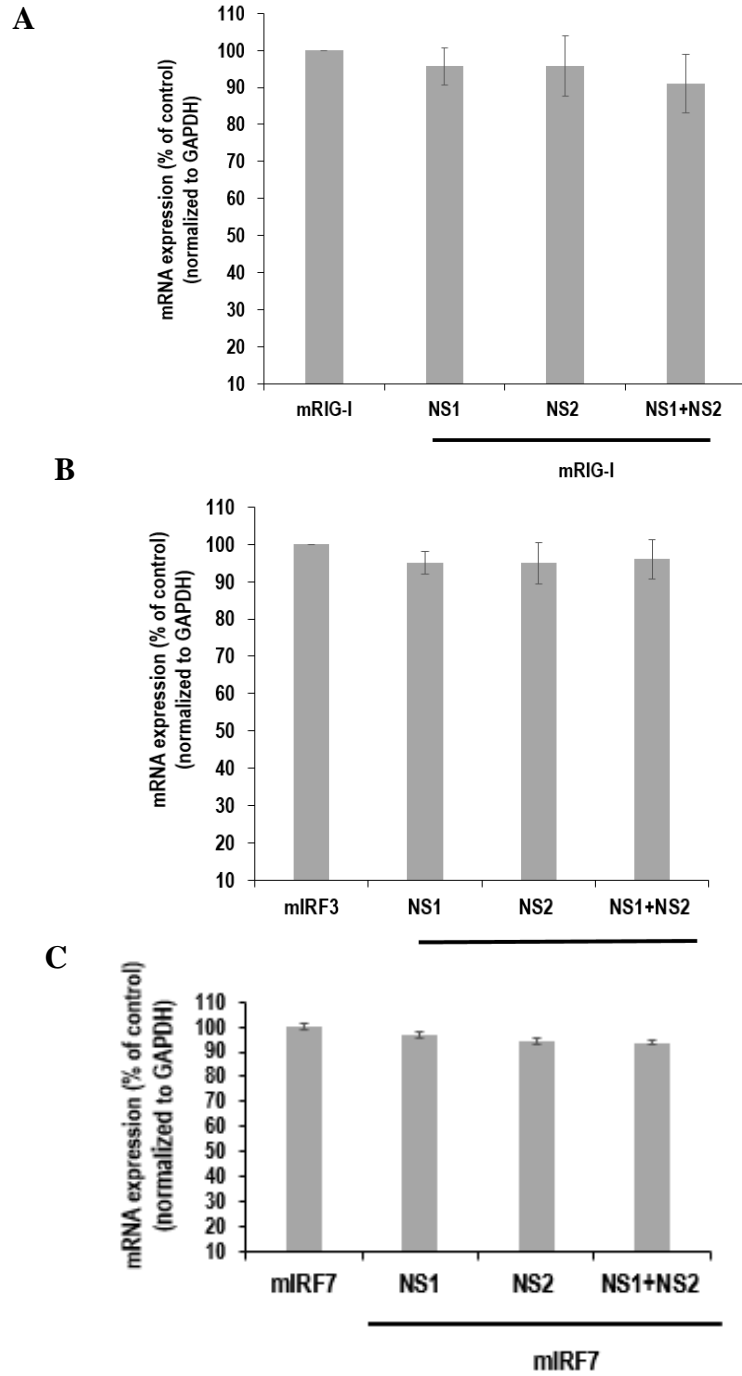
**Figure 12: Transcript levels of mRIGI-I, mIRF3 and mIRF7 are not effected by PVM
NS. A.** There is no effect of PVM NS on mRIG-I mRNA level. **B.** No change is seen on
mIRF3 level. **C.** mIRF7 level is not affected**.**

2.4.7. Reduction of steady-state level of mRIG-I and mIRF3 is proteasome-dependent

Reports showed that RSV NS proteins can degrade several components of the IFN pathway by proteasomal-mediated degradation [Ramaswamy, M. et al. 2006, Spann, K. M. 2004]. As documented in the above observations, PVM NS proteins are also responsible for the reduction of some components of the IFN induction pathway, such as mRIGI-I and mIRF3. Our next attempt was to focus on the mechanism of these reduced levels. We tested the role of proteasome in the reduction of mRIG-I and mIRF3 steady-state levels. MEF cells were co-transfected with recombinant FLAG-NS1 and NS2, empty pCAGGS vector, FLAG/Myc mRIG-I, and V5-mIRF3 in two different experiments, respectively, for mRIG-I and mIRF3. After 8 hrs of transfection, cells were treated with 10 µM of MG132 which is a proteasomal inhibitor. Cells were harvested after 24 hrs of transfection and mRIG-I and mIRF3 levels were measured with IB with specific antibodies for both [Figure 13 A, B]. Results show MG132 can restore back the amount of mRIG-I level [Figure 13A] and also for mIRF3 [Figure 13B]. As a proteasomal inhibitor, MG132 rescues the mRIG-I and mIRF3 levels, that establish that the mechanism of this rapid degradation of these two IFN induction pathway proteins, mRIG-I and mIRF3 by PVM NS proteins is proteasome-dependent.

Proteasomal degradation is ubiquitin mediated. Proteins which need to be degraded are tagged with small proteins ubiquitin for the degradation via proteasome. Subsequently, the ubiquitinated form of the proteins of interest must be detected if the degradation is proteasome-dependent. To delineate this possibility of the ubiquitinated form of both mRIG-I and mIRF3, pull down (IP) experiments for these two proteins were performed. In one experiment, MEF cells were co-transfected with recombinant NS proteins and mRIG-

I, and in another experiment with recombinant NS proteins and mIRF3. Cell were also treated with MG132 as before. After 24 hrs of transfection IP was performed using Myc antibody for mRIG-I followed by IB using the ubiquitin antibody [Figure 14A]. In another experiment, similar experimental procedures were followed where mIRF3 was pulled down using the V5 antibody and IB was done using the ubiquitin antibody [Figure 14B]. Figures 12A and B both display the ubiquitinated forms of mRIG-I and mIRF3, respectively. The slower migration form of both proteins appeared as a smear in IB analyses. We have also used one positive control for these IP experiments, that is TNF-α induced IkBα ubiquitination which is known to be ubiquitinated for proteasomal-dependent degradation [Figure 14C].
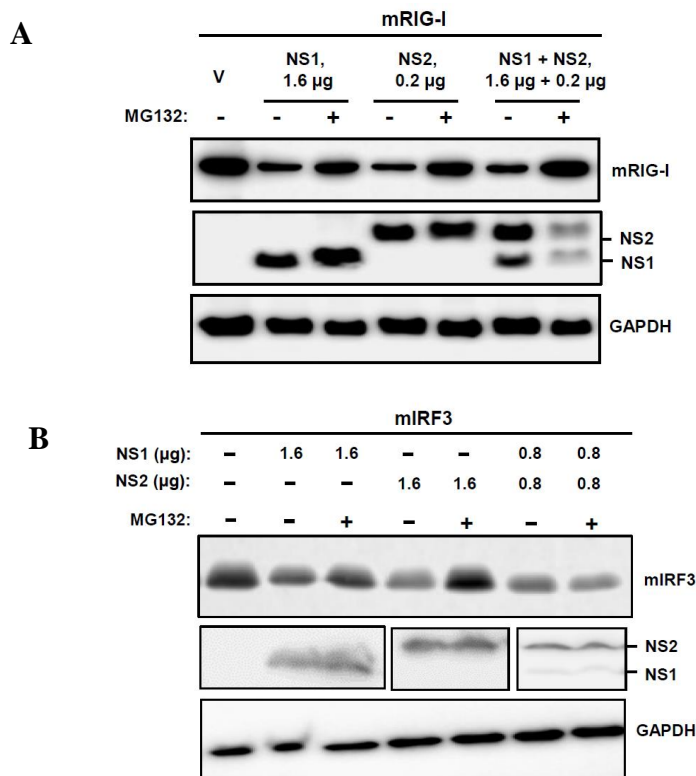
Figure 13: Reduction of steady-state level of mRIG-I and mIRF3 is proteasome-dependent. A. The steady-state level of mRIG-I is restored back in the presence of MG132. **B.** mIRF3 shows a similar effect, MG132 can rescued the amount of mIRF3.
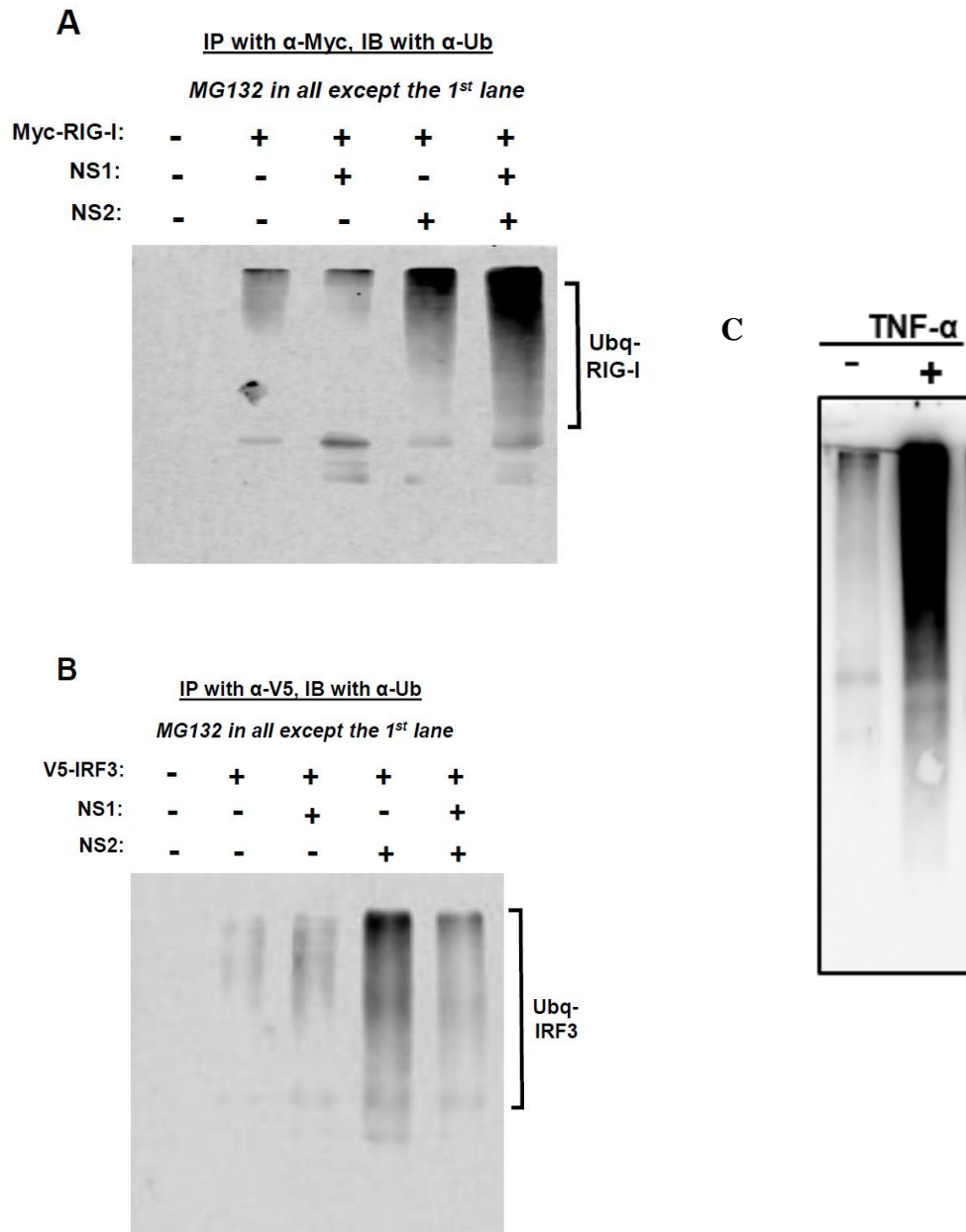
Figure 14: PVM NS proteins mediated ubiquitination of mRIG-I and mIRF3. A. Pull down with Myc antibody and IB with Ub antibody shows ubiquitinated form of mRIG-I. **B.** IP with V5 antibody and IB with Ub antibody shows ubiquitination of mIRF3. **C.** TNF-α mediated IkBα serves as a positive control.

2.4.8. PVM NS proteins can decrease the steady-state level of mSTAT2

After IFN induction, IFN signals through the JAK-STAT pathway and produces many ISGs. This pathway is known as the IFN response pathway. Our previous luciferase assay experiment demonstrates that PVM NS proteins can suppress the IFN response pathway. STAT2 is a key component of the IFN response pathway. Previous reports showed that STAT2 is the sole substrate of the IFN response pathway, which is targeted by RSV NS proteins [Elliott J. et al. 2007, Ramaswamy, M. et al. 2006, Lo MS. et al. 2005]. To determine the mechanism behind the suppression of the response pathway, we have tested the effect of PVM NS proteins on mSTAT2. A similar overexpression approach with recombinant plasmid experiment was performed. MEF cells were transfected with different amounts of FLAG-NS1, FLAG-NS2 and both NS1and NS2 with FLAG-mSTAT2 followed by IB after 24 hrs of transfection using FLAG antibody. Actin was used as a loading control. The molecular weight of mSTAT2 is 105 kD. Results from the above dose dependent study show that levels of mSTAT2 are lowered by both PVM NS1 and NS2 efficiently alone [Figure 15A, B] and also when added together [Figure 15C]. Densitometric analyses of the above results also show significant reductions of mSTAT2 levels by both PVM NS1 and NS2 proteins.

We could not test the native mSTAT2 level, as mSTAT2 antibody is not available commercially.
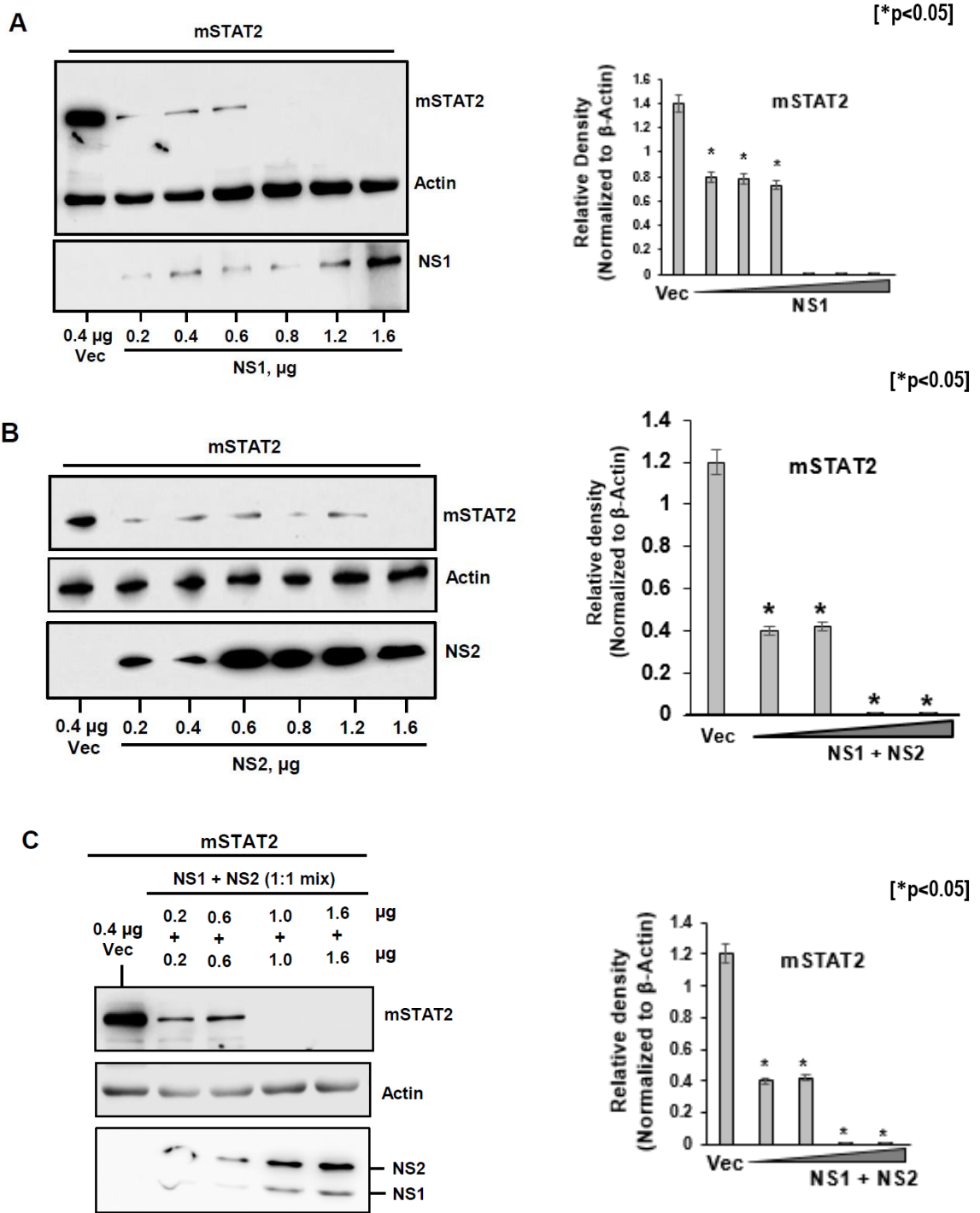
Figure 15: PVM NS proteins decrease the steady-state level of mSTAT2 efficiently.
A. PVM NS1, **B.** PVM NS2 and **C.** PVM NS1 and NS2 both efficiently decrease the level of mSTAT2. Densitometric analyses of A, B and C, where, N = 3 and $p<0.05$ is significant.

