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Regulation of IRF-3-Dependent Innate Immune Signaling Pathway by the PLpro Domain of Non-Structural Protein 3 (NSP3) of Severe Acute Respiratory Syndrome (SARS) Coronavirus

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Abstract

The induction of Type I Interferons (IFNs) is a powerful and rapid innate defense mechanism against viral infection, and many viruses have developed elaborate strategies to overcome the antiviral effects of IFN, ensuring their survival and replication. Severe acute respiratory syndrome coronavirus (SARS-CoV) is a highly pathogenic virus that causes severe lung disease in humans and is associated with high mortality rates. SARS-CoV, like all other successful viruses, encode proteins that counteract the innate immune response. A number of reports have indicated the papain-like protease (PLpro) domain of SARS-CoV Non-Structural Protein 3 (NSP3) as a powerful interferon antagonist, by suppressing interferon regulatory factor 3 (IRF3) dependent innate antiviral defenses. IRF3 plays a key role in viral-induced type I IFN induction pathway. Thus, viruses are well-known to evade the establishment of an antiviral state by regulating the activation of IRF3. However, functional studies detailing the PLpro IFN antagonistic abilities, are not describe in the context of the full length nsp3 protein, in which it is contained in virus infected cells. Nsp3 is the largest replicase gene product in the coronavirus genome, which contains several functional domains that are required for coronavirus replication. Establishment of a stable and controllable CoV-nsp3 expression system will allow the physiological relevant study of the PLpro mediated function of this protein. Here, I described the development of tetracycline-inducible mammalian cell lines for stable expression of the full length nsp3 of HCoV-OC43, HCoV-NL63, MERS-CoV, and SARS-CoV, respectively. Although these cell lines exhibited stable and tight control of nsp3 expression in the presence of tetracycline, I observed a variation in CoV's nsp3 protein expression levels. However, HeLa-Fit-SCoV-nsp3 and HeLa-Fit-SCoV-nsp3-delPLP stable cell lines expressed SARS-nsp3 and SARS-nsp3-delPLP robustly and at comparable levels. I found that expression of SARS-CoV nsp3 compromised virus-induced expression of IRF-3-dependent antiviral genes and that such ability depended on the PLpro domain. In agreement with our previous study examining the effects of the PLpro domain, the inhibitory effect was downstream of the IRF-3 kinases while upstream of IRF-3. Overall, my data demonstrates that SARS-CoV nsp3 is a bona fide interferon antagonist, which acts through PLpro-mediated suppression of IRF-3 activation.

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Regulation of IRF-3-Dependent Innate Immune Signaling Pathway by the PLpro Domain of Non-Structural Protein 3 (NSP3) of Severe Acute Respiratory Syndrome (SARS) Coronavirus

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From The University of Tennessee

By
Sandra Nicole Lester
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DEDICATION

This dissertation is dedicated to my beautiful daughter Mariah Pullins, my parents Barbara and James Lester and my family and friends, all whom have encouraged me to do my best, believed that my best has no limitations and provided me with unconditional love and support.

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ABSTRACT

The induction of Type I Interferons (IFNs) is a powerful and rapid innate defense mechanism against viral infection, and many viruses have developed elaborate strategies to overcome the antiviral effects of IFN, ensuring their survival and replication. Severe acute respiratory syndrome coronavirus (SARS-CoV) is a highly pathogenic virus that causes severe lung disease in humans and is associated with high mortality rates. SARS-CoV, like all other successful viruses, encode proteins that counteract the innate immune response. A number of reports have indicated the papain-like protease (PLpro) domain of SARS-CoV Non-Structural Protein 3 (NSP3) as a powerful interferon antagonist, by suppressing interferon regulatory factor 3 (IRF3) dependent innate antiviral defenses. IRF3 plays a key role in viral-induced type I IFN induction pathway. Thus, viruses are well-known to evade the establishment of an antiviral state by regulating the activation of IRF3. However, functional studies detailing the PLpro IFN antagonistic abilities, are not describe in the context of the full length nsp3 protein, in which it is contained in virus infected cells. Nsp3 is the largest replicase gene product in the coronavirus genome, which contains several functional domains that are required for coronavirus replication. Establishment of a stable and controllable CoV-nsp3 expression system will allow the physiological relevant study of the PLpro mediated function of this protein. Here, I described the development of tetracycline-inducible mammalian cell lines for stable expression of the full length nsp3 of HCoV-OC43, HCoV-NL63, MERS-CoV, and SARS-CoV, respectively. Although these cell lines exhibited stable and tight control of nsp3 expression in the presence of tetracycline, I observed a variation in CoV's nsp3 protein expression levels. However, HeLa-Fit-SCoV-nsp3 and HeLa-Fit-SCoV-nsp3-delPLP stable cell lines expressed SARS-nsp3 and SARS-nsp3-delPLP robustly and at comparable levels. I found that expression of SARS-CoV nsp3 compromised virus-induced expression of IRF-3-dependent antiviral genes and that such ability depended on the PLpro domain. In agreement with our previous study examining the effects of the PLpro domain, the inhibitory effect was downstream of the IRF-3 kinases while upstream of IRF-3. Overall, my data demonstrates that SARS-CoV nsp3 is a bona fide interferon antagonist, which acts through PLpro-mediated suppression of IRF-3 activation.

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LIST OF ABBREVIATIONS

ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
ACE2	Angiotensin Converting Enzyme 2
CoV	Coronavirus
DUB	Deubiquitination
DDX3	Dead Box Helicase 3
DC-	SIGNDendritic Cell-Specific Intercellular Adhesion Molecule-3 Grabbing-Non-Integrin
DMV	Double Membrane Vesicle
dsRBM	dsRNA Binding Motifs
dsRNA	Double Stranded RNA
E	Envelope
eIF-2a	Eukaryotic Translation Initiation Factor 2a
FRT	Flp Recombination Target
GOI	Gene of Interest
HE	Hemagglutinin Esterase
HAU	Hemagglutinin Units
HCoV	Human Coronavirus
HPV	Human Papillomavirus
IB	Immunoblot
IP	Immunoprecipitation
IFITs	Interferon-induced Protein with Tetratricopeptide Repeats