2.4.9. Transcript level of mSTAT2 remains unchanged

To rule out the effect of PVM NS on mRNA level of mSTAT2, we have tested the transcript level of mSTAT2. RT-PCR data showed that NS proteins cannot alter the RNA level. This observation also suggests a different mechanism for reduction of mSTAT2 by PVM NS.
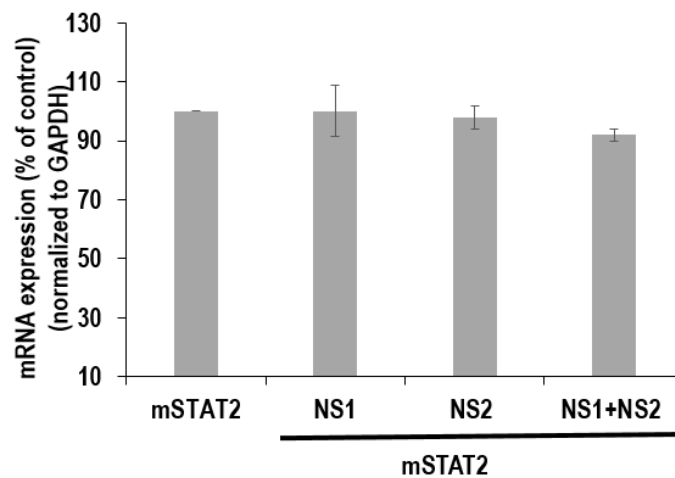
Figure 16: No effect on mRNA level of mSTAT2 by PVM NS. After normalization with GAPDH, there is no change on the RNA level of mSTAT2.

2.4.10. Reduction of mSTAT2 level by PVM NS proteins is proteasome-dependent followed by ubiquitination

RSV NS2 can degrade mSTAT2 in a proteasome-dependent way, and the degradation can be prevented by a proteasomal inhibitor [Ramaswamy, M. L. et al. 2006 (72). We have found that PVM NS proteins are also able to reduce the level of mSTAT2 very efficiently, so we tested it for proteasomal degradation. MEF cells were transfected as before and as a

proteasomal inhibitor MG132 was used. Observation from this experiment showed that MG132 can restore back the mSTAT2 level [Figure 17A]. To explore that the degradation is ubiquitin mediated, we have followed the similar experimental procedure as the IFN induction pathway components. MEF cells were cotransfected with FLAG-NS and FLAG-STAT2 and also treated with MG132. IP with FLAG antibody and IB with ubiquitin antibody were performed. The result showed that mSTAT2 degradation by PVM NS is proteasome-dependent, and the IB showed the ubiquitinated form of mSTAT2 [Figure 17B].
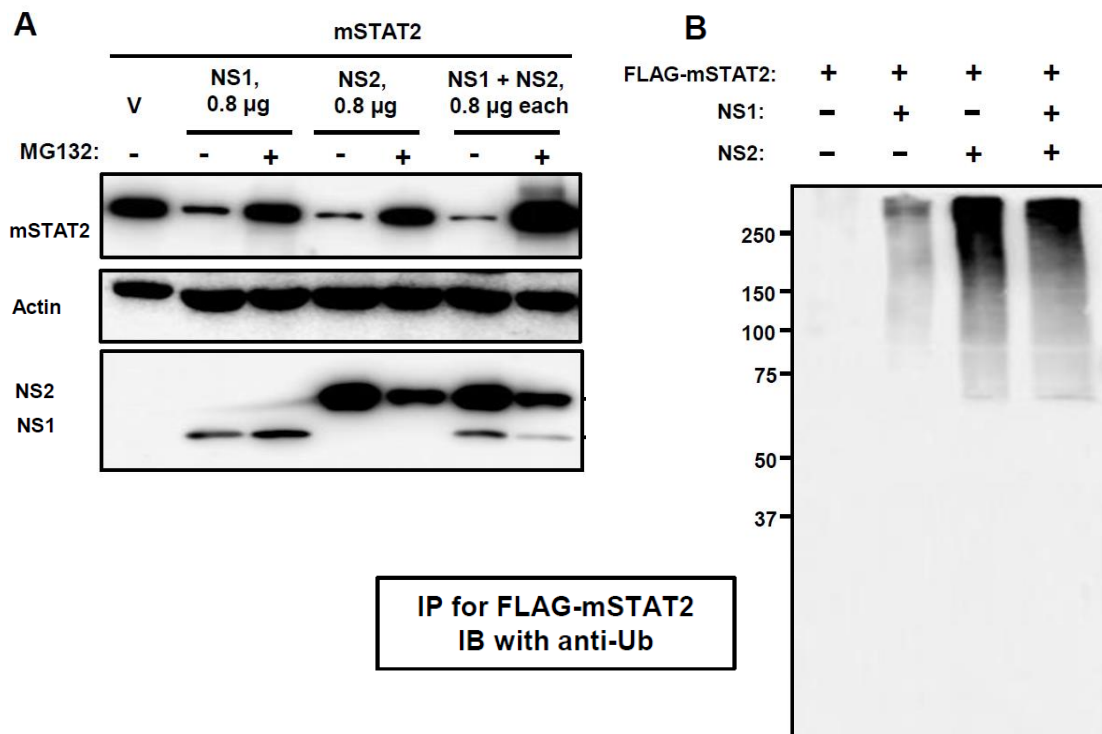
Figure 17: Ubiquitination and proteasome-dependent degradation of mSTAT2. A. MEF cells were cotranfected with FLAG-NS and FLAG-mSTAT2. MG132 treatment can restore mSTAT2 level. **B.** IP assay followed by IB shows ubiquitinated form of mSTAT2 protein.

2.4.11. The reduction of the steady-state level of the IFN pathway specific components with PVM infection

All the above experiments represent overexpression strategies of recombinant PVM NS proteins. To determine if these observations of our study can be replicated in PVM infected cells, we have performed some virus experiments. Here we have used three IFN pathway components as representative positive and negative targets of PVM NS. IRF3 and STAT2 were represented as positive targets from the IFN induction and the response pathway, respectively, and IRF7 was represented as a resistant target from the IFN induction pathway. MEF cells were transfected with these three IFN pathway components: V5-IRF3, FLAG-STAT2, FLAG-IRF7, in three different experiments. The cells were also infected with PVM at two different m.o.i.: 0.8 and 1.5. The cells were incubated for two different time points: 12 hrs and 24 hrs. Subsequently, cells were harvested and levels of different IFN pathway components were analyzed by IB with protein specific antibodies. Results showed that the levels of IRF3 and STAT2 are reduced in a dose-dependent and time-dependent treatment with PVM [Figure 18 A, B]. As expected, no change was seen in IRF7 level [Figure 18C]. GAPDH was used as a loading control. The growth of PVM was measured by antibody against PVM nucleocapsid (N) protein. These observations validate our previous findings with recombinant overexpression studies. Thus we can conclude that PVM can suppress the IFN pathway targeting different pathway specific proteins.
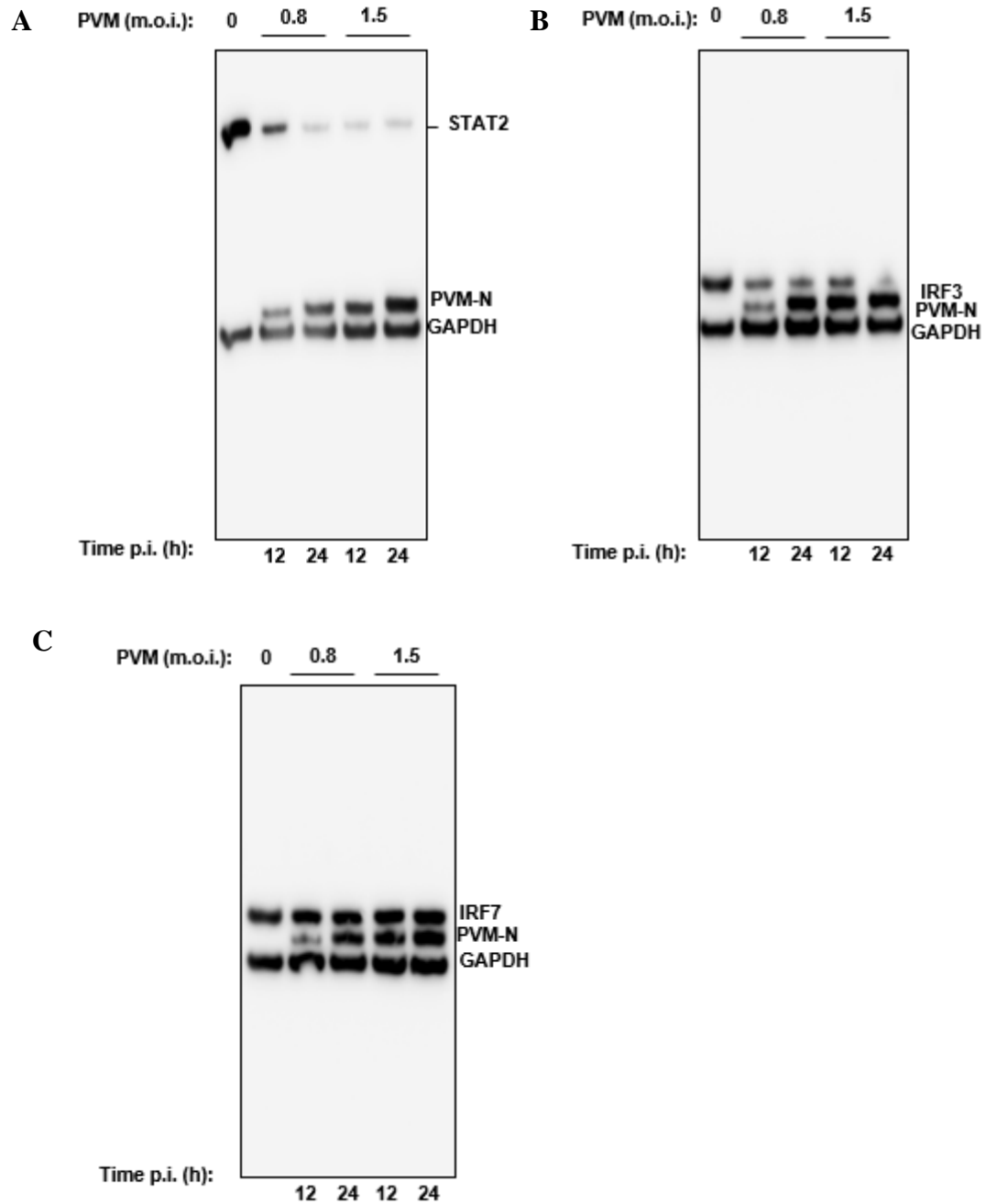
Figure 18: PVM infection lowers the level of IFN pathway proteins. A. PVM infection at different time points and at different m.o.i. reduce the mSTAT2 level. **B.** PVM infection also promotes loss of mIRF3. **C.** There is no effect on mIRF7. GAPDH is used as a loading control. Virus growth was measured by an antibody against PVM N protein. U= uninfected cell

2.4.12. Reduction of steady-state level of specific ISGs by PVM NS

The IFN signaling cascade concludes with the production of several ISGs. ISGs have different functions, some of them are known to have antiviral activities. Though it is still not known which ISG(s) has antiviral activity against PVM, Our second part of this manuscript is all about one ISG known as OASL. This OASL can inhibit RSV and counteract its antiviral activity, RSV NS proteins can also degrade OASL [Zhu, J. 2014, Dhar, J. 2015]. In an attempt to find out what PVM NS further does to circumvent the host immune system and complete shutdown of the defense mechanism of the host cells, we have assayed some of the available ISGs with PVM NS. These ISGs expressing cell lines are the kind gifts from Dr. J. T. Gao laboratory. The ISGs are Tetracycline induced, expressed in HEK 293 cell lines [Jiang, D. 2008]. These experiments allowed us to delineate the effect of PVM NS on these ISGs. The ISGs we have used in our experiments are all FLAG-tagged. ISG expressing cells were induced with Tetracycline and transfected with PVM NS as before. For proper selection, cells were also treated with two antibiotics, Blasticidin and Hygromycin. The steady-state levels of these ISGs were measured by IB using FLAG antibody. Results showed significant reduction in four ISGs: TRAFD1 (65kD), IFITM1 (14kD), ISG20 (20kD) and IDO (45kD) [Figure 19A, B, C, D]. We also, show here two substrates that were resistant to PVM NS treatment: IFIT3 (56kD) and Viperin (42kD) [Figure 19 E, F]. These observations explore the substrate repertoire of PVM in ISG family.
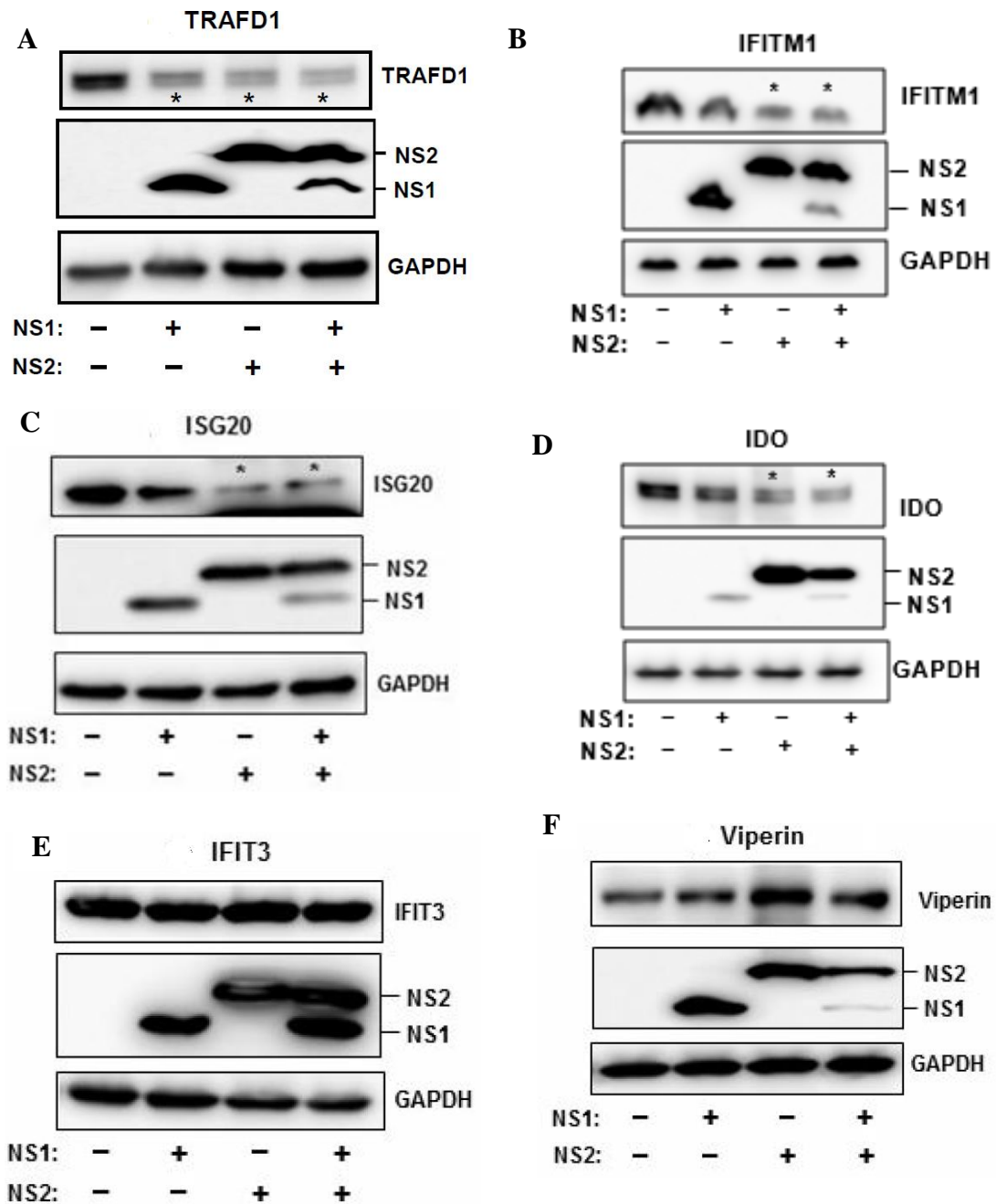
Figure 19: Reduction of levels of specific ISGs by PVM NS. Tet-induced ISGs are also targets of PVM NS. **A.** TRAFD1 level is reduced with NS1, NS2 and both NS1 and NS2. **B.** PVM NS1 seems more effective on IFITM1 than NS2. The molecular weight of IFITM1 is 14kD and it runs closely between NS1 and NS2, so NS bands are presented separately. **C.** Both for ISG20 and **D.** IDO PVM NS2 is more effective. **E.** IFIT3 and **F.** Viperin are the two representatives of resistant substrates for PVM NS.

2.4.13. NS proteins can interact with IFN pathway proteins

The above observations demonstrate that mRIG-I, mIRF3 and mSTAT2 are the targets of PVM NS to suppress the IFN pathway. Previous studies showed that NS proteins of RSV can co-localize with the target substrates for their degradation [Boyapalle, S. et al. 2012, Swedan S. et al. 2011, Goswami, R. et al. 2013]. To determine that PVM NS proteins can truly interact with the IFN pathway components destined them for degradation, we performed co-localization studies. We have tested colocalization of two positive substrates mRIG-I and mIRF3 with NS proteins, as an antibody for native mSTAT2 is not available. MEF cells were cotransfected with V5-mIRF3 and FLAG-tagged PVM NS for one experiment. In another experiment, interaction was measured with native mRIG-I and FLAG-tagged PVM NS. To induce native RIG-I, we have used RSV at m.o.i. 2. Cells were processed for immunofluorescence and confocal microscopy. MG132 was added to these experiments to stabilize the degradation complex. Observations obtained from these experiments demonstrate that both mRIG-I and mIRF3 co-localize in a perinuclear position in the cytoplasm with PVM NS proteins [Figure 20A, B].

To validate these results we have performed another independent Co-IP experiment. MEF cells were transfected with V5-mIRF3 and PVM NS and treated with MG132. IRF3 was pulled down with V5 antibody and that was followed by IB. FLAG-tagged NS1 was detected in IB [Figure 20C], which demonstrates the interaction of NS proteins with IRF3. Apart from the above result, this IB also showed two dark upper and lower bands. Those bands are heavy and light chain bands for V5 antibody, which was used for IP. Together,

these two experiments showed the direct interaction of PVM NS with the IFN pathway substrates.
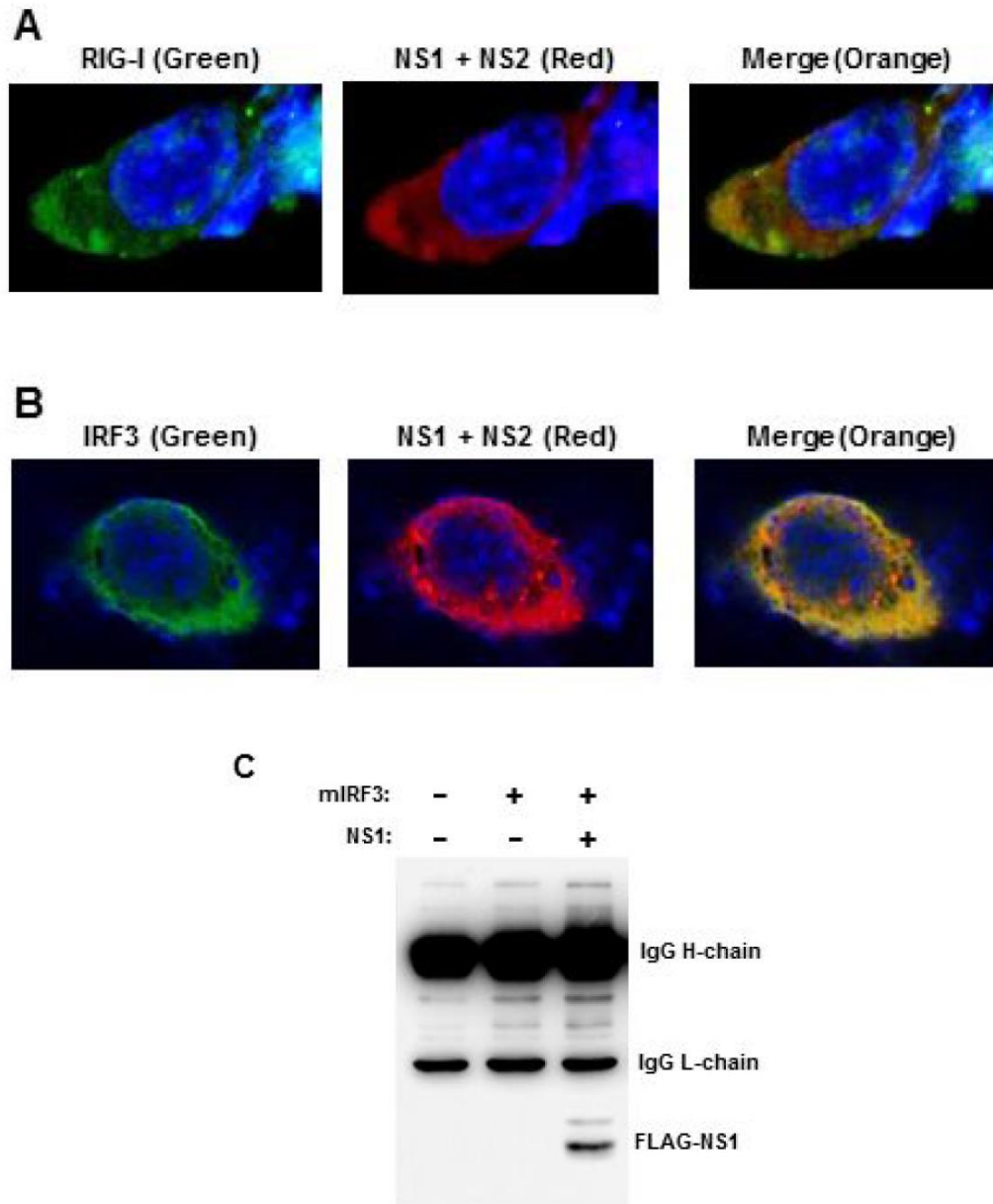
Figure 20: Co-localizations of NS proteins with mRIG-I and mIRF3. A. mRIG-I (green) colocalizes with PVM NS (red); the merged panel shows the orange color. **B.** mIRF3 (green) co-localizes with PVM NS (red) indicated by the orange color. DAPI (blue) was used for nuclear staining. **C.** IP of V5-mIRF3 can detect FLAG-NS1 in IB.

2.4.14. PVM NS proteins have no effect on other signaling pathway proteins

PVM NS proteins are IFN antagonists and can alter the steady state level of the IFN pathway specific proteins, leading them to proteasomal-mediated degradation. To determine whether NS proteins of PVM have any effect on other signaling molecules from different pathways, we have tested a few of their representatives. We have tested AKT, CIITA and S6 kinase. AKT is a serine/threonine kinase and plays an essential role in glucose metabolism, apoptosis, cell proliferation and migration. CIITA is a transcriptional co-activator that encodes class II Major Histocompatibility Complex (MHC). Ribosomal S6 kinase is a signal transduction protein kinase activated by MAPK/ERK pathway.

To test the effect of PVM NS on these proteins, MEF cells were transfected with PVM NS1 and NS2 as before. Cell lysates were collected and subjected to SDS-PAGE followed by WB, probed with different protein specific antibodies. The molecular weights of AKT is 62 kD, CIITA is 97 kD and S6K is 65 kD. Results [Figure 21] showed from the above experiment that there is no effect of PVM NS proteins on these signaling pathway proteins which are not related to IFN signaling. GAPDH was used as a loading control.
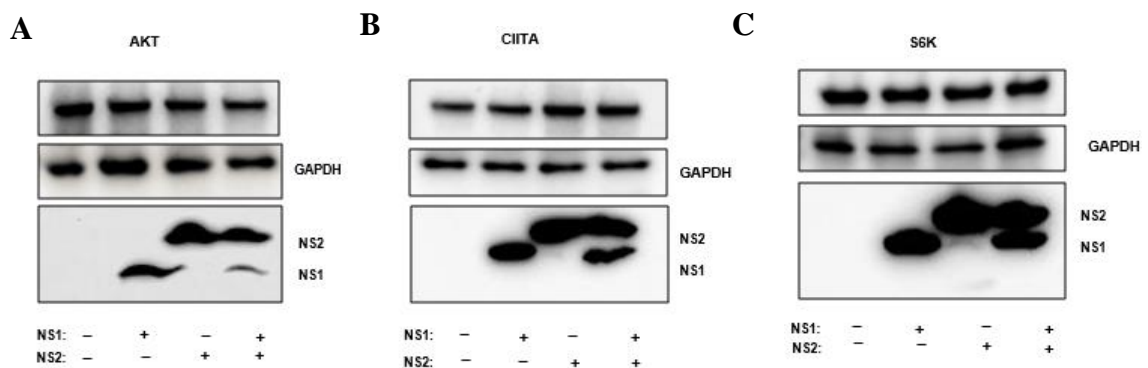
Figure 21: Effect of PVM NS on different signaling pathway components. After normalized with GAPDH, PVM NS proteins have no effect on AKT, CIITA and S6K.

2.4.15. PVM P protein

PVM NS proteins selectively decrease different components of the type I IFN pathway. To confirm the specificity of NS protein's functions on IFN pathway components we have tested another structural protein of PVM; that is PVM P protein. We have commercially cloned it in pCAGGS vector and it is FLAG-tagged. We have tested the effect of this PVM-P protein on different representative substrates, such as RIGI, IRF3, and TRAFD1 of type I IFN pathway specific proteins. MEF cells were cotransfected with FLAG-tagged PVM-P and FLAG-RIGI, V5-IRF3. Tet-induced HEK293 cells were plated for TRAFD1 experiment. Transfection was done as before by using FLAG-TRAFD1 and FLAG-PVM-P. Cell lysates were collected and subjected them to SDS-PAGE followed by IB with substrate specific antibodies. The molecular weight of PVM-P protein is 43kD. Observation [Figure 22] showed that PVM-P has no effect on IRF3 and TRAFD1. Although for RIG-I it shows an insignificant decrease of its steady state level and that is consistent for two experiments.
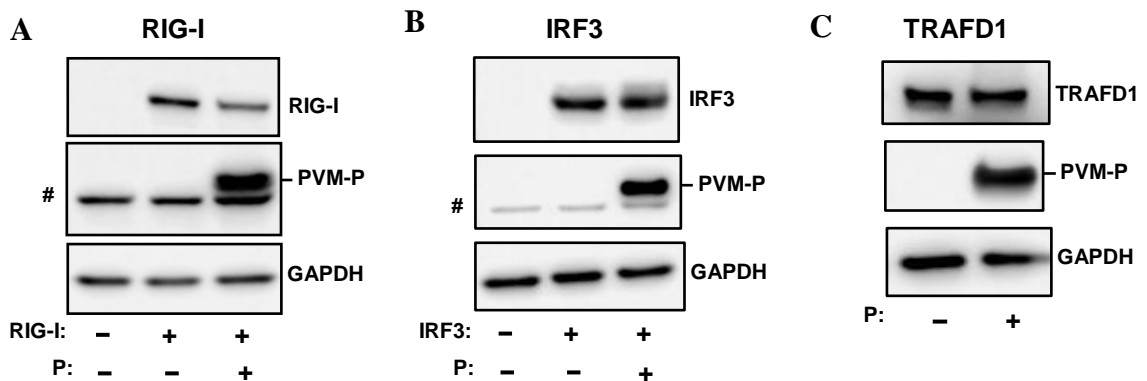
Figure 22: Effect of PVM-P protein on different representative substrates of IFN pathway components. There is no effect of PVM-P on IRF3 (B) and TRAFD1 (C). A little but insignificant effect is seen on RIG-I. # stands for a nonspecific band just below P, which is only seen in MEF cell lines but not in HEK293 cells.

Supplement Data

2.4.16. PVM NS C-terminal deletions mutants functionally active as the wild type NS

Both NS proteins of RSV have C-terminal tetra-peptide DLNP sequences. Studies showed that C-terminal 10 amino acid sequences including the DLNP region are important for the degradation property of RSV NS and suppression of IFN [Swedan S. et al. 2009]. Although our study demonstrates that there is no homology between the two NS sequences of PVM and RSV [Figure 6], we have still tried to determine the significance of C-terminal amino acids for PVM to show functional similarity with RSV. We have commercially made three deletions mutants for both PVM NS1 and NS2: 5, 10 and 15 amino acids, respectively, from the C-terminus end. We have tested the expression of these mutants; they were well expressed. The only exception is NS1Δ10 mutant; in all the experiments, the levels of expressions for this specific mutant are constantly less than other mutants. Then we have tested their functional ability to degrade the IFN pathway components: mRIG-I, mIRF3 and mSTAT2. MEF cells were transfected with different deletions mutants and subjected to IB. The observations showed that these deletions of NS are as active as the wild type [Figure 23]. We can conclude from the above observations that these C-terminal amino acids sequences have no specific effect on the degradation properties of both PVM NS proteins.
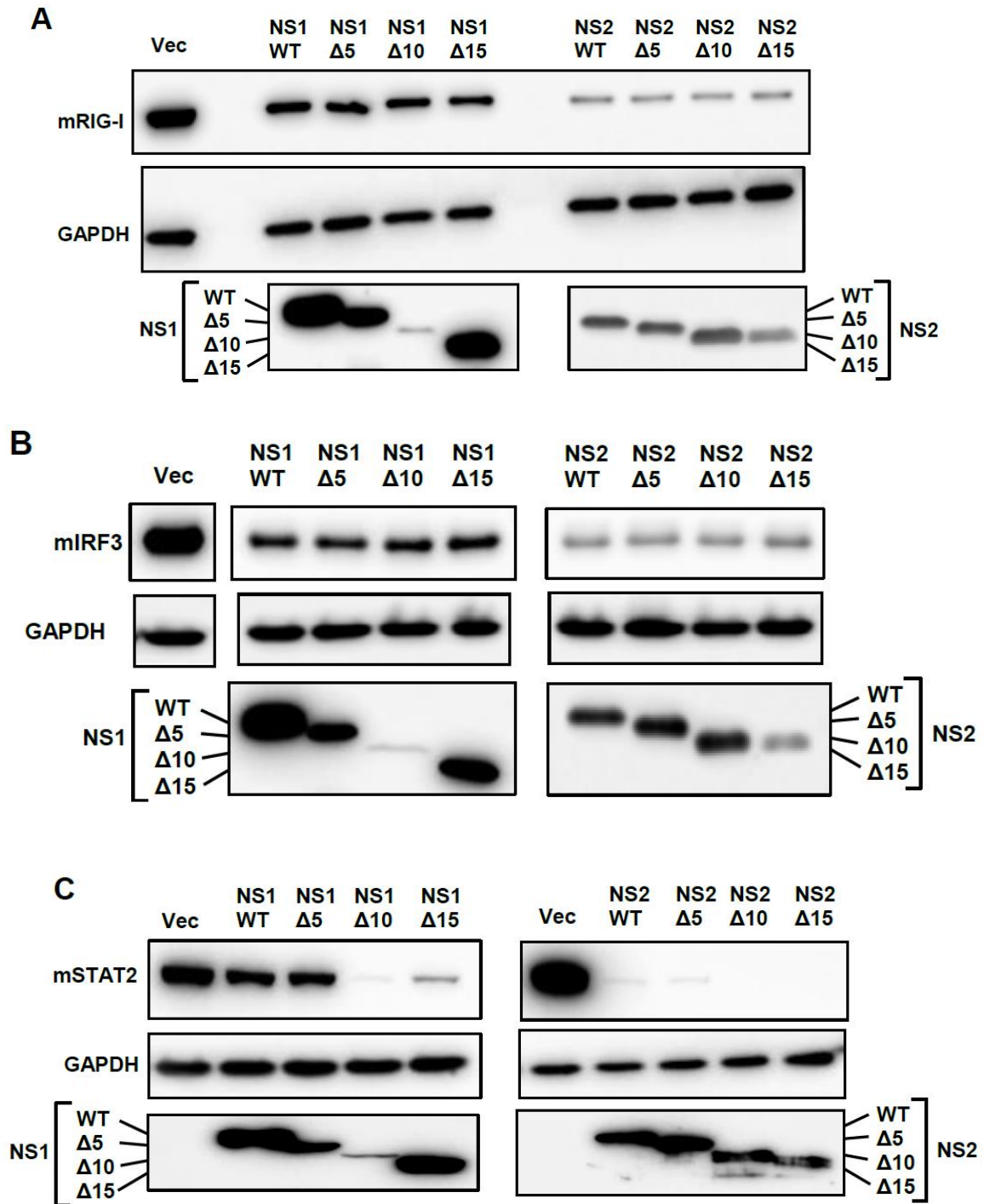
Figure 23: The effect of PVM NS C-terminal deletion mutants on IFN pathway substrates. A. The C-terminal deletions of 5, 10, and 15 amino acids of NS1 and NS2 can decrease mRIG-I. **B.** Deletions are as effective as the wild type for mIRF3. **C.** mSTAT2 level is also decreased with PVM NS deletions. GAPDH was used as a loading control.