IBV	Infectious Bronchitis Virus
IFN	Interferon
IRF3	Interferon Regulatory Factor 3
ISG56	Interferon Stimulated Genes 56
ISGF3	Interferon Stimulated Gene Factor 3
ISRE	Interferon Stimulated Response Element
I	Internal
JAK	Janus Kinase
LACV	Lacrosse Virus
L-SIGN	Liver/Lymph Node-Specific Intercellular Adhesion Molecule 3-Grabbing Integrins
MDA5	Melanoma Differentiation-Associated Protein-5
M	Membrane
mRNAs	Messenger RNAs
MERS	Middle East Respiratory Syndrome
MHV	Mouse Hepatitis Virus
Mx	Mxyovirus
NF-κB	NF-kappaB
NSP	Nonstructural Protein
N	Nucleocapsid
2'-5' OAS	2'-5' Oligoadenylate Synthesases
ORF	Open Reading Frame
PLpro & PLP	Papain like Protease
PACT	PKR Activator

PAMPs	Pathogen Associated Molecular Patterns
PRRs	Pattern Recognition Receptors
PBS	Phosphate Buffered Saline
Poly (I-C)	Polyinosinic-polycytidylic Acid
Pp	Polyprotein
PKR	Protein Kinase RNA-activated
PRD	Positive Regulatory Domain
RAS	Renin-Angiotensin System
RIG-I	Retinoic-Acid Inducible Gene I
RLRs	RIG-I like Receptors
RNase L	Ribonuclease L
SeV	Sendai Virus
SARS	Severe Acute Respiratory Syndrome
STAT1	Signal Tranducers and Activators of Transcription 1
S	Spike
STING	Stimulator of Interferon Genes
Sg	Subgenomic
Tet	Tetracycline
TLRs	Toll-Like Receptors
Ub	Ubiquitin
Ubl	Ubiquitin-like
UTR	Untranslated Region
VSV	Vesicular Stomatitis Virus

WHO   World Health Organization

CHAPTER 1.   INTRODUCTION

Brief Overview of Coronaviruses

Coronaviruses (CoVs) are the largest RNA viruses known to date, with non-segmented positive sense ssRNA genomes of up to 27-32 kb in length (1). CoVs belong to the family Coronaviridae in the Nidovirales order. The basic genomic organization is similar for all coronaviruses, an organization that consists of several genes encoding several nonstructural and structural proteins and a set of accessory proteins that are unique to each virus species (1, 2). The genomes of several CoVs are illustrated **Figure 1.1**. All CoVs replicate by a similar and unique mechanism, which is associated with the synthesis of an extensive 3'-nested set of multiple subgenomic mRNAs for transcription during infection (1-3). Historically, CoVs were divided into three distinct groups, based on serological analysis, later confirmed by genome sequencing (1, 4, 5). In 2009, a new taxonomic nomenclature was adopted, as such CoVs are now divided into genera (*alpha-*, *beta-*, and *gammacoronaviruses*) corresponding to groups 1, 2, and 3 (6). All mammalian CoVs, including all bat coronaviruses, SARS-CoV, and MERS-CoV, belong to the first and second genera, *Alphacoronaviruses* and *Betacoronaviruses*, whereas all avian CoVs belong to the *Deltacoronaviruses* and *Gammacoronaviruses* genera. However, within the *Gammacoronavirus* genus, there is only one exception, represented by the Beluga whale coronavirus SW1 strain, which was identified in this aquatic mammal (7). Recently, three novel CoVs in birds were identified (8). This discovery formed a distinctive genera of CoVs and now represents a novel genus, *Deltacoronavirus* (8, 9) **Table 1.1**. Coronavirus diversity can be attributed to several factors. First, their large genome size, coupled with the lack of proof reading in RNA polymerases, leads to a high mutation rate. Second, with their unique replication mechanism of random template switching, CoVs have a high recombination frequency, thus promoting their remarkable ability to jump between species and readily adapt from animal to human hosts (1, 10, 11).

Coronaviruses infect a variety of animal species, including humans, causing mostly respiratory and enteric pathologies, and in some infrequent cases hepatic and neurologic pathologies (1,5). Infection can be acute or chronic (1, 5). Many of the animal coronaviruses, such as infectious bronchitis virus (IBV), porcine epidemic diarrhea virus (PEDV) and bovine coronavirus (BCoV), are of economic importance and therefore are very valuable to veterinary research. Human coronaviruses (HCoVs) were first isolated in the 1960s (1, 12-15). Initially, there were very few studies examining the role of CoVs in humans, most likely because of their lack of substantiated severe disease-forming capabilities. HCoVs 229E and OC43 were the first HCoVs to be identified. Since the late 1960s, they have been recognized as being the causative agent of upper respiratory tract infections such as the common cold (1, 12-15). However, as an opportunistic pathogen in more susceptible individuals, including infants, the elderly, and the immunocompromised, infection can be more severe (16-20).

In late 2002, a previously uncharacterized virus that was associated with the development of an atypical pneumonia, which often progressed to severe lung disease
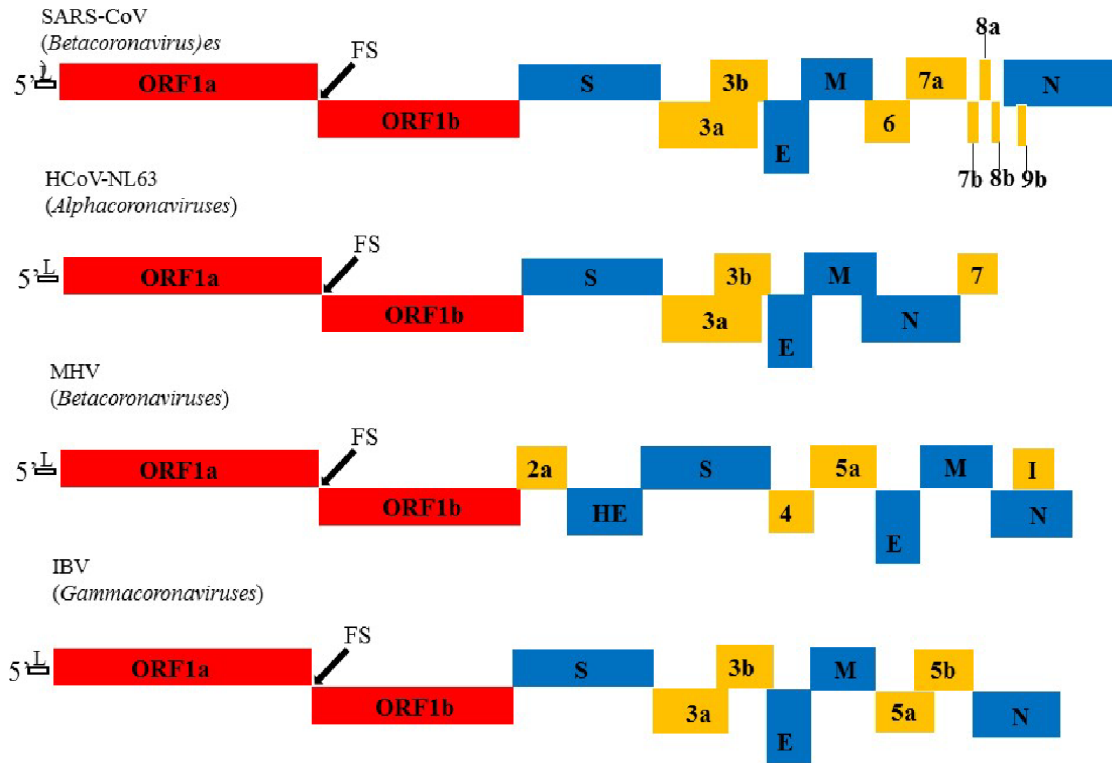
Figure 1.1.   Genomic organization of coronaviruses.