2.4.17. Cysteine mutants of NS proteins

Ubiquitination followed by proteasomal degradation required a chain of enzymatic reactions. It includes three enzymes: E1-activating enzyme, E2-conjugating enzyme and E3-ligating enzyme. The ECS (Elongin C–Cullin–SOCS box)-type E3 ubiquitin ligases interact with elongin C through BC box. This interaction helps to recruit and assemble the multisubunit complex and interact with E2 enzyme and attaches the ubiquitin chains to the substrate that followed the proteasomal degradation. Studies have shown RSV NS1 has the potential to acts as E3 ligase. RSV NS1 has the consensus binding sequences that can assemble Elongin C and Cullin 2 [Elliot J. et al. 2007]. The deletion studies of PVM NS have shown that they are as active as the wild type, so we next focused whether PVM NS has any potential ubiquitin E3 ligase motifs [Figure 24A]. We have tried to search for the potential BC box by mutating all available Cysteine residues for PVM NS1. We have followed the procedure from Site Directed Mutagenesis Kit-Agilent, and mutated 3 of these Cysteines to Alanine; Cys40, Cys57, and Cys104 [Figure 22B]. There is also another Cysteine at position 3; we have ignored it because of its close proximity to the N-terminus end. MEF cells were cotransfected with these three mutants and mRIG-I, mIRF3, and mSTAT2 in three different experiments followed by IB. The degradative activity of these Cys→Ala mutants are also as active as the wild type NS [Figure 25A]. We have also tried to mutate one Cysteine to Alanine for PVM NS2; that is Cys 137 because of its location similarity with RSV NS2 BC box [Figure 6]. A similar experimental procedure was followed as above for the three substrates. This Cys 137→Ala mutant [Figure 25B] also shows no defect on the degradative property of PVM NS2 [Figure 25B]. Our experiments

for the search of potential BC box Cysteine turned out negative, which also supports the recent advances in finding the structural motif that is responsible for the proteasomal degradation of the IFN pathway components for other *Pneumovirus* RSV [Whelan, J. N., et al. 2016]. We conclude that PVM NS proteins have unique proteasome recruitment mechanisms of degradations of the IFN pathway components.

A

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ASB-2        PLAHLCR601        PVM-NS1-1   TILRGARVCNTWQD45
E1B55K       ALRPDCKYKI187     PVM-NS1-2   SVYLVGRDCNLLRP63
SOCS3        NVATLQHLCRKTVN198 PVM-NS1-3  TEPPIDPTCELLVE109
SOCS2        SAPSLQHLCRLTIN171 PVM-NS2-1  INWNFIRICNDRLK78
SOCS1        RVRPLQELCRQRIV183 PVM-NS2-2  EADSIEWKCIRRQE115
VHL          PVYTLKERCLQVVR167 PVM-NS2-3  QHKEDWIECTGCIP142
Vif          QVRSLQYLALTVYT157 PVM-NS2-4  EDWIECTGCIPYPG145
E4Orf6       SPGSLQCIAGGQVL141
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B

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PVM-NS1      MGANVMMELDYGGRAAWLAFHITNFDRSDLETILRGARVCNTWQDQRLSVYLV 53
PVM-NS1      GRDCNLLRPFVQAAKFIHNTRRGQTLTHWFTKNIVFSSTGQETEP 98
PVM-NS1      PIDPTCELLVELISG---- 113