The structures of four coronavirus genomic RNAs are shown. The SARS-CoV genome
and the genomes for human coronavirus Netherlands-63 (HCoV-NL63), mouse hepatitis
virus (MHV) and avian infectious bronchitis virus (IBV) respectively. 5'end consists of a
cap and a leader sequence (L). Red boxes represent ORFs encoding nonstructural
proteins; blue boxes represent ORFs encoding structural proteins; yellow boxes
represents ORFs encoding accessory proteins ORFs; open reading frames. FS: frameshift.

Table 1.1.   **Coronavirus classification.**

Genus	Species
Alphacoronavirus	Transmissible Gastroenteritis Virus (TGEV)
	Porcine Epidemic Diarrhea Coronavirus (PEDV)
	Human Coronavirus 229E
	Human Coronavirus NL-63
Betacoronavirus	Mouse Hepatitis Virus (MHV)
	Human Coronavirus OC43
	Human Coronavirus HKU-1
	Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)
	Middle East Respiratory Syndrome Coronavirus (MERS-CoV)
Deltacoronavirus	Bulbul Coronavirus HKU11 (BuCoV-HKU11)
	Thrush Coronavirus HKU12 (ThCoV-HKU12)
	Munia Coronavirus HKU13 (MuCoV-HKU13)
Gammacoronavirus	Infectious Bronchitis Virus (IBV)
	Beluga Whale Coronavirus [BWCoV] SW1

and mortality, was isolated from humans in Guangdong, China (5). The rapid spread of the disease to over 30 countries in a relatively short period of time represented a major public health threat. The disease was named severe acute respiratory syndrome (SARS), and the etiological agent was quickly identified as a highly contagious novel coronavirus (CoV), designated SARS-CoV (5). The severity of the disease was correlated with increasing age, with mortality reaching 50% for patients over 60 (6). SARS-CoV is a betacoronavirus whose genomic sequence, although similar to other betacoronaviruses, was different enough to make this a member of a new coronavirus subgenera (6). By the end of the SARS-CoV worldwide epidemic, more than 8000 SARS cases and around 800 deaths due to SARs had been recorded (5). Serological studies suggested that SARS-CoV had recently emerged in the human population and that cross-species transmission from an animal to human host seemed the most plausible reason for its emergence (21). SARS-CoV caused the first epidemic of the 21st century and is the first paradigm of serious illness in humans caused by a coronavirus. The fact that a coronavirus could cause severe disease in humans sparked an interest in the scientific community to understand SARS-CoV and the new disease.

Subsequently, in 2004 and 2005, two previously unknown HCoVs, NL63 and HKU1, were discovered and found to cause mild upper respiratory tract infections worldwide (22, 23). Less than ten years after the SARS epidemic, another novel human CoV was identified in 2012 to cause clinical pathologies similar to those described in SARS disease. Many names have been used to refer to this newly identified CoV; however, due to its origin, it was later named Middle East Respiratory Syndrome (MERS) CoV (24). In contrast to SARS, MERS progresses to a severe respiratory infection much more rapidly. MERS-CoV mostly with an origin in the Middle East has spread to 23 countries in Europe, Asia and the United States of America (25, 26). Similar to the scenario of the SARS epidemic, it is suggested that MERS-CoV represents another series of interspecies-transmission events in CoVs (25, 26). To date, the World Health Organization (WHO) has reported 1621 confirmed cases of MERS-COV infection globally, resulting in 584 deaths (http://www.who.int/csr/don/4-december-2015-mers-saudi-arabia/en/website). Although the number of infected people who are confirmed to have MERS is less than the number of SARS confirmed cases, the mortality rate resulting from MERS is a lot higher than the mortality rate resulting from SARS. Nonetheless, the rather large number of global SARS cases, which spread rapidly in a short amount of time, highlights the propensity of human CoVs to adapt readily to the human host, allowing efficient human-to-human transmission. Although there have not been any new infections reported since 2004, resurgence of SARS or of related viruses from zoonotic sources remains a distinct possibility, as exemplified by the recent emergence of another highly pathogenic HCoV, MERS-CoV. Thus far, there are no clinically approved vaccines or antiviral therapeutics for any of the HCoV infections. Therefore, understanding how CoVs emerge, infect, and cause disease in the human host is essential.

Coronaviruses as Emerging Pathogens

All through history, infectious diseases have emerged worldwide and tremendously affected the well-being of human populations. Many of the emerging pathogens that impact or threaten human health have emerged from unknown zoonotic reservoirs, creating a unique challenge to the scientific and medical community. There have been two known occurrences of emergence of highly pathogenic coronaviruses. SARS-CoV crossed the species barrier to cause the first pandemic of the 21st century. Evidence suggests that SARS-CoV emerged from bats to infect palm civets, sold live in open markets, acting as the intermediate host, facilitating animal-to- human and human-to-human transmission. Examinations of a range of domestic and wild mammals were conducted in Guandong, China, after the outbreak of SARS, and these examinations support the hypothesis that marketplace animals may have been the source of the virus found in humans (27, 28). Animal traders working with live animals in these markets had high seroprevalence for both the human and animal SARS-CoV, although no prior history of disease could be detected (27). Furthermore, there is a significant amount of serological data suggesting that SARS-CoV had not previously been endemic in humans (21).

Guan et al published evidence establishing that Himalayan palm civets, Chinese ferret badgers and raccoon dogs all carried SARS-like viruses that were genetically and antigenically related to human SARS-CoV (27). In particular, it was seen that the viruses collected from masked palm civets in the early phase of the epidemic and in 2003 exhibited deletions in open reading frame 8 (ORF 8), which differed in length from 29 nucleotides to larger deletions that resulted in the loss of the entire ORF 8 region at the late phase of the epidemic in 2004, suggesting two separate animal-to-human transmission events (5, 21, 27, 29, 30). The fact that SARS-CoV was present only in market or farmed animals, but not in those from the wild (7), suggests that the palm civets and other marketplace animals are unlikely to have been the natural reservoir hosts of SARS-CoV, but merely a secondary host bridging the gap between bat SARS-like CoV and SARS-CoV. The presence of SARS-CoV was detected in different species of horseshoe bats (31, 32). Sequencing of genomes from bat SARS-like CoVs revealed an overall nucleotide sequence similarity of 88% to 92% for all bat SARS-like CoVs isolates to that of the SARS-CoVs isolated from humans or civets (33). The bat viruses also lack the nucleotide deletion, suggesting that SARS-CoVs and SARS-like CoVs share a common ancestor (33). These findings indicate that SARS-CoV or SARS-like CoV may still persist in different unknown animal reservoirs in nature and that a SARS epidemic may recur in the future.

Bat species have also been implicated as primary reservoirs of MERS-CoV, but these species are distinct from those that are suggested to have been involved in the emergence of SARS-CoV (34). Interestingly, in addition to being reported in human infections, high seropositivity for MERS-CoV has been reported in dromedary camels (35, 36). Furthermore, viral sequences obtained from these dromedary camels were almost identical to sequences from two human MERS-CoV cases linked to this farm (37).

Other animal species such as sheep, goats, and cows were found to be negative for MERS-CoV neutralizing antibodies (38, 39). Thus, it is very likely that dromedary camels acquired the virus from bats and that the virus has successively spread between animals in the Middle East region, a spread representing another zoonotic event occurring among CoVs to humans. Recently, several reports have demonstrated human-to-human transmission of MERS-CoV (37, 40). Contrary to the SARS epidemic that was rapidly controlled, MERS-CoV still spreads more than 3 years after its identification, with its most recent large outbreak in South Korea (http://www.who.int/csr/don/25-october-2015-mers-korea/en/). Also, there is evidence that other human CoVs have emerged from bats, including HCoV 229E and HCoV NL63 (34, 41-42). These findings and others indicate that CoVs persist in bats and that these viruses have the ability to mutate, recombine, and cross species barriers to emerge as novel severe disease-causing pathogens, further complicating the development of vaccines and antiviral therapeutics.

Currently, there are no-FDA approved vaccines or treatment for SARS or MERS, so clinical management of patients infected with these highly pathogenic viruses is mostly dependent on supportive treatment and prevention of complications. Efforts to develop a vaccine for SARS-CoV have utilized a number of strategies, including vaccines based on inactivated whole SARS-CoV (1, 43-46), spike subunits (47-49), recombinant viruses expressing SARS-CoV proteins (50-53), DNA plasmids expressing SARS-CoV structural proteins (54-55), or virus-like particles (VLPs) (56), which have all been tested in vitro and in vivo. Although MERS-CoV vaccination approaches are in the initial stages, Song et al demonstrated a recombinant modified vaccinia virus Ankara (MVA) expressing the full-length MERS-CoV spike (S) protein (MVA-MERS-S) that could induce high MERS-CoV neutralizing antibodies in mice (57). Despite the tremendous efforts given to developing a vaccine for highly pathogenic coronaviruses, their extensive genetic diversity and high frequency of recombination present challenges to developing an effective vaccine. For example, vaccination for animal coronavirus IBV is only partially successful due to the recombination of IBV vaccine viruses and virulent wild-type strains, resulting in the emergence of antigenic variant viruses that cause outbreaks of disease in chickens (1, 58). This has been observed in vaccine strains in the field and in a natural outbreak of IBV (1, 58). Furthermore, enhanced disease was observed in vaccinated animals that subsequently became naturally infected with feline infectious peritonitis virus, suggesting that other coronavirus vaccines might also enhance rather than protect from disease (1).

Since the SARS epidemic, several studies have been conducted to identify potent antiviral therapeutics for SAR-CoV infection. Attempts have been made to use antiviral compounds that target specific viral molecules or pathways important to the viral life cycle. Other attempts have been made to use drugs that enhance the immune response or provide specific antibodies using passive immunization (1, 59-63). Antiviral drugs that can control viral loads, thus regulating tissue damage and inflammation, will be most effective for highly pathogenic coronaviruses. However, at present, there are no specific antiviral drugs that have been proven effective for any HCoV infection. Within a period of ten years, two highly pathogenic novel HCoVs, SARS-CoV and MERS-CoV, emerged from zoonotic reservoirs to cause severe acute respiratory distress syndrome (ARDS) in

humans and are associated with high mortality rates. Thus, such emergence emphasizes the need to further understand the virus and host interactions that regulate disease severity and infection outcome.

SARS Pathogenesis and Acute Respiratory Distress Syndrome

Among the HCoVs known to date, the pathogenesis of SARS appears to be distinguished by a complex mechanism, which seems to consist of direct and indirect influences. Specifically, damage to the lung appears to occur directly from SARS-CoV infection and replication in target cells, as well as a subsequent indirect injury to the lung mediated by an ill-regulated and aberrant immune response. Prior to the SARS epidemic, HCoV 229E and OC43 were the only two CoVs known to infect humans (1, 5). Recognition as pathogens of upper respiratory tract infections, and causing minimum mortality, made them less interesting, thus generating minimal concern in identifying the determinants that regulate disease outcomes (1, 12-15). The emergence of SARS rekindled an interest in CoVs' molecular and cellular basis of pathogenesis. Furthermore, it demonstrated that there may be molecular determinants that account for the dramatic differences in pathogenesis between other HCoVs and SARS-CoV. The most recent emergence of a novel CoV, MERS CoV, which also causes severe disease in humans, illustrates the need to understand the pathogenic mechanisms to disease, as well as the development of therapeutics and vaccines for controlling and preventing CoVs that cause severe disease in humans.