PVM-NS2      MSTAMNKFTQTISKPATILNISDSEESGDEAGVGKVSRTTQSSERWLDLLIEKFQPSLQN 60
PVM-NS2      ITRYINWNFIRICNDRLKKEKMGYIEAKQYVEDMAWMVIASEADSIEWKCIRRQEKVTGV 120
PVM-NS2      KYPKFFFVQHKEDWIECTGCIPYPGHDLIYDEDDDD 156
```

Figure 24: BC box sequences. A. Known and potential BC box sequences for other viral proteins. **B.** Cys 40, Cys 57, and Cys 104 residues are highlighted for PVM NS1 and Cys 137 for PVM NS2.
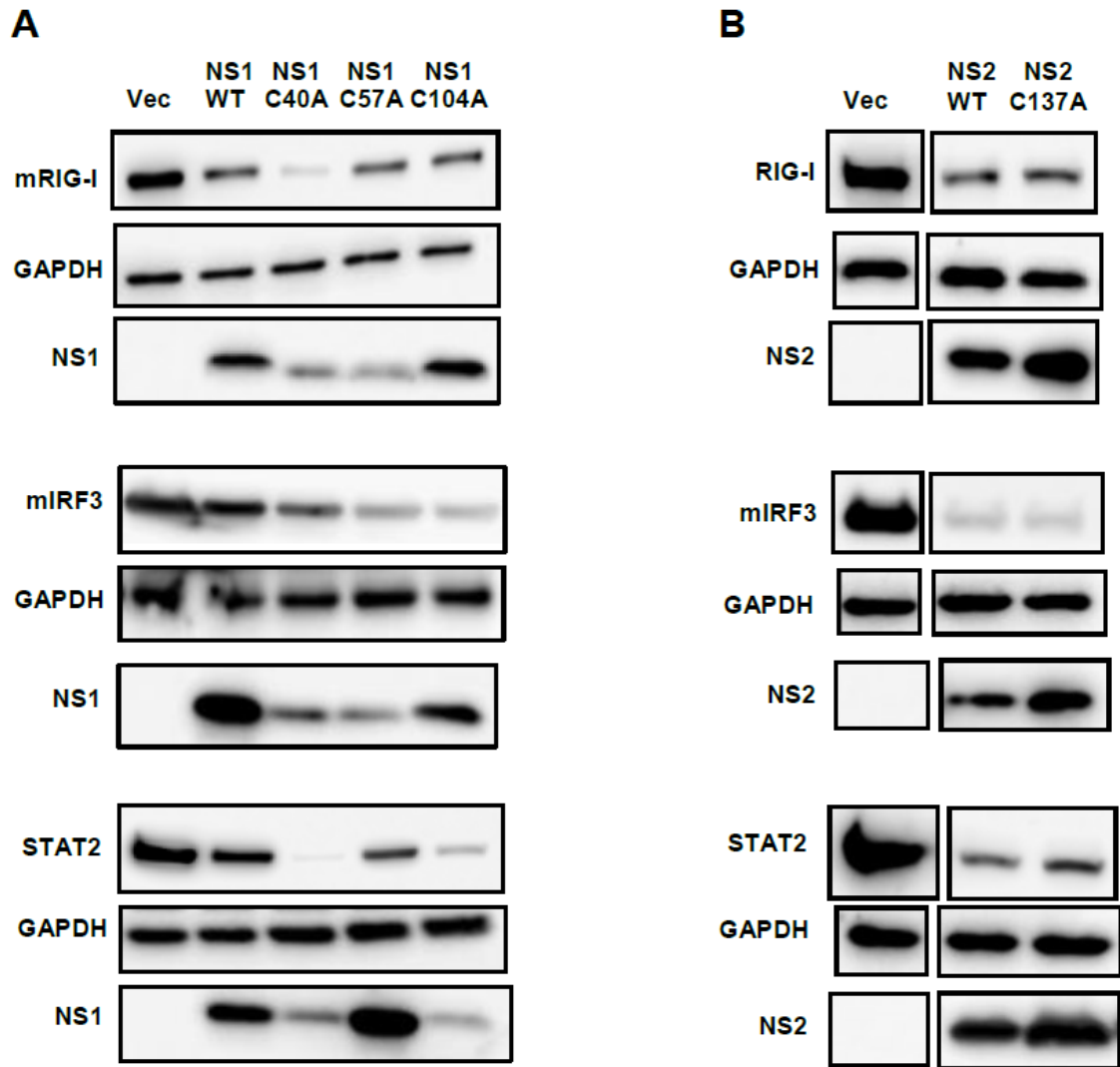
Figure 25: PVM NS Cys mutants are as effective as wild type NS. A. Cys 40, Cys 57 and Cys 104 mutants to Alanine of PVM NS1 can decrease the steady-state level of mRIG-I, mIRF3 and mSTAT2. **B.** PVM NS2 Cys137 to Ala mutant also can decrease the level of mRIG-I, mIRF3 and mSTAT2.

2.4.18. PVM NS proteins can decrease the steady-state level of human IFN pathway components

We have mentioned before that PVM causes the similar respiratory illnesses in mice as another *Pneumovirus* RSV does in human. Studies showed that PVM has the potential to act as a future human pathogen [Brock, L. G. et al. 2012]. However, there is no definite evidence for that yet. But the functional similarities between RSV and PVM NS make the prediction more relevant. Studies showed that RSV NS can degrade specific components of the IFN pathway from human origin, where this study shows that PVM NS are also functionally similar and degrade a few components of the IFN pathway from mouse origin. Next, to delineate if these mouse PVM virus NS proteins have any role on the human homolog of IFN pathway components, we have tried to test the effect of PVM NS proteins on human RIG-I, IRF3, IRF7, and STAT2. Human A549 cells were cotransfected with recombinant FLAG-PVM NS and FLAG-hRIG-I, V5-hIRF3, V5-hIRF7 and FLAG-hSTAT2 in four different experiments. Lysates were subjected to IB with specific antibodies. Observations from the above experiments showed that PVM NS also are able to decrease the human homologs of the IFN pathway components. But the targets are different [Figure 24]. PVM NS can degrade mRIG-I, mIRF3 from the IFN induction pathway and mSTAT2 from the IFN response pathway. mIRF7 is unaffected by PVM NS. But these results show that for human homologs, the targets are hIRF3 and hIRF7 from the IFN induction pathway and STAT2 as the universal target for both human and mouse from the response pathway. There is no effect on hRIG-I by PVM NS [Figure 24].
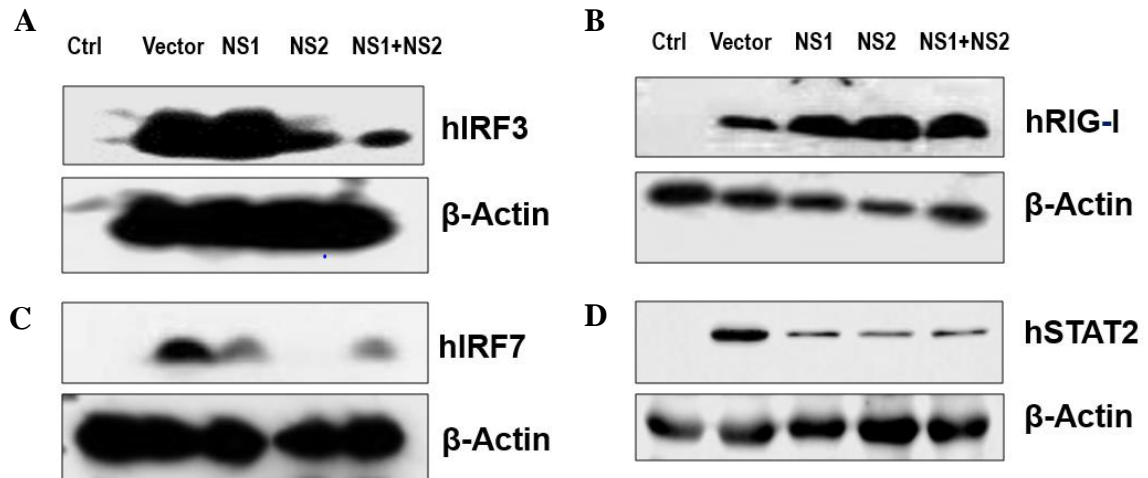
Figure 26: Effect of PVM NS on human homologs of the IFN pathway components.
A. PVM NS2 can decrease the level of hIRF3. **B.** No effect is seen on hRIG-I. **C.** Both NS1 and NS2 are able to decrease the level of hIRF7. **D.** hSTAT2 is affected by both PVM NS proteins.

2.5. Discussion

PVM infection in mice suppresses IFN signaling causing respiratory illness. It allows viruses to circumvent the host immune response and successfully replicate inside the host cells. This literature establishes that two nonstructural proteins of PVM, those are known to be IFN antagonists, are primarily responsible for this suppression. However, the exact mechanism of this type I IFN suppression by PVM NS is unknown. The NS sequence alignment study of two Pneumoviruses, RSV and PVM, reveals that there is no sequence homology between the two NS proteins of these two viruses. We also considered the possibility that the higher order structure of the NS proteins may show some similarity. We note that structures of neither RSV nor PVM NS proteins have been experimentally determined. Moreover, the NS proteins do not exhibit any similarity to any known sequence (in the GenBank protein sequence database) in biology, and thus homology-based structure modeling was also not possible. We, therefore, used several bioinformatic structure prediction tools that are publicly available, such as HHPred and JPred. However, these predicted structures did not reveal any appreciable similarity at the secondary structural level. That makes our study more rational. Instead of these dissimilarities in their structures, our study reveals that the NS proteins of both the viruses are functionally homologous. This is the first report where we are showing PVM NS proteins can suppress IFN pathway specific components to circumvent the host immune system. This dissertation explores the cytoplasmic cell signaling components of both induction and response pathways of the type I IFN signaling that are targeted by PVM NS proteins. The first evidence of PVM NS proteins targeting the specific ISGs, which is the distal arm of the

type I IFN signaling, is revealed by our study. However, the specific antiviral ISGs that can inhibit PVM is currently not known. There are hundreds of ISGs produced from IFN signaling. It will be always interesting to delineate the mechanism of action in detail. This dissertation aims to clarify the IFN signaling components that are targeted by PVM NS proteins and the substrate specificity of NS action.

To summarize the major accomplishments of this dissertation, the first thing we have shown is that PVM NS proteins are IFN antagonists. Though many other reports have shown similar things, but our effort to study the details of the IFN pathway in relation with PVM recombinant NS plasmids is unique. The full length gene clones of both NS proteins of PVM were made and these recombinant tagged NS were well expressed with transient transfection. The cell lines we have used for most of the experiments were MEF cell lines, which produce a similar phenotype as in PVM natural infection. We have also used human A549 cells that are known to induce a positive feedback loop of the type I IFN signaling. We have shown PVM NS can target both the induction and response pathway components. RIG-I and IRF3 are degraded by both NS proteins, where IRF7 is totally unaffected in IFN induction pathway. STAT2 becomes the sole target of the IFN response pathway and efficiently degraded by both NS. We have seen similar mechanism of action for RSV NS proteins targeting different component of the IFN pathways. RSV NS were found to form homo and hetero dimers, for PVM NS it is not known. But PVM NS induces proteasomal-mediated degradation of these target components of IFN pathways at different extents. Proteasomal-mediated degradation is always preceded by ubiquitination of the substrates. IP analyses by pulling down specific substrates with substrate specific antibodies identified ubiquitinated higher migrated forms of RIG-I, IRF3, and STAT2. Our study with native

RIG-I or IRF3 also validate these findings showing similar reproducible results as recombinants. We cannot study the native STAT2 in this respect because of the absence of reliable and a specific cellular antibody against native STAT2. Apart from that, to establish the credibility of our studies, the actual PVM virus experiments were performed for validations. The reproducible decreased levels of specific representatives of the IFN pathway components with virus treatment authenticate and confirm our previous observations. In all our experiments, NS2 expression level was always higher than the same amount of NS1.

The distal arm of IFN pathway is the production of several hundreds of ISGs from which some are known to have antiviral activities. Recent reports, including ours, have shown the antiviral activities of OASL and Viperin against RSV. It is reasonable to predict that NS proteins of viruses also can counteract the antiviral activities by targeting different members of the ISG family. For example, RSV NS1 can degrade OASL, allowing effective viral growth and replication inside host cells. We tested a few of the available Tet-induced ISGs for PVM. Both PVM NS proteins can reduce TRAFD1 level; for IFITM1, NS2 is effective to decrease the level more efficiently than NS1. For ISG20, both the NS proteins effectively reduce the level. IDO level is reduced by PVM NS2 efficiently than NS1. There are a few hundreds of ISGs available (exact no is not known). Our study showed that to establish complete shutdown of the immune response, NS proteins not only target the induction and response pathway components but also the IFN induced ISGs. We also found a few other ISG that are not targeted by PVM NS proteins, such as Viperin, IFIT3, NN-1, PKR-M, RIG-B, USP18, IFITM2, NNMT. Exploring the full repertoire of ISGs that have antiviral functions against Pneumoviruses and by NS proteins to counteract the antiviral

activities will help further to clear the whole picture of the infection mechanism of Pneumoviruses for allowing optimal viral growth and resulting pathogenesis.

Substrate specific degradation of IFN pathway components indicates direct interaction and colocalization of NS proteins with RIG-I, IRF3, and STAT2. Our studies with two representatives of IFN pathway components (IRF3 and RIG-I) showed that they colocalizes with PVM NS proteins in cellular cytoplasm with a perinuclear preference. That can explain the mechanism of substrate specific degradation by NS. In RSV, NS proteins can form homo and heterodimers and targeting specific substrate. For PVM it is not known. But our results demonstrate that PVM NS proteins targets different substrate to different extents. RIG-I, STAT2, TRAFD1 are degraded by both PVM NS efficiently, whereas ISG20, IDO, IFITM1 are targeted by PVM NS2. For IRF3, NS2 is more efficient. It appears that NS1 can inhibit NS2 function when added together, indicating the formation of heterodimer between NS1 and NS2. The stoichiometry of forming the NS1 and NS2 heterodimer depends on the amount of protein expressions that sequestering the effect of NS2 when added together.   We have shown RSV and PVM NS proteins are structurally dissimilar but functionally homologous. Our laboratory has shown C-terminal DLNP domain of RSV NS which is common for both RSV NS1 and NS2. In a search for the functional domain of PVM NS that is responsible for this degradation, we deleted 5, 10, and 15 amino acids from the C-terminal of both NS1 and NS2 of PVM. Our attempt to locate the specific domain failed as these deletions are also as effective as the wild type NS proteins. The expression level of PVM NS1Δ10 deletion mutant was always less. The reason is unknown, but we can predict that it maybe these specific 5 amino acids change the folding or secondary or tertiary structure of the NS1 that makes it unstable. Our

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conclusion from these above observations is these C-terminal amino acids domains are not important for the degradation property of NS proteins.

The ubiquitination system followed by proteasomal degradation is a common phenomenon for degradation of the cellular substrates of *Pneumovirus* genus. Reports documented for RSV and our study also prove the authentication for PVM. The ubiquitination process requires three enzymes: E1-activating enzyme, E2-conjugating enzyme and E3-ligating enzyme. A previous report showed that RSV NS1 can be a potential E3 ligase which acts in ubiquitin mediated proteasome-dependent degradation of STAT2 [Elliott J. et al. 2007]. There are many types of ubiquitin ligases. One of them is canonical cellular Elongin-Cullin-SOCS box ubiquitin ligase. This ligase is composed of Elongin B (EloB), Elongin C (EloC), Cullin 5 (Cul5), Rbx2, and a SOCS box protein. The recruitment of these complex initiates binding of E3 ligase with the substrate followed by proteasomal mediated degradation. This SOCS box has a BC box consensus sequence that recognizes and binds to EloB, EloC (EloBC) and the Cullin box with Cul5 or Cul2. There are many paralogs available for Cullin box. One report showed that RSV NS1 has a consensus BC box sequence. It can recruit EloC, Cul2, and an Rbx subunit. This proposed SOCS box motif predicted is important for the degradation of STAT2 for the RSV NS1 [Elliott J. et al. 2007]. Although the dissimilarities between the NS of RSV and PVM are intriguing, the functional homology to suppress the IFN signaling between these two viruses led us to investigate a potential BC box in PVM NS proteins which may be important for the proteasomal degradation of IFN pathway components by PVM NS. Though deletions mutants of C-terminal amino acids are also effective in the degradation of the IFN pathway components for PVM NS, we tested specific Cysteine residues for both