SARS-CoV infection causes a wide spectrum of disease, varying from "influenza-like" symptoms, such as malaise, fatigue, and high fevers, to a worsening atypical pneumonia (5, 64-69). In addition, gastrointestinal manifestations and diarrhea were frequently reported in SARS cases (1, 5, 65-66). Globally, the fatality rate was approximately 10%, but approached 50% in people 65 years of age or older and those with underlying illness (WHO update49050703). SARS-CoV-induced mortality is mainly characterized by progressive respiratory failure, due to a massive inflammatory response within the lung (ARDS) and a systemic component with widespread extrapulmonary dissemination, resulting in virus shedding in respiratory secretions, stools, urine, and possibly even sweat (65, 69-73). Lung-pathology findings in fatal SARS cases were dominated by diffuse alveolar damage, epithelial cell proliferation, an increase in macrophages and other severe pulmonary appearances (69, 72, 74-75). Interestingly, laboratory findings in infected individuals included lymphopenia and neutrophilia (66, 76-78). In contrast to the incubation period of other respiratory pathogens, SARS-CoV typical incubation period is 4-6 days, yet sometimes as short as 2 days, and peaks at around day 10 and subsequently declines (1). Transmission of SARS has appeared to occur primarily by direct person-to-person contact, droplet and airborne routes (5). Equally important, the virus is shed in the feces and urine, making it plausible that fecal-oral transmission can occur (65).

For a virus to cause disease, viral attachment proteins must first bind to specific receptors and, in some cases, co-receptors on the host cell surface, allowing entry into the

cell. Thus, the discovery of virus receptors can provide insight into mechanisms of pathogenesis. Studies using pseudotyped retroviral and lentiviral vectors, containing the SARS-CoV spike (S), membrane (M), and envelope (E) proteins independently and in combination, revealed that the S protein is both indispensable and necessary for virus attachment to target cells (79-82). SARS-CoV S protein also induces membrane fusion of the viral envelope with host cell membranes, using a mechanism similar to that of class I fusion proteins such as human immunodeficiency virus (HIV) gp160, influenza virus hemagglutinin (HA), and paramyxovirus F protein (83, 84), a process associated with conformational changes of the S protein. The functional receptor for SARS-CoV has been shown to be a type I transmembrane metallopeptidase, angiotensin-converting enzyme 2 (ACE2) (85).

SARS-CoV spike protein plays an essential role in the pathogenesis of SARS. SARS-CoV is believed to have been transmitted from palm civets to humans during an interspecies- jumping event. Characterization of human and palm civet receptor usage revealed that human SARS-CoV can bind both human and palm civet ACE2 whereas the palm civet virus can only bind to palm civet ACE2. Li et al reported mutations of two key residues in the receptor-binding domain of the SARS-CoV S protein, responsible for the adaptation of the virus to human hosts (86). In comparison of the spike protein from palm civets and humans, molecular analysis of key residues in the receptor-binding domain showed an accumulation of mutations over a 2-year period, suggesting that changes in the receptor- binding domain of the S protein can alter host cell specificity and viral pathogenesis, resulting in pathogenic viruses that cause severe disease in humans (28, 87).

The major targets of SARS-CoV infection are alveolar Type II pneumocytes and ciliated cells of the airway epithelium (1, 5, 88, 89). ACE2 is expressed in the lungs, heart, kidney, and small intestines as well as other tissues (90, 91). Overall, the receptor expression pattern explains the tissue tropism of SARS-CoV for the lung, small intestine and the kidney. Still, noteworthy contradictions include the absence of virus in endothelial cells, where ACE2 is abundantly expressed, compared to the presence of SARS-CoV in colonic epithelium and hepatocytes that lack ACE2 expression (73, 91). These inconsistencies suggest that the presence of ACE2 may not be the only determining factor for tropism of SARS-CoV. The proteolytic enzyme cathepsin L seems to play an important role in the interaction of SARS-CoV and ACE2 expressing cells. Simmons et al demonstrated that SARS-CoV infection was obstructed by specific inhibitors of the pH-sensitive endosomal protease cathepsin L (92). In spite of high expression of ACE2 on endothelial cells, they express low levels of cathespin L, suggesting that differences in expression of cathespin L in various cell types may explain these inconsistencies in SARS-CoV infection in relation to ACE2 expression patterns (91, 92).

DC-SIGN (CD209) and its homolog L-SIGN (also called DC-SIGN-R,CD209L) are C-type lectins that recognize high mannose-containing carbohydrate residues, present on viral-enveloped glycoproteins. DC-SIGN is abundantly expressed at the surface of dendritic cells (DCs) localized in the lymphoid tissues and certain macrophages (93, 94).

L-SIGN is mostly expressed on endothelial cells in the liver, as well as those in lymph nodes and the lungs (95). DC-SIGN and L-SIGN function as viral attachment factors by enhancing viral entry and facilitating infection of cells that express the cognate entry receptor (in-*cis*). They are also able to capture viruses and transfer viral infections to other target cells (in-*trans*) (96-99). Both have been reported to be additional receptors for SARS-CoV, yet cells expressing DC-SIGN or L-SIGN in the absence of ACE2 are not susceptible or are only partly susceptible to SARS-CoV infection, suggesting that binding to these molecules enhances SARS-CoV infection of ACE2 expressing cells (5, 92, 100, 101). Several reports demonstrate in-trans transmission of SARS-CoV by dendritic cells to susceptible target cells (79,101). Even though the dendritic cells examined were capable of transferring infectious virions via a synapse-like structure, permissiveness for SARS-CoV infection has not been shown (79). A similar mechanism has been described for HIV, in which HIV-1 travels with its target cells to lymph nodes where the virus is transferred to T cells at the immunological synapse (99). Viral exploitation of hijacking these two calcium-dependent lectins subverts the host's innate immune defenses and supports virus survival, an observation that may be relevant to SARS pathogenesis.