NS1 and NS2 for PVM, because Cysteine is the most invariant amino acid and ubiquitin molecule that catalytically attaches through Cysteine via a thioester bond. We mutated all the available Cysteines for PVM NS1 (Cys 40, Cys 57, and Cys 104), except that N-terminal first Cysteine because of its position. Our search for any specific Cysteine residue is not effective for the degradation property of PVM NS1. We have also tried to mutate one specific Cysteine from NS2, Cys 137, because of its distant similarity with the BC box domain of RSV NS2.This Cys137 mutant of PVM NS is also found to be effective for the degradation. Our effort to search for a functional motif with mutations of the Cysteine residues was ineffective though it indicates different mechanism of degradation for PVM NS. That also signifies the importance of our study. Two Pneumoviruses, RSV and PVM, have commonalities between them for being from the same genus, but no similarities between their NS sequences. They are functionally similar, but follow different mechanism to circumvent the host immune system. Studies reported that not only Cysteine but also other amino acids such as Alanine and Serine can be a major part of the uncanonical BC box motif. A nonstructural protein of HIV-1, Vif, promotes ubiquitin mediated degradation of antiviral proteins. It is able to assemble the EloBC-Cul-Rbx E3 ligase complex like RSV NS1 BC box. Noteworthy here is that Alanine plays the similar role as Cysteine in RSV NS1 with differences in Cullin box and Rbx binding motifs. The crystal structure of Vif protein identifies the BC box with alanine in the middle that forms the multisubunit structure and acts as E3 ligase [Yu, Y. et al. 2004]. This can be true for PVM NS proteins also. Our prediction is the presence of such new motif apart from the canonical Cysteine residue may be true for PVM NS proteins mediated degradation. So, our conclusion is PVM NS can be a potential E3 ligase with a unique BC box motif or can help to assemble

the components needed for ubiquitin mediated proteasomal degradation. Figure 27 A, B is the schematic representation of our proposed model. A detailed analysis is needed of the structural complexity of the NS proteins to find such noncanonical motif responsible for the degradation property of PVM. Another possibility is to locate the specific common domain of the target substrates of PVM NS proteins that may be important for the specificity of NS target.
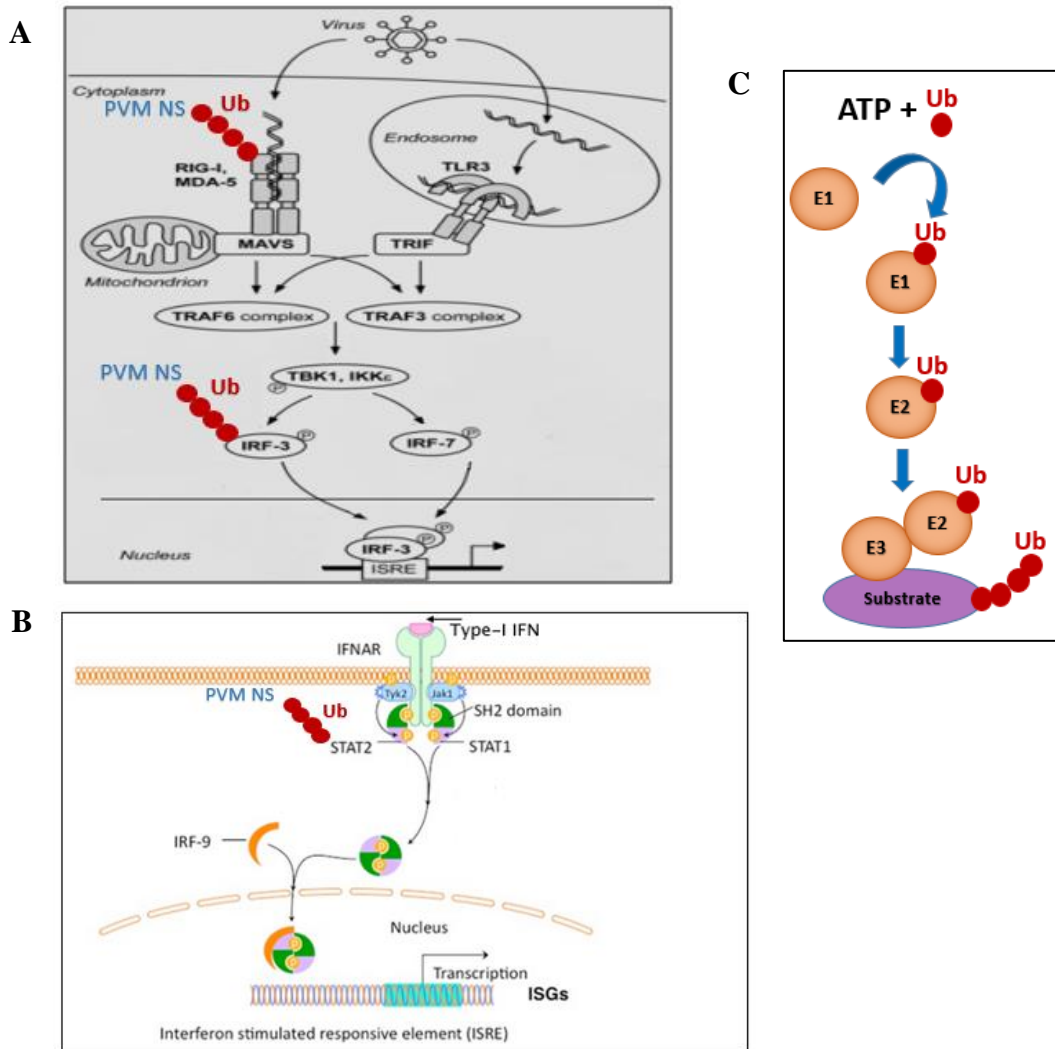
Figure 27: Proposed model for PVM NS mediated suppression of the IFN signaling pathway. A. Suppression of IFN induction pathway. PVM NS proteins suppress IFN induction pathway by proteasomal degradation of pathway specific components RIG-I and

IRF3. **B. Suppression of IFN response pathway.** The sole target of NS proteins in the response pathway is STAT2. PVM NS degrades STAT2 in proteasomal dependent manner. **C. Schematic diagram of Ubiquitination process.**

We mainly delineated the effect of PVM NS proteins on degrading different components of the IFN pathway. There is also possibility that PVM NS can target the synthesis of their target proteins which can be further determine the mechanism of NS action on IFN pathway components.

Our effort to compare one human virus with the mouse one causing a similar kind of disease also reveals different target specificities between them [Table 5]. Even the results for the same PVM virus with different target repertoire, indicate specific motifs responsible for target recognition and degradation. For instance PVM can degrade the mouse RIG-I very efficiently, whereas it has no effect on its human homolog. NCBI BLAST search reveals 77% sequence homology between human and mouse RIG-I. This substrate specificity made PVM more interesting to study. On the contrary PVM can efficiently decrease human homologs of one induction pathway component, i.e. IRF3 and one response pathway component STAT2. That observations indicate that the fundamental role of suppression of the IFN pathway is achieved, though the targets are different. That makes PVM even more significant and gives us the possibility to develop PVM as a potential human pathogen in the future.

Table 5: Lists of the type I IFN signaling pathway targets by Pneumoviruses

Virus	Origin	Targets		Host
		IFN Induction pathway	**IFN response pathway**	
PVM	Mouse	RIG-I (By NS1, NS2) IRF3 (By NS1, NS2)	STAT2 (By NS1, NS2)	Mouse
		IRF3 (By NS2) IRF7 (By NS1, NS2)	STAT2 (By NS1, NS2)	Human
RSV	Human	RIG-I (By NS1, NS2) TRAF3 (By NS1, NS2) IKKε (By NS1) IRF7 (By NS1, NS2)	STAT2 (By NS2)	Human

We have also shown the substrate specificity of PVM NS proteins. The results with different signaling pathway components with PVM NS proteins reveal that NS proteins can only target IFN pathway specific components. There are 8 structural proteins of PVM. To further confirm the substrate repertoire of PVM we have taken on account one of these structural protein, i.e. PVM-P proteins. The observations showed that this structural PVM-P protein also does not have any role on NS targeted IFN pathway specific substrates. Both of these above observations validate the specific role of PVM NS proteins as IFN antagonists.

Taken together, our study shows that NS proteins interfere with multiple components of the IFN induction and signaling pathway to suppress the type I FN signaling. This suppression needs the cooperative activity of NS1 and NS2 through direct interaction with

the specific substrates. In summary, results from this dissertation implicate the complexity and novelty of interaction between PVM NS proteins and their target substrates. There is no effective antiviral therapy or vaccines available for human RSV which is a leading cause of pediatric illness. The lack of animal model for RSV is one of the reason which hampered vaccine development. In contrast PVM which mimics the similar pathogenesis of RSV in mouse model can be used as workable animal model. The knowledge from our study is not only helps us to understand the virulence of PVM, but also that will help us to delineate the prevention strategies for the whole *Pneomovirus* genus specially for RSV. Moreover, those observations might be used for new vaccines development.

CHAPTER III

2'-5'-OLIGOADENYLATE SYNTHETASE-LIKE PROTEIN INHIBITS

RESPIRATORY SYNCYTIAL VIRUS REPLICATION AND IS TARGETED BY

THE VIRA NONSTRUCTURAL PROTEIN 1[2]

3.1 Abstract

Respiratory Syncytial Virus (RSV) is a human *Pneumovirus* from the *Paramyxoviridae* family. It is an enveloped, negative sense, single-stranded RNA virus. RSV causes severe respiratory tract inflammation and illness in infants with a high rate of hospitalization and mortality. Adults are also affected with RSV infection, but there is currently no effective antiviral therapy for hRSV. The RSV genome encodes 11 proteins, in which two non-structural proteins are responsible for inhibiting the type-I IFN (IFN α/β), induced by viral infection. IFN signaling establishes an antiviral state by producing several ISGs to protect the host cells from viral infection. Studies show that RSV NS1 and NS2 working singly or

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or together can degrade specific cellular components such as, RIG-I, IRF3, IRF7, TBK1, and STAT2 of the IFN pathway, both induction and response, and thus suppress type I IFN mediated innate immunity. Our work reveals the effect of NS proteins of RSV on the distal arm of the IFN pathway, on specific ISGs to evade and complete shutdown of the host immune system. 2′-5′-Oligoadenylate Synthetase-Like protein (OASL) is one of these interferon-inducible antiviral proteins or ISGs belonging to OAS family. OASL can induce degradation of RNA by activating RNase L by synthesizing 2′-5′-oligoadenylates (2-5A). There are one human and two isoforms of mouse OASL: Oasl1 and Oasl2 with their unique features. The C-terminal end of OASL has two tandem ubiquitin-like (UBL) domains, which is unique to human OASL only and absent in any of the other members of the OAS family. This report reveals the antiviral properties of hOASL and one mouse OASL (mOASL2). They both can inhibit RSV growth. On the contrary, to establish virus growth, replication, and pathogenesis inside the host cells, RSV NS1 can degrade the hOASL and mOASL2 in a ubiquitin mediated proteasomal dependent process.

3.2. Introduction

Respiratory Syncytial Virus (RSV) is a single stranded, negative sense, non-segmented RNA virus. The human *Pneumovirus*, RSV, is a leading cause of pediatric illness. RSV causes bronchiolitis and pneumonia mainly in infants and also in the elderly. The reports highlighted an estimation of 34 million new cases of RSV infections with lower respiratory tract infection in children under 5 years of age. RSV infection causes 66,000– 199,000 deaths every year [Nair, H. et al. 2010]. Earlier infection cannot prevent new RSV infection because of its high mutation rate. Consequently, the repeated infection of RSV causes the significant mortality rate. The two drugs against RSV, Ribavirin, an aerosol drug and Palvizumab, a monoclonal antibody, are not effective to reduce the risk of severe infection [Chen, C.H. et al. 2008, Ohmit, S.E. et al. 1996, Nokes, J.D. et al. 2008, Forbes, M. et al. 2008]. Thus, currently there is no effective antiviral therapy available for RSV.

Two nonstructural proteins of RSV, NS1 and NS2 play the major role in suppressing IFN signaling and establish viral pathogenesis inside the host cells. Our laboratory has shown that RSV NS proteins promote proteasome-mediated degradation of multiple members of the IFN pathway to circumvent the IFN mediated immune response. NS proteins can target both induction and response pathway components to establish the viral pathogenesis completely. The major targets of NS proteins of RSV are RIG-I, IRF3, IRF7, TBK1, and STAT2 [Sweden S. et al. 2009, 2011].

The distal arm of the IFN pathway is the production of ISGs. There are hundreds of ISGs available with diverse functional features and antiviral activities. Viral infections promote the type I IFN mediated signaling cascade to prevent infection by production of

these antiviral proteins. Recent studies have highlighted one of these ISGs, Viperin, that has antiviral activity. Viperin has an important functional domain and it can limit viral infection by creating unfavorable conditions for viral replication and growth though the exact mechanism still remains unknown [Karla, J. H. et al. 2014]. In respect to Pneumoviruses, Viperin has shown to play a major role in inhibiting RSV. RSV infection upregulates the expression of Viperin and thus lowers the titers of human RSV in nasopharyngeal cells [McGillivary G. et al. 2013]. Recent reports also demonstrated the antiviral activities of some ISGs: IFIT family proteins, IFIT6 and cGAS [Diamond, M. S. et al. 2013, Schattgen, S. A. et al. 2011, Xiao, T. S. et al. 2013].

2′-5′ Oligoadenylate Synthetase-Like protein (OASL) is one of the vital antiviral factors which is induced by IFN signaling. Chapter 3 of this dissertation is about OASL antiviral activity in reference to RSV. OASL belongs to OAS family. There are also other members of in the OAS family such as cyclic GMP-AMP synthetase (cGAS) [Sun, L. et al. 2013]. The members of OAS family have unique features of synthesizing 2′–5′ oligoadenylate, which activates latent RNase L and promotes RNA degradation. There is one human OAS-like (OASL) protein that belongs to the same family. hOASL has the N terminal OAS like domain, but lacks the 2′–5′ oligoadenylate synthetase activity. Instead of that, hOASL has a unique feature having two tandem ubiquitin-like domains (UBL) at their C-terminus end, which is absent in any other members of OAS family [Fig 258] [Kristiansen, H. et al. 2011]. Viral infection in host cells induces type I IFN signaling and therefore induces IRF3-mediated rapid induction of the OASL gene [Marques, J. et al. 2008]. Studies revealed the antiviral activities of OASL in some viruses such as Hepatitis C Virus and West Nile Virus [Ishibashi, M. et al. 2010 (97), Su, X. et al. 2008, Yakub, I. et al. 2005].

There are two *OASL* orthologs present in mouse, such as *Oasl1* and *Oasl2*. Mouse *Oasl1* is enzymatically inactive and does not have the 2′–5′ activity. On the contrary, m*Oasl2* has two Asp residues in its N-terminus and that have the enzymatic activities like OAS family [Figure 28] [Eskildsen, S. et al. 2003]. These two mouse *OASL* show 70% and 48% sequence similarities with human *OASL*. Studies have shown functional homology between the mOASL2 and hOASL [Zhu J et al. 2014].
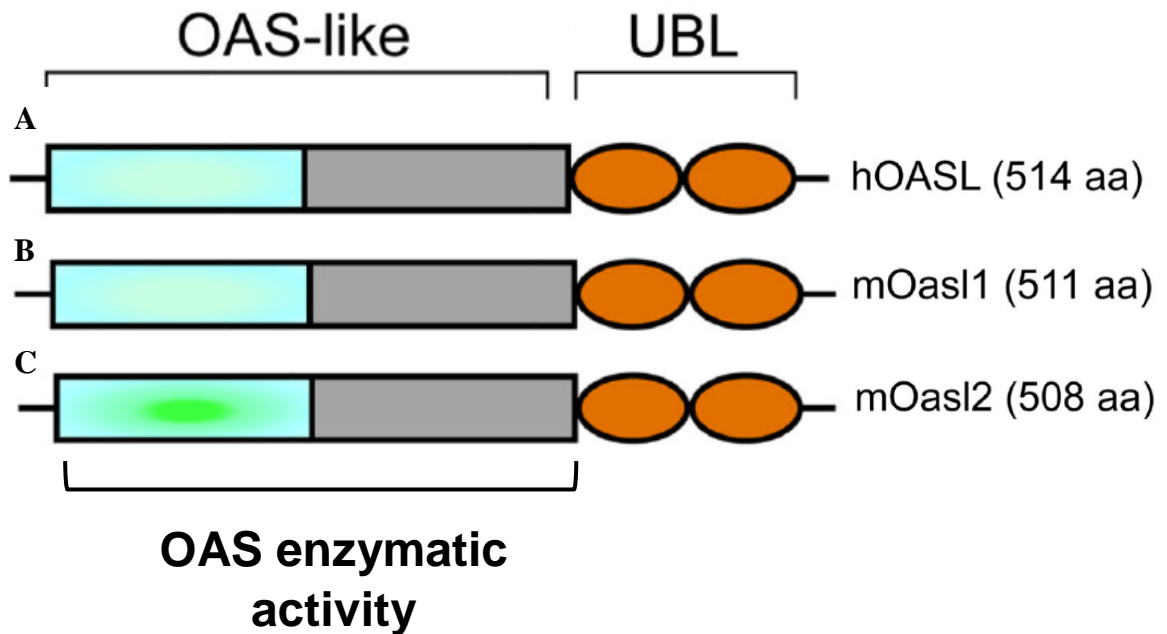
Figure 28: Domain organization of OASL proteins. A. Human OASL protein has N-terminal OAS domain but devoid of Adenylate Cyclase activity. It has C-terminal UBL activity. **B.** Mouse OASL1 is similar with human OASL, no N-terminal enzymatic activity, but has C-terminal UBL domain. **C.** Mouse OASL2 has OAS enzymatic activity at its N-terminal end and also has C-terminal UBL domain.

Recent reports showed that OASL can promotes activation of RIG-I [O'Neill, L. A. Et al. 2011, Ibsen, M. S. et al. 2015]. The presence of OASL can activate RIG-I without the

ubiquitin ligase, TRIM 25, which is necessary for K63-linked polyubiquitination of RIG-I. The proposed model showed that OASL can mimic pUb and makes the RIG-I more sensitive to viral recognition [Gack, M. U. et al. 2009, Zeng, W. et al. 2010]. Our laboratorypreviously has shown that RIG-I is targeted by RSV NS proteins to circumvent the host immune response [Barik, S. 2013].

As OASL can itself activate RIG-I signaling, we hypothesized that OASL can be also targeted by NS proteins. This part of the dissertation is about the effect of RSV NS proteins on OASL and also the antiviral activity of OASL on RSV. OASL can boost the sensitivity of RIG-I on viral recognition, introducing OASL ectopically can help us to combat RSV infection in a novel way. Knowledge from this study will delineate novel strategies in viral recognition and combat the infection more efficiently.

This study is a collaborative effort with Dr. Soumendra Nath Sarkar's laboratory in The Department of Microbiology and Molecular Genetics, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA [Dhar, J., Cuevas RA et al 2015].

3.3. Materials and methods

3.3.1. Cell lines

HEK293 cells were used principally in most of the experiments. These cells were originated from human embryonic kidney cells in tissue culture. Cells were cultured in monolayers in the incubator at 37°C with 5% CO_2. Dulbecco's minimum essential media (DMEM) was used, supplemented with, Penicillin (100 IU/ml) and Streptomycin (100 µg/ml) and heat-inactivated Fetal Bovine Serum (10% FBS).

Human epidermoid cancer cells (HEp-2) cells were used for RSV growth. The similar DMEM media was used supplemented with 10% FBS and antibiotics.

Human colorectal carcinoma (HCT-116) cells were obtained from Dr. Sarkar's laboratory. Cells were cultured in McCoy's 5a Medium (Life Technologies, Grand Island, NY) with 10% FBS and antibiotics.

The immortalized Mouse Embryonic Fibroblast (MEF) cells were used principally for in mouse-related experiments. These cell lines were obtained from Dr. Ganes Sen's laboratory in the Lerner Research Institute, Cleveland Clinic.

Bone marrow derived macrophage (BMDM) cells were used from different mice origins [WT *Oasl*, *Oasl2-/-* etc.]. These cells were also obtained from Dr. Sarkar's laboratory.

HCT116 OASL expressing and OASL deficient cells were obtained from Dr. Sarkar's laboratory.

3.3.2. Virus strains

Human RSV serotype A was used. HEp-2 cells were used for growth and maintenance of RSV. The storage condition is laboratory -80ºC freezer.

3.3.3. Antibodies

For WB and IF analysis different antibodies were used. Antibody against RSV Nucleoprotein (N) (Abnova clone B023) was used from mouse origin. IgG-HRP anti-mouse (Santa cruz, sc-2354) antibody was used as secondary antibody. Mouse anti-FLAG antibody (Sigma, SLBF6631/F1804), rabbit anti-FLAG (Sigma, F7425) antibodies were used to detect FLAG-tagged recombinant NS proteins of RSV. All the recombinant OASLs are V5-tagged. Mouse V5-tagged (Thermo scientific, MA5-15253) antibody was used to detect OASL proteins. Mouse β-actin was used as a loading control (Santa cruz, sc-81178).

The secondary antibodies which are conjugated with fluorophores for microscopy experiments are: Alex Fluor 610-conjugated donkey anti-mouse IgG (Life Technologies).

3.3.4. Immunofluorescence (IF): Confocal microscopy

HEK293 cells were grown in on square coverslip in 6-well plates. These cells were stably transfected with V5-OASL. For control, stably pcDNA transfected HEK 293 cells were used. Cells were treated with RSV at m.o.i. 3 for 18 hrs. The next day cells were washed with PBS and fixed with ice-cold 4% paraformaldehyde for 20 minutes. Fixed cells were followed by blocking for 1 hr with PBS containing 1% BSA and immunostained with mouse anti-RSV Nucleoprotein (N) solutions made in PBS for 2 hrs. As secondary

antibody Alex Fluor 610-conjugated donkey anti-mouse IgG was used. The mounting medium was Prolong Gold (Invitrogen). Slides were kept in the dark at room temperature for overnight and then stored at -20°C. Images were captured in a Nikon A1RSI Confocal Microscope with NIS element 2.3 software at 60x magnification.

3.3.5. Western blotting (WB) or Immunoblotting (IB)

A monolayer of stably transfected cells (HEK293, HCT116) were infected with RSV at m.o.i. 3 or with recombinant FLAG tagged RSV NS proteins. After 18 hrs of post-infection or 24 hrs of post-transfection, cell lysates were harvested using 1X Laemmli Buffer. Lysates were then processed for sonication heating and centrifugation as described before (material methods Chapter 2). Samples were then subjected to SDS-PAGE followed by immunoblot. Blocking of the membrane for 2 hrs with 6% non-fat dry milk solution in TBST was done. The membranes were then probed with specific antibodies. ECL prime was used as detection reagent (GE Healthcare). Bands were developed in LI-COR Odyssey Fc Imaging System machine along with the densitometric analysis. β-actin was used as a loading control. As a proteasomal inhibitor, Lactacystin [LC (10 µM)] was used.

3.3.6. Quantitative RT-PCR

Cells were grown in 100 mm cell-culture dishes and transfected with either of Oasl, Oasl-ΔUBL, Oasl1, Oasl2 recombinant plasmids or infected with RSV at m.o.i. 3. The total RNA were extracted after 24 hrs post-transfection or 18 hrs of post-infection using Trizol. Quantitative real time PCR reaction was performed using Real-Time Detection System and

SYBR-GREEN Supermix (BioRad). Primers for RSV-N was used for the PCR are listed in the table 6. β-actin was used as a loading control.

Table 6: Lists of primers: All primers are written 5' to 3' (qRT-PCR)

RSV-N	Forward: TGCAGGGCAAGTGATGTTAC Reverse: TTCCATTTCTGCTTGCACAC
hOASL	Forward: AAAGAGAGGCCCATCATCC Reverse: ATCTGGGTAACCCCTCTGC
Actin	Forward: AGAAAATCTGGCACCACACC Reverse: GGGGTGTTGAAGGTCTCAAA

3.3.7. Plaque assay

HEp-2 cells were plated at $1.5 * 10^5$ cells/well in 6-well plate. Viral dilutions (RSV) from 10^{-1} to 10^{-5} were prepared in media free from antibiotics and FBS. At about 80-90% confluency, cells were treated with 100 µl of RSV dilutions. Cells were incubated in 37ºC for one hour with gentle rocking of the plate in every 15 minutes of interval. Next the plate was overlaid with 1.5 ml/well of autoclaved Carboxymethylcellulose supplemented with FBS and incubated for 5 days. The Carboxymethylcellulose layer was loosened and aspirated by adding PBS in each well for 15 minutes. Ice cold methanol was added and kept it in -20ºC for 1 hour. Methanol was then removed and the plate was stained with 0.1% crystal violet in ethanol solution for 30 seconds at room temperature. The solution was removed, washed, and the plate was dried upside down on a paper towel. Then the holes or Plaque Forming Unit (PFU) in purple monolayers on the plate was counted under microscope in PFU/ml.

$$\text{PFU/ml (Original stock)} = \frac{\text{Number of plaques}}{\text{Dilution factor x (ml of inoculum/plate)}}$$

3.3.8. Statistical analysis

All results presented here are mean $\pm$ standard deviation from three separate experiments. Statistical analyses were performed using GraphPad Prism 5.0 software. All statistical analyses were calculated by using two tailed student's T-test analysis. The statistical significance was considered as $p < 0.001$.

3.4. Results

3.4.1. Human OASL inhibits RSV growth and replication

Our previous study [Zhu, J. et al. 2014] showed that human OASL has the antiviral activity and can enhance the RNA mediated RIG-I signaling. So in this part of our literature we first wanted to delineate the antiviral activities of hOASL against RSV. HEK293 cells that were stably transfected with V5-tagged OASL were grown in monolayers on coverslips. Cells were then infected with RSV at m.o.i. 3. After 18 hrs of post-infection cells were processed for immunostaining with anti-RSV-N protein and Alexa fluor 610 (Red) and observed under Confocal Microscope. As a control we have used empty vector pcDNA transfected HEK293 cells. Figure 29 shows OASL expressing cells (left) shows no RSV growth where only the vector panel (right) shows enormous growth of RSV denoted with red color. The result suggests hOASL can inhibit RSV growth.
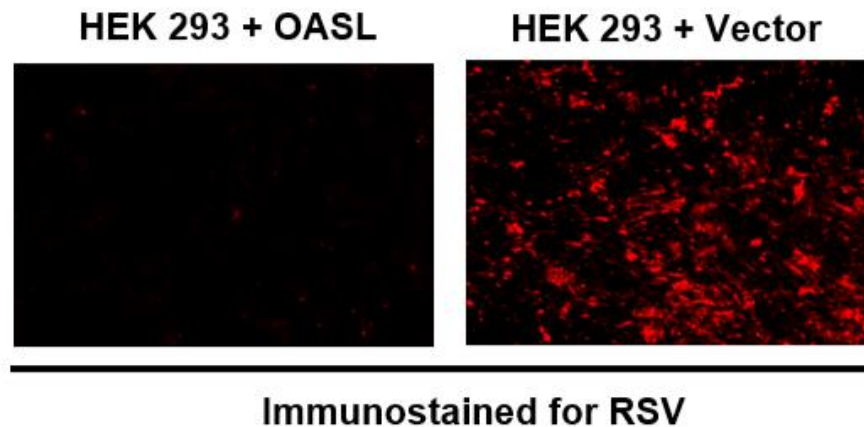
Figure 29: Inhibition of RSV growth by hOASL. Stably transfected OASL expressing cells show no RSV growth compared to only empty vector (pcDNA) transfected cells under Confocal Microscope.

3.4.2. Human OASL can reduce the level of intracellular RSV protein and RNA (Nucleoprotein)

Our earlier finding demonstrated the inhibitory effect of OASL on RSV growth. Our next aim was to conduct a detailed analysis of the effect of hOASL on RSV protein and mRNA level.

HEK293 V5-OASL cells were infected with RSV at m.o.i. 3. Cell lysates were collected at different time points (0, 12, 24, 36 hr) and subjected then to IB with anti-RSV-N antibody. Empty vector (pcDNA) treated cells were used to compare the effect. β-actin was used as a loading control. Figure 30A shows OASL expressing cells reduce the steady-state level of RSV protein level in a time-dependent way.

In a separate study to measure the mRNA level of RSV, we have followed the similar procedure described above up to the infection stage. Then total RNA was collected and RNA level was quantified by qRT-PCR. Figure 30B shows the graph from three data sets from three time points. Graphs indicate that hOASL also decreases the intracellular RNA level of RSV.

The quantification of total viruses liberated after 24 hrs of post RSV infection was measured by plaque assay. The PFU/ml count shows the virus count [Figure 30C]. The plotted graph demonstrates that OASL expressing cells show less viral count compare to the empty vector treated cells [Figure 2730C].

Together these results [30ABC] validate our previous findings that hOASL has antiviral properties against RSV and it can efficiently inhibit RSV growth and replication. This also establish RSV inhibition by human OASL at the genome level.
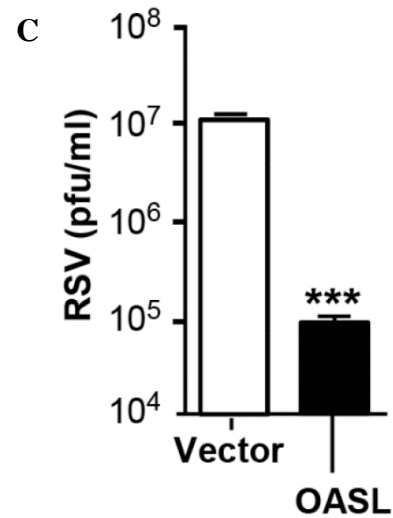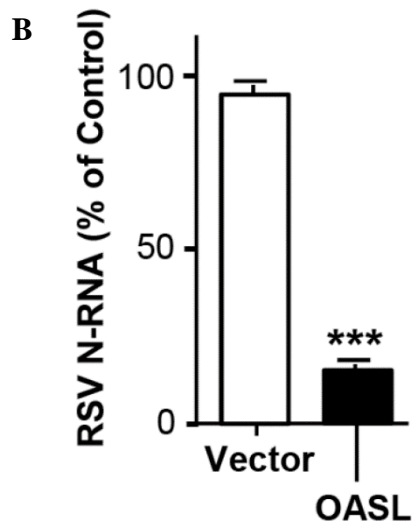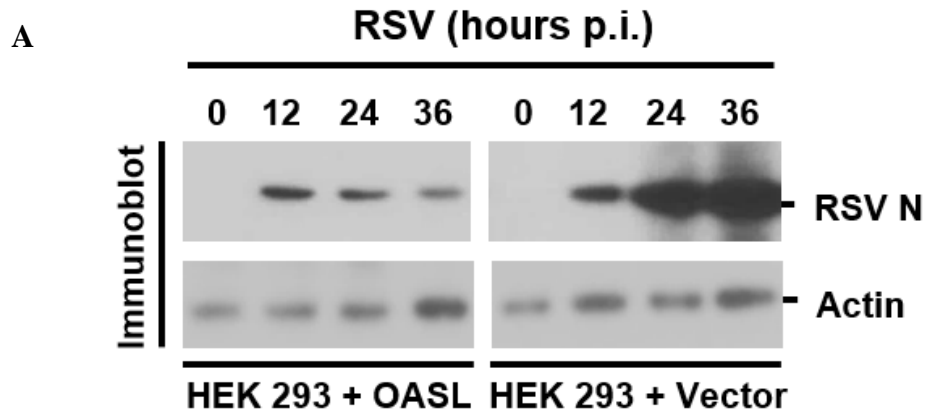
Figure 30: hOASL reduces intracellular RSV protein and RNA level and total viral count. A. A time dependent study (0, 12, 24, 36 hr) shows that hOASL reduces the level of RSV protein, in an empty vector panel, no reduction is seen. β-actin is the loading control. **B.** hOASL can reduce the RNA level significantly compared to the vector control. **C.** The viral plaque assay total PFU count shows significant reduction in RSV amount in hOASL expressing cells. The asterisks * indicate level of significance where p<0.001.

3.4.3. The antiviral activity of hOASL against RSV is not cell specific

Our next aim was to demonstrate that these inhibitory activities of hOASL against RSV are not cell specific. With this aim we next used another different cell line, HCT116; OASL-expressing cells, where stably transfected OASL expressed and OASL-deficient cells, where expressions of OASL was silenced by using shRNA. Similar experimental procedures were followed. Both cells were infected with RSV at m.o.i. 3 and at different time points, cell lysates were collected and subjected to IB. The results demonstrate a similar observation of reduction of RSV protein level in OASL-expressing cells [Figure 31A], but no reduction in OASL-deficient cells [Figure 31B].

The RNA level and total viral count were also measured by similar qRT-PCR and plaque assay studies respectively for both of these OASL-expressing and OASL-deficient cells. The observations [Figure 31B, C] from the experiments validate the previous findings that hOASL can inhibit RSV growth and replication regardless of cells.
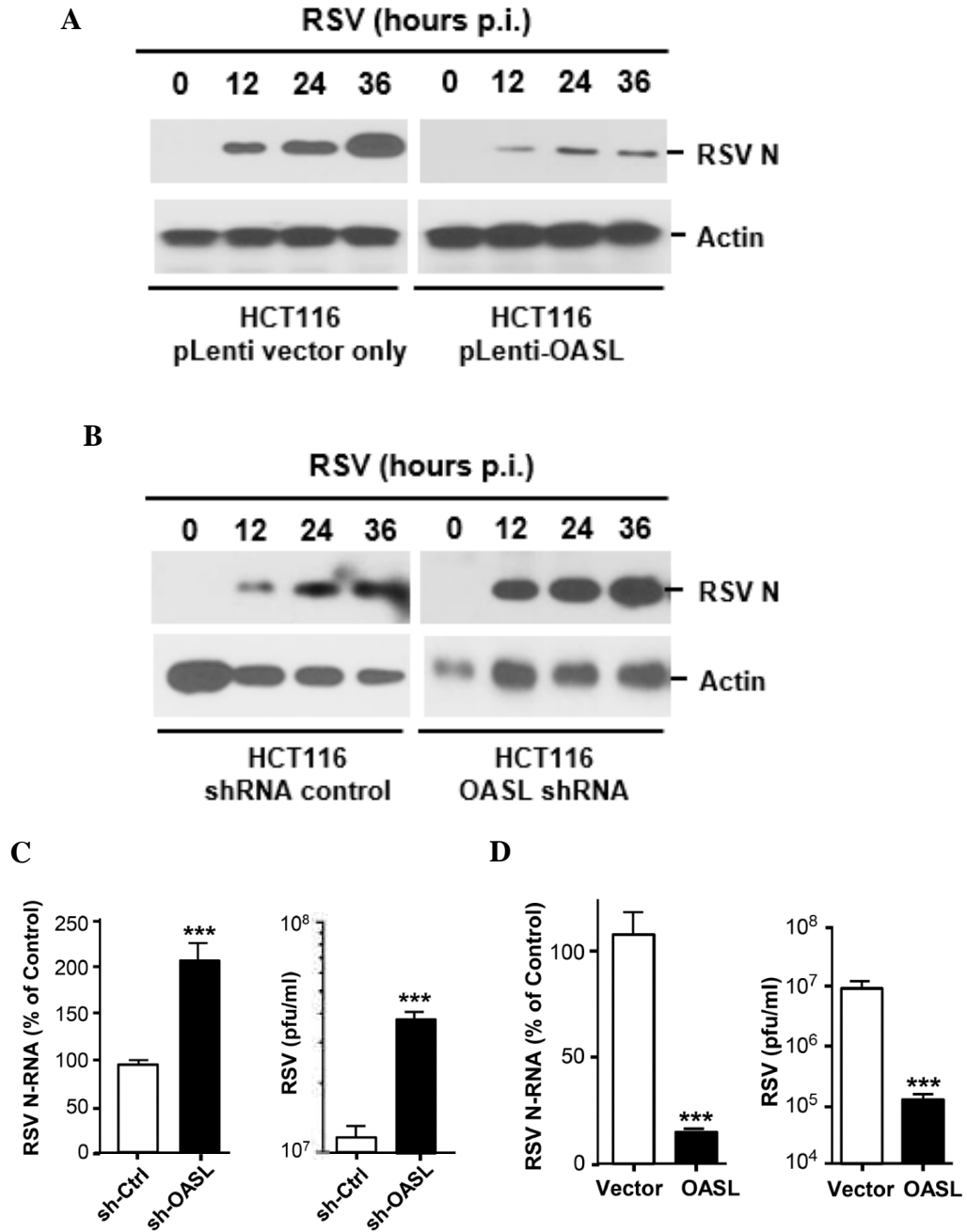