The main cause of death in SARS patients is the development of ARDS. ARDS is the most severe clinical form of acute lung injury (ALI) caused by a mixture of indirect or direct processes that injure the lung (102). ARDS is best characterized by diffuse alveolar damage, which is the principle lung pathology observed in fatal SARS cases (69, 74-77,102). Interestingly, in addition to functioning as SARS-CoV receptor, ACE2 plays an essential role in the renin-angiotensin system (RAS), which may contribute to the pathogenesis of SARS. The renin-angiotensin system (RAS) plays a central role in the regulation of cardiovascular and renal functions by maintaining blood-pressure homeostasis and electrolyte balance (103,104). ACE2 is the only human homologue of the key regulator of blood-pressure angiotensin-converting enzyme (ACE). ACE is a metalloproteinase that converts angiotensin I (Ang I) into the potent vasoconstrictor angiotensin II (Ang II), inducing hypertension while also inducing cell proliferation and fibrosis (105,106). Contrary to ACE, ACE2 functions as a carboxypeptidase and negatively regulates the renin-angiotensin system by processing angiotensin I and angiotensin II (Ang II) into Ang-(1-9) and Ang-(1-7) respectively. Ang-(1-7) is known to act as a vasodilator, with anti-proliferative and apoptotic functions, as a result, antagonizing the actions of Ang II (105,106).

Animal experiments using a murine model of ALI demonstrated that ACE2 protects mice from severe acute lung injury induced by acid aspiration or sepsis, which was facilitated by inactivation of Ang II (107). Furthermore, it was reported that mice deficient for ACE show significantly improved disease (107). Kuba et al reported that binding of SARS-CoV S protein to ACE2 considerably reduces the expression of ACE2 in the lung, resulting in a diminished protective role of ACE2 and subsequently acute respiratory failure (108). In addition, intraperitoneal injection of recombinant SARS-CoV S protein intensified ALI in mice, and this effect was ACE2 specific (108). Viral determinants of SARS-CoV pathogenesis remain to be elucidated. Interestingly, these

observations provide a possible molecular explanation for the acute respiratory distress syndrome and lethality associated with severe SARS disease.

SARS-CoV Genomic Organization and Expression Strategy

Coronaviruses' virions are spherical enveloped particles about 100 to 160 nm in diameter (1). The association of the large single-stranded positive-sense RNA genome and the N phosphoprotein forms the long, helical nucleocapsid, inside the virion (1,5, 6). The virion surface is surrounded by one or two types of projecting spikes. All CoVs possess the large, petal-shaped spikes, formed by the spike (S) glycoprotein, giving them their distinctive crown-like morphology, which can be seen under the electron microscope (1,2,5,6). The S glycoprotein mediates binding to host cell receptors and membrane fusion whereas the smaller spikes that span the surface of the virion consist of the hemagglutinin-esterase (HE) glycoproteins and are only present in some *Betacoronaviruses,* such as HCoV OC43 and Mouse Hepatitis Virus (MHV) (1,5,109,110) **Figure 1.2.** The virion envelope also contains the transmembrane (M) glycoprotein, a triple-spanning integral membrane protein, which spans the lipid bilayer three times (1,6,113), and a small envelope (E) protein, which is the least abundant viral protein present on the virion envelope but has been shown to play a major role in viral assembly (1,5,6).

The genomic organization of CoVs is very well conserved among all known coronaviruses. The coronavirus genome is a non-segmented positive-stranded, 5'-capped, 3'-polyadenylated RNA molecule that can function as messenger RNAs (mRNAs) (1). Encoded within the 5' end, approximately two thirds of the polycistronic genome is the replicase gene, which consists of two large, overlapping, open reading frames (ORF) 1a and 1b that specify the viral nonstructural proteins (nsps). Encoded in the 3' end, approximately one third of the genome is the structural proteins arranged in the order hemagglutinin esterase (HE), if present, spike (S), envelope (E), membrane (M) and nucleocapsid (N) and internal (I) protein encoded within the N gene for some CoVs(1,2,5,6). Interspersed between the structural genes is a series of ORFs, encoding group-specific accessory proteins, which differ among CoVs in number, nucleotide sequence, and gene order, but are conserved within the same genera (1). Although these gene products are dispensable for virus replication, it is suggested that these proteins play a role in virus-host interactions or interfere with the host's innate immune responses (6, 112-115).

Fourteen functional pen reading frames have been identified in the genome of SARS-CoV (4, 116). Conserved in all CoVs are homologs of proteins encoded by the overlapping ORFs 1a and 1b, and by ORFs 2, 4, 5, 6 and 9a (4). Interestingly, neither SARS-CoV nor MERS-CoV genomic sequence contains a gene for the HE protein, which is present in most *Betacoronaviruses* (3, 4, 109, 110, 116-117). Translation of ORF1a and ORF1ab into two large precursor polyproteins (pp), pp1a and pp1ab, is dependent on a ribosomal frame-shifting signal encoded near the end of ORF1a (1,3,4,116,118). The signal regulating the frame-shift consists of a slippery sequence and
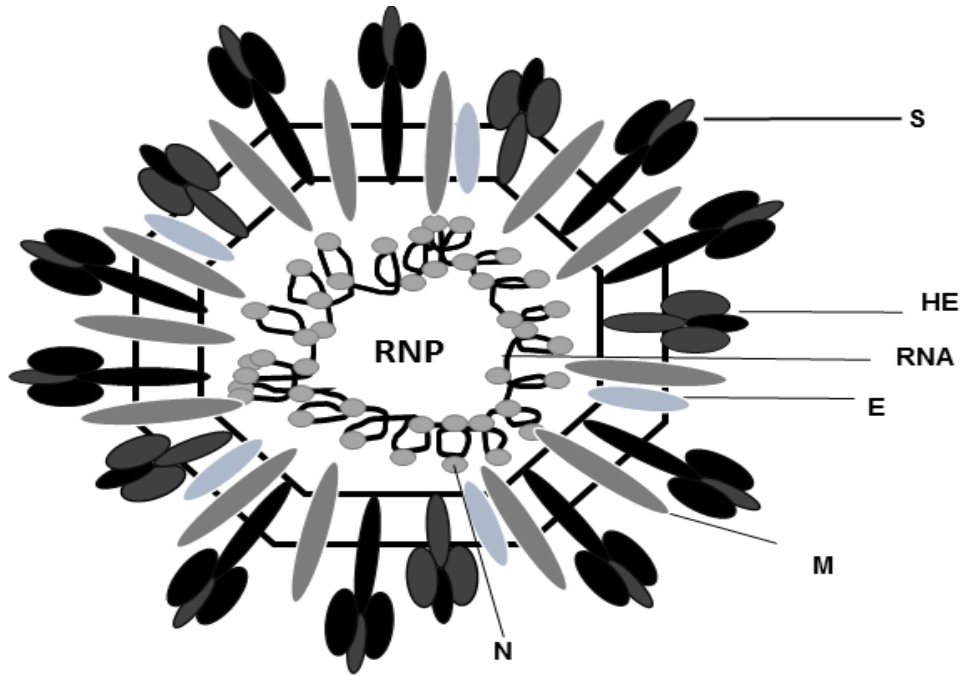
Figure 1.2.   Schematic of coronavirus virion structure.