Figure 31: Growth and replication of RSV in OASL-expressing and OASL-deficient cells. A. IB results show that OASL expressing cells have less amount of RSV protein level in HCT116 cells. **B.** OASL-deficient cells show enhanced RSV growth with time. **C.** OASL expressing cells show less RNA level and less viral count. **D.** OASL-deficient cells show enhanced level of RSV RNA and also increasing viral count. [P<0.001].

3.4.4. Mouse OASL2 can inhibit RSV growth and replication

Mouse has two OASL orthologs, OASL1 and OASL2. OASL1 is catalytically inactive like hOASL lacking the enzymatic activity of OAS family proteins. In contrast OASL2 has the catalytic activity. So our first aim was to test the effect of mOASLs on RSV growth and replication. HEK293 cells were transfected with recombinant OASL1 and OASL2 plasmids and also infected with RSV at m.o.i. 3. After 18 hrs of post-transfection, the total RNA were collected. RSV N gene mRNA level was measured by qRT-PCR. The result from the above experiment demonstrates that mOASL1 has no effect on RSV mRNA level. But mOASL2 can reduce the level of RNA very efficiently [Figure 32A]. The viral count assay was done with the similar experimental set up for mOASL1 and mOASL2. The observation shows that mOASL2 can reduce the viral count also but there is no effect with mOASL1 [Figure 32B].

To validate our findings, that the inhibitory effect is only with mOASL2, we have used bone marrow derived macrophage (BMDM) cells from wild type (WT) and $Oasl^{-/-}$ mouse. RSV RNA level was tested in both of these cells. The results show that in $Oasl2^{-/-}$ cells the RSV RNA level is more compared to the WT cells [Figure 32C].
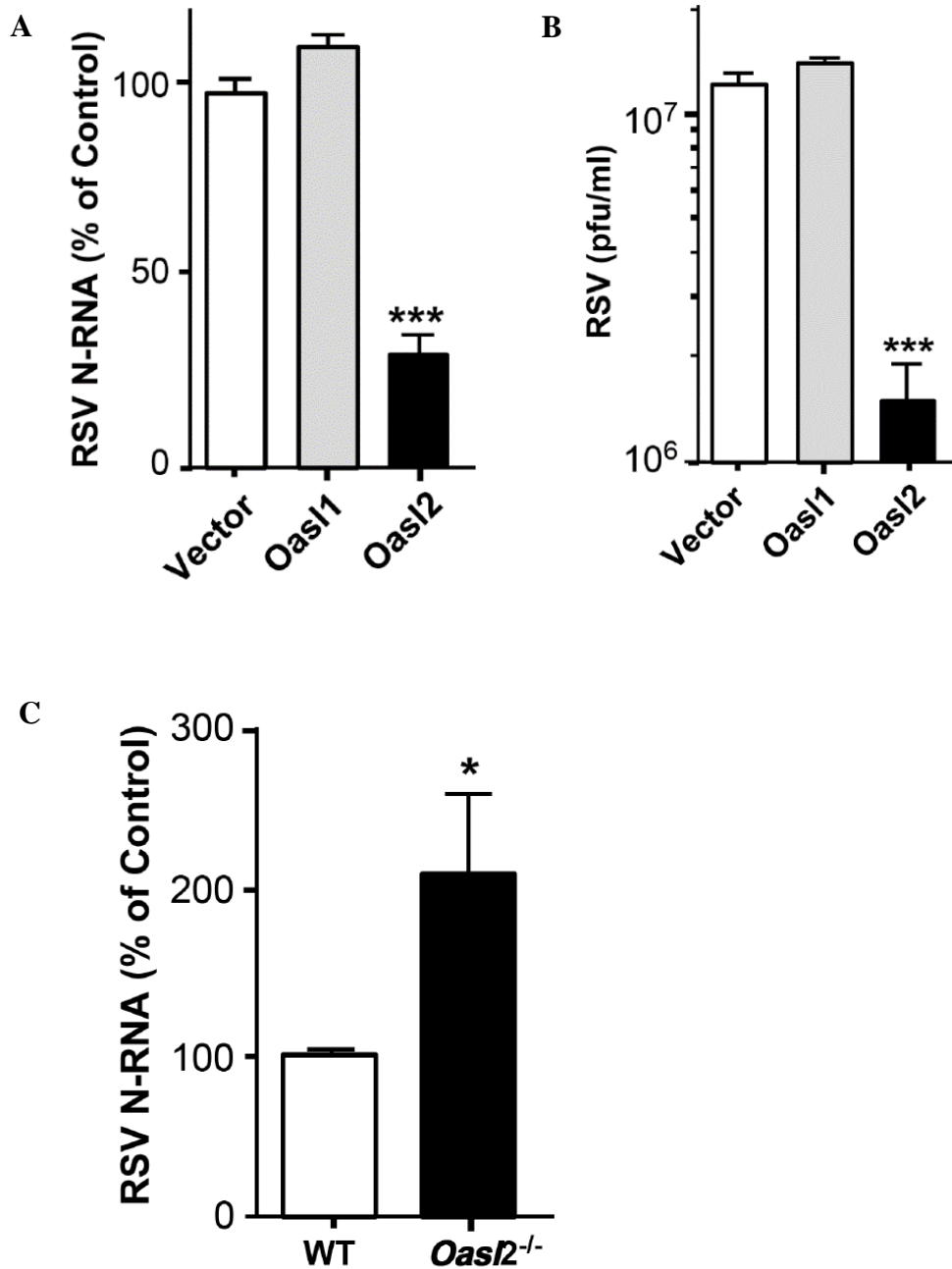
Figure 32: Effect of mOASL on RSV growth and replication. A. mOASL1 does not have any effect on RSV RNA level, mOASL2 can reduce the level significantly, $p<0.0001$. **B.** Viral count assay demonstrates mOASL2 can inhibit RSV growth, where no inhibitory effect is shown with mOASL1, $p<0.05$. **C.** BMDM cells from *Oasl2*[-/-] have no effect on RNA level of RSV, $p<0.001$.

3.4.5. The antiviral activity of OASL against RSV is dependent on C-terminal ubiquitin domain of OASL, but independent of N-terminal catalytic domain

As discussed before, human OASL differs from the OAS family lacking the catalytic N-terminal domain. Instead of that, it has C-terminal UBL domain. Studies revealed that this UBL domain is important for antiviral activities of OASL against Vesicular Stomatitis Virus (VSV) and Sendai Virus (SeV) [Zhu, J. et al 2014]. In this regard to explore the mechanism of RSV inhibition by OASL, our first aim was to test the two important N and C-terminal domains of OASL. Mouse OASL2 has the N-terminal catalytic domain. To abolish this catalytic activity of mOASL2, two Asp residues were mutated from the catalytic conserved triad domain [Eskildsen, S. et al. 2002]. The mutant was named as DADA mutant. Next the C-terminal UBL domain was also mutated from hOASL to explore whether it has the similar effect on RSV like VSV and SeV. Next we have tested all the mutants and WT versions of human and mouse OASL on RSV growth and replication. MEF cell lines were used. Cells were transfected with hOASL, hOASL-ΔUBL, mOASL1, mOASL2 and mOASL2-DADA. Effects of these WT and mutant recombinant proteins on RSV mRNA level were quantified with qRT-PCR. The results from this study demonstrate that though mOASL2 is enzymatically active having this N terminal catalytic property, but this 2′-5′A is not important for the inhibitory activity of OASL on RSV. So OASL2-DADA mutant is as effective as WT OASL2. On the other hand, the UBL mutant loses its antiviral activity completely [Figure 33A]. So we can conclude from these observations that N-terminal 2′-5′A of OASL is not important for the antiviral activity against RSV but requires UBL domain.

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To validate these findings, we have also measured the total viral count (PFU) with these WT and mutant OASL. The results show a similar conclusion where antiviral activity of OASL protein is independent of the N-terminal catalytic activity but depends on C-terminal UBL domain [Figure 33B].

[hOASL-ΔUBL and mOASL2-DADA - both of these mutants were made by Dr. Soumen Sarkar's laboratory].
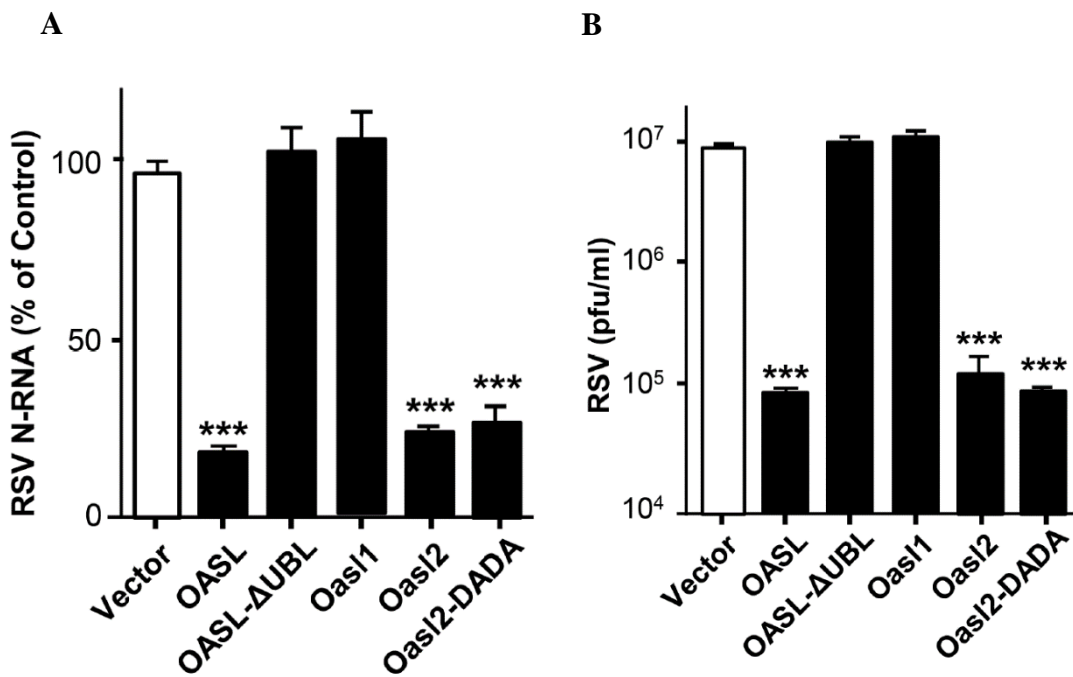
Figure 33: Effect of human and mouse WT and mutant OASLs on RSV growth and replication. A. qRT-PCR assay with hOASL, hOASL-ΔUBL, mOASL1, mOASL2 and mOASL2-DADA shows that hOASL-ΔUBL is defective in antiviral activity against RSV, mOASL2-DADA is as effective as WT mOASL2. **B.** Plaque assay for measuring total viral count shows the similar results, where hOASL-ΔUBL has no effect on RSV growth, but mOASL2-DADA can inhibit RSV growth significantly. [p<0.001]

3.4.6. RSV NS1 can degrade OASL to counteract its antiviral activity

Previous reports showed that RSV NS proteins are IFN antagonists. To circumvent and suppress the host immune system completely, NS proteins can target multiple components of IFN pathway specific proteins, such as, RIG-I, IRF3, IRF7, and STAT2. NS proteins degrade these components in a proteasome dependent way. OASL has antiviral activity against RSV and it inhibits the growth and replication of RSV. We hypothesize that to counteract the antiviral activity of OASL, RSV can also target the OASL protein. With this aim, we tested the effect of RSV NS1 on hOASL. HEK293 cells were transfected with FLAG-tagged recombinant RSV NS proteins and V5-tagged hOASL. After 24 hrs of transfection cell lysates were collected and subjected to IB with specific antibodies. Figure 31A shows that RSV NS1 can decrease the level of hOASL significantly, but NS2 is ineffective [Figure 34A]. β-actin was used as loading control. qRT-PCR analysis of the samples shows that there is no effect on mRNA level (DATA not shown).

To explore whether the decreased level of OASL mediated by RSV NS1 is due to proteasomal-mediated degradation, we have tested the effect of RSV NS1 on hOASL with a proteasomal inhibitor lactacystin (10μM). HEK293 cells were cotransfected with FLAG-tagged RSV NS1 and V5-tagged hOASL. After 8 hrs of transfection, LC were added to the medium. Cell lysates were collected after 24 hrs of transfection and subjected them to IB with specific antibodies. Results show LC have restored the amount of OASL when added [Figure 34B]. The above observations conclude that RSV can also target OASL in a proteasomal-mediated way for degradation. To validate that UBL domain is important for this degradation, we have done a similar approach where V5-OASL was replaced with V5-OASL-ΔUBL. IB result with this specific UBL domain is important for the RSV OASL

interaction. RSV cannot degrade the OASL protein when UBL domain is deleted [Figure

34B]. β-actin was used as a loading control.
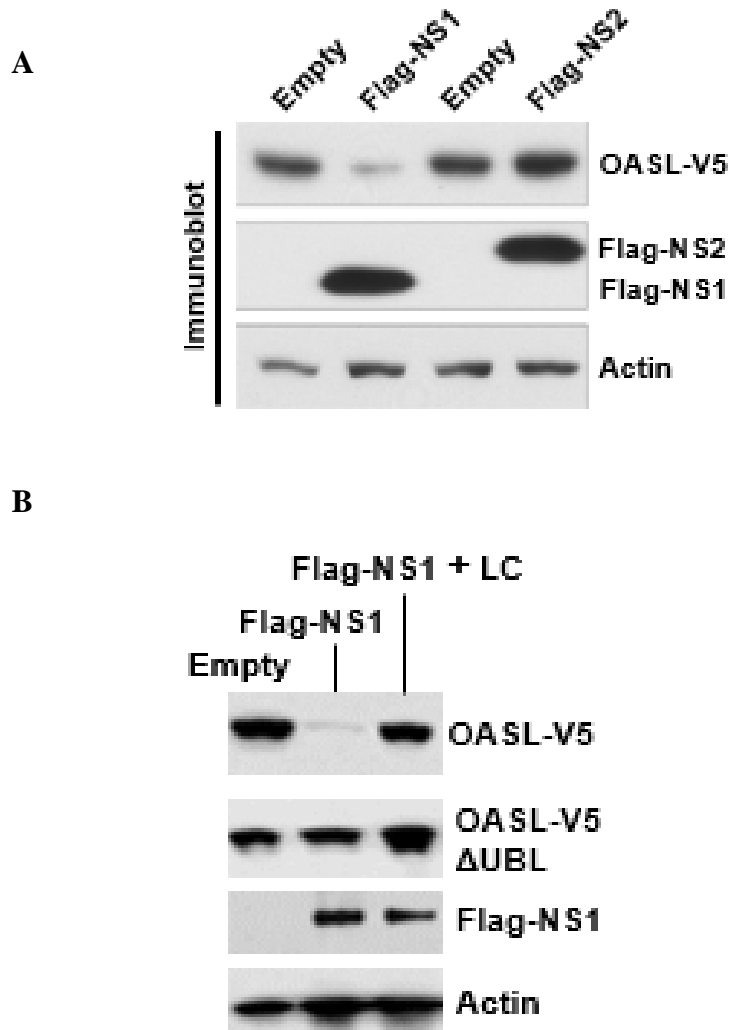
Figure 34: Effect of RSV NS1 on hOASL. A. RSV NS1 can decrease the steady state level of hOASL. B. RSV NS1 can degrade hOASL in a proteasome-dependent way. RSV NS1 has no effect on OASL-UBL mutant. β-actin is used as a loading control.

3.4.7. Effect of RSV NS1 on human and mouse OASL WT and mutants

To explore the effect on RSV NS1 on different WT and mutant of human and mouse OASL, cells were transfected with FLAG-tagged recombinant RSV NS proteins and V5-tagged hOASL, hOASL-ΔUBL, mOASL1, mOASL2 and mOASL2-DADA. After 24 hrs of transfection cell lysates were collected and subjected to IB with specific antibodies. Figure 35 shows NS1 can decrease the steady state level of hOASL, mOASL2 and OASL2-DADA, but no effect on OASL-ΔUBL and mOASL1 [Figure 35]. So our findings reveal that UBL domain is important for NS protein to target OASL.
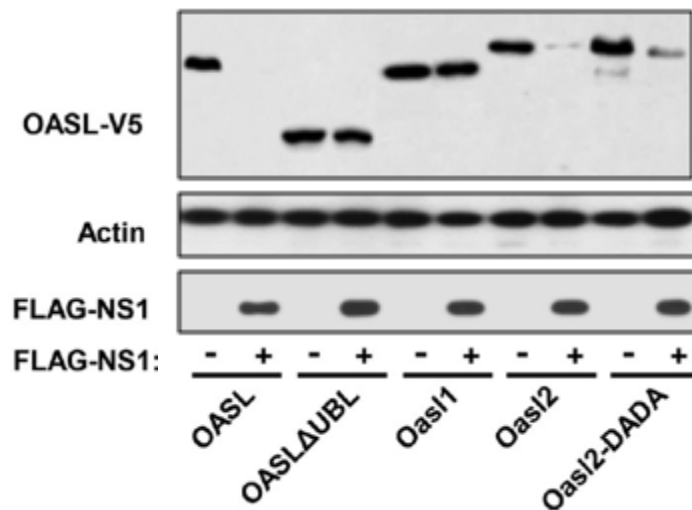
Figure 35: Effect of RSV NS1 on human and mouse OASL WT and mutants. NS1 can decrease the steady state level of hOASL, no effect on UBL mutant. For mouse, mOASL2 and OASL2-DADA both were effected by RSV NS1, but no effect on OASL-ΔUBL and mOASL1. β-actin is as a loading control.

3.5. Discussion

Viral infection induces type I IFN signaling as an antiviral defense mechanism. This signaling induces several ISGs. Some of them are antivirals acting directly against viruses. Our results demonstrate a unique ISGs, OASL, that modulates virus growth and replication significantly. The full name of OASL is 2′-5′ Oligoadenylate Synthetase Like protein which belongs to OAS family. OAS family proteins have a special characteristic; they have N terminal OAS domain that can synthesize 2′-5′ oligoadenylates. It can activate endonuclease RNase L and mediates antiviral effects by direct cleavage of viral RNA. But OASL family proteins does not have this catalytic activity. Previous reports showed that OASL has the antiviral properties that can act against two viruses, VSV and SeV. OASL mainly acts on cytoplasmic RNA sensor RIG-I and induces its antiviral signaling robustly. There are one human OASL and two mouse orthologs of OASL proteins, OASL1 and OASL2. Reports showed that both human OASL and mouse OASL2 have the antiviral properties. This part of my dissertation is on antiviral property of OASL against hRSV.

hRSV can suppress IFN signaling. The two nonstructural proteins NS1 and NS2 are mainly responsible for this suppression. Our previous reports showed that NS proteins of RSV are IFN antagonists and they target many of the IFN pathway components to circumvent the innate immune system. The distal part of the immune system is the production of ISGs. The biochemical properties of a few of them are delineated. In this chapter, we have shown the effect of one commonly expressed ISG OASL and its antiviral

effect on RSV growth and replication, which is a unique and noble report of any ISG interaction with RSV.

Our results demonstrate human OASL and mouse OASL2 can inhibit RSV growth and replication. But there is no effect of mouse OASL1 on RSV. OASL not only inhibits the growth, but the protein and RNA level of RSV are also significantly affected by hOASL and mOASL2. Different cell related studies showed that these effects are not also cells specific. A detailed analysis of the antiviral activities of both OASLs from human and mouse origin raised a question about their mechanism of action. hOASL and mOASL2 differ in their structural organization; hOASL does not have the N-terminal enzymatic catalytic activity as the OAS family of proteins. But mOASL2 has this catalytic domain. And both of these OASLs from human and mouse origin have C-terminal ubiquitin-like domain. Our observation showed that for the antiviral inhibitory activity of both the hOASL and mOASL2, the UBL domain is important. Mutation of this specific UBL domain causes loss of the inhibitory activity for both of these hOASL and mOASL2. Though the two OASLs are structurally dissimilar, they have functional homology between them and the mechanism of action is similar. Though mOASL1 has the similar UBL domain at its C-terminal end, it has no antiviral effect. The reason behind this is unknown. Our DADA (2 Asp residues) mutant of mOASl2 shows that though N-terminal catalytic activity is a unique characteristic of OAS family protein it is not responsible for the antiviral activity of OASL against RSV.

Reports have shown, RSV can circumvent the host immune system efficiently by acting on RIG-I, IRF3, IRF7 and STAT2. Here we have shown to counteract the antiviral activity of OASL, RSV can decrease the steady state level of hOASL. This decreased level is due

to proteasomal dependent degradation of OASL by NS1 protein of RSV. NS2 has no effect on OASL. The effect is on translational level of OASL. The half-life determined by pulse chase assay showed NS1 can reduce the half-life of OASL significantly.

The antiviral activity of human OASL and mouse OASL2 is independent of OAS domain but requires the UBL domain. UBL domain of OASL is also required for the interaction with RSV NS1 protein. Mouse OASL1 also has the UBL domain but does not share the antiviral property of human OASL and mouse oASL2. These observations point out the unique properties of ubiquitin domain of both mouse OASL1 and OASL2.

In summary, results from this report implicate that NS proteins of RSV not only target the multiple components of IFN pathway specific proteins, but also target the distal arm of IFN signaling. This reveals a new insight in the field of RSV and develops new knowledge about the complexity of the host-pathogen interaction. In the future, which may provide a basis for the design and development of new vaccine strategies against RSV.

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