S, spike glycoprotein; HE, hemagglutinin-esterase glycoprotein; M, membrane glycoprotein; E, small envelope protein. RNP; RNA-nucleocapsid protein.

a downstream pseudoknot structure formed by the genomic RNA (5, 119). Encoded within the polyproteins are two virally encoded cysteine proteases, the papain-like protease (PLpro) and a 3C-like protease (3CLpro), which in some cases is referred to as main protease (Mpro). These cysteine proteases are excised by their own proteolytic activity and are responsible for further processing the pp1a and pp1ab polyproteins (3,118 120). Processing of the two large polyproteins is necessary for the release and maturation of 16 nonstructural proteins (nsp1-16) (120).

These nonstructural proteins form a membrane-associated, large multi-subunit protein replicase complex, which utilizes a reticulovesicular network of double membrane vesicles (DMV), originating from the rearrangements of the endoplasmic reticulum (121,122). Along with many unknown cellular factors, this replicase protein complex is responsible for the replication of the viral genome from a full-length negative-stranded template, and the transcription of a nested set of eight subgenomic (sg) mRNAs, used for translation of structural and accessory proteins (3,4). It has been suggested that the establishment of DMVs is initiated by the recruitment of replicase proteins to host membranes, a process facilitated by several transmembrane domain-containing replicase products, such as nsp3, nsp4 and nsp6 (122-125). This has also been seen with arteriviruses, another family within *Nidovirales* order (126, 127). This viral replication strategy may provide a shielded environment for the protection of viral double-stranded RNA intermediates from the host cell's innate immune-detection sensors.

SARS-CoV Multi-Domain Non-Structural Protein 3 (NSP3)

Among the 16 nsps encoded within SARS-CoV replicase polyprotein, special attention has been given to nsp3, since it is becoming apparent that protein domains within nsp3 may serve roles in pathogenesis that are distinctive from viral replication. The SARS-CoV nsp3 protein is the largest nsp in the genome, with 1922 amino acids, containing several structural and functional domains (3, 4). Nsp3 of SARS-CoV has been well characterized structurally by either X-ray crystallography or NMR. It has been estimated that SARS-CoV nsp3 has about 14 domains: UB1, AC, ADRP, SUD-N, SUD-M, SUD-C, UB2, PL2pro, NAB, G2M, TM1, ZF, TM2, and Y, which may have 3 structural domains (128). NMR studies were used to determine the structure of the highly conserved N-terminal region of nsp3, exhibiting an ubiquitin-like 1 (UBL1) globular fold, followed by an acidic domain (AC domain) rich in glutamic acid (129). The UBL1 domain is structurally similar to Ras-binding proteins and interferon-stimulated gene 15 (ISG15) (128). Although the functional significance of the UBL1 domain has yet to be experimentally validated, these structural similarities may suggest that this domain may be important for regulating the host cell responses, thus promoting survival. Following this is the ADP-ribose-1"-phosphatase (ADRP) domain, also called the X domain or macro domain, thought to play a role during SARS life cycle (130). The SARS Unique Domain (SUD) follows next and has been considered a domain unique to SARS-CoV (4). It has been shown that the SUD domain is made up of three domains, named by their location the N-terminal SUD-N, the middle SUD-M and the C-terminal SUD-C (131).

Recently a new domain was identified in MHV nsp3. Interestingly, the authors show that this novel domain has close structural homology to the SARS-CoV unique domain C (SUD-C), suggesting that the SUD domain may not be unique to only SARS-CoV (132).

Following the SUD domain are an additional UBL domain (UBL2) and the catalytically active PLpro. Unlike other CoVs that encode two different PLPs, SARS-CoV encodes only one PLP domain termed PLpro within nsp3, which is essential for processing the amino terminal end of the replicase polyprotein at 3 junctions, through the recognition of LXGG motif, releasing nsp1-nsp3 mature proteins respectively (133). It is well known that when two PLP domains are encoded within the coronavirus replicase polyprotein, the PLP2 recognizes the LXGG motif and has similar characteristics to SARS-CoV PLpro (134). Several studies have revealed that SARS-CoV PLpro is a deubiquitinating (DUB) enzyme and has de-ISGylating activity (134-139). The host cell uses both ubiquitin (Ub) and ISG15 as unique signaling components to promote the antiviral innate immune responses during SARS-CoV infection. The fact that the PLpro can cleave and disrupt important host cell innate immune elements illustrates the multifunctional nature of this protease and suggests that it is an excellent drug target for the development of antiviral treatments. Downstream of the PLpro domain are a nucleic acid-binding domain (NAB), which may function with RNA chaperone activity, and one unfamiliar domain named the marker domain (G2M)(125). Subsequent to the G2M domain are two transmembrane domains, a putative metal- binding region (ZN) and the Y domain, whose function is unclear (3, 4,125).

Due to the large size of the nsp3 protein, in vitro studies examining the functional role of domains contained in nsp3 during SARS-CoV infection have predominantly made use of truncated domain constructs. Specifically, it is unclear whether the protein domains within nsp3 impact the innate immune responses when expressed in the context of the full-length nsp3 protein, which is the case in virus-infected cells. Therefore, an enhanced understanding of viral components that are critical for efficient replication and negative regulation of the host innate immune responses, ultimately regulating pathogenesis and virulence, is essential to understanding SARS pathogenesis.

The Effect of Virus Infection on Host Innate Immunity

Virus infection represents an evolutionary arms race between virus and the host. This antagonistic relationship leads to the host utilizing its intrinsic defense mechanisms in an attempt to restrict and eliminate virus infection. Viruses, in turn, have adapted multiple strategies to subvert or even manipulate the host defenses to promote its own infection. The host innate immune system is the first line of defense against invading pathogens, including viruses. Hosts have to sense viral pathogens and induce immune responses for protection. Upon the detection of invading viruses, host cells mount an immediate antiviral response. While viral recognition triggers early antiviral immune defenses promoting virus elimination, a consequence of this strong, immediate, innate

immune response may be an aberrant inflammatory response that could cause severe disease in the host. Understanding virus and host interactions is critical to the prevention and treatment of viral diseases.

The host innate immune system is equipped with cellular pattern-recognition receptors (PRRs) that recognize conserved viral structural components that are recognized as foreign to the host, called pathogen-associated molecular patterns (PAMPs) (140). Toll- like receptors (TLRs) were the first PRRs to be identified and remain the best studied. TLRs are expressed in many cell types including immune cells, such as macrophages and dendritic cells, and are localized on the cell surface or endosomal compartments (141, 142). There are 5 TLRs that are key in the detection and control of viral infection. TLR3 recognizes dsRNA, a replication intermediate generated during the life cycle of many RNA viruses. TLR2 and TLR4 sense viral structural proteins or glycoproteins, TLR7 and TLR8 recognize viral ssRNA, and TLR9 senses non-methylated viral CpG containing DNA (140-142). Thus, viral attachment to the host cell is sensed by external TLRs, while viral invasion is recognized by TLRs localized internally. Although no TLR has been directly implicated in the recognition of SARS-CoV, a protective role for TLRs adaptor proteins was established in MA15-SARS-CoV infection. Wild-type mice infected with MA15-SARS-CoV exhibited transient weight loss, from which they recovered after 7 days; however, MyD88-deficient mice lost significantly more weight, all of which died by day 6 post infection (143). Most recently, Totura et al demonstrated that mice deficient in the TLR3/TLR4 adaptor TRIF are highly susceptible to SARS-CoV infection, observing a lung pathology that was similar to those seen in human patients with severe SARS disease (144). The TRIF-deficient mice also exhibited increases in weight loss, mortality, and higher viral titers (144). Additionally, TLR4 was identified as a protective host factor against *Betacoronavirus* Mouse Hepatitis Virus-1 (MHV-1) in a respiratory model of SARS disease (145). These findings highlight the significance of TLR3/TLR4 adaptor proteins in mediating a protective antiviral innate immune response to highly pathogenic coronavirus infections. TLRs recognize a broad range of PAMPs; however, it is noteworthy to mention that PRRs other than TLRs are involved in PAMP recognition and in the regulation of the innate immune response.

Retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), such as RIG-I and MDA5, are also an important class of PRRs that have been shown to recognize conserved viral-specific components (146). These PRRs are ubiquitously expressed and are localized in the cytosol where they survey the cytoplasm for viral dsRNA (147). Although RIG-I and MDA5 both recognize viral RNA, they play differential roles in the viruses they sense. The difference in the recognition of RNA viruses by RIG-I and MDA5 has been credited to their distinctive preferences for viral RNA ligands. For RIG-I, 5′-triphosphate (ppp)-containing RNAs at least 20 base pairs in length—as well as short, blunt-ended dsRNAs—have been shown to be the most favorable RIG-I agonist (148,149). Although MDA5 ligands are less defined, it has been established that their role is more dependent on long and branched-structure dsRNAs (150). Overall, RLR and TLR pathways are not redundant, but rather allow the host to combat the virus infection more efficiently by sensing viruses through multiple pathways by multiple mechanisms in different cell types.

In vitro studies indicate that RIG-I and MDA5 are transcribed during SARS-CoV infection; however, it is not known whether SARS-CoV is recognized by these PPRs (151). On the contrary, MHV has been shown to be recognized by MDA5 and RIG-I in a cell-specific manner (152,153). Considering that MHV and SARS-CoV replicate in the cytoplasm where they produce large amounts of dsRNA, it is likely that SARS-CoV could be detected by the same sensors. TLRs and RLRs recognize viral PAMPs during virus infection to activate the host intracellular defense signaling cascades, resulting in the production of Type I Interferons, inflammatory cytokines and chemokines, and the subsequent activation of adaptive immunity (146).

One of the most efficient and fundamental events in the induction of the early-phase antiviral innate immune response is the production of Type I Interferons, named for their ability to interfere with viral replication (154). There are two essential events of the Type I IFN system: first, the synthesis of Type I IFNs; second, the response to secreted Type I IFN's, Type I IFN-mediated signaling. Type I IFNs can be classified into two principal classes: IFN-β, the immediate early genes expressed by the initial response to invading viruses, and IFN-α, the delayed set of genes expressed by a secondary de novo protein-synthesis pathway (155). At the molecular level, the induction of type I IFNs is initiated by sensing the invading viral pathogen through the appropriate PRR(s), which triggers multiple and distinct intracellular signal transduction. Although the pathways may differ initially, they all converge to activate the latent transcription factors such as nuclear factor-kappa B and the interferon regulatory factors (IRFs).

The IRF transcription factors are key regulators in the synthesis of type I IFNs. In particular, IRF-3 and IRF-7 are vital. IRF-3 is constitutively expressed in most cell types and plays a central role in the production of IFN-β (156). In the absence of virus infection, this transcriptional factor resides in the cytosol in an inactive state. Upon virus infection, IRF-3 undergoes serine phosphorylation in its C-terminal region by kinases TBK1 and IKK□, allowing its dimerization (157,158). The dimeric form of IRF-3, either a homodimer or a heterodimer with IRF-7, then translocates to the nucleus, where it recruits the transcriptional co-activators CBP and p300, and binds to its target IFN-β promoter along with other transcriptional factors to initiate IFN-β mRNA synthesis (156). This initial wave of IFN triggers expression of a highly homologous IRF, IRF-7. IRF-7 is expressed constitutively at low levels mainly in immune cells. However, it can be strongly induced by type I IFN-mediated signaling, making it an important element in the IFN-α/β positive feedback loop, where IFN-α/β enhances its own expression (159). This phenomenon occurs in a two-step process where IRF-3 induces the early expression of IFN-β and IFN-α. These genes subsequently signal through the IFN-α/β receptor and the JAK-STAT pathway to induce IRF-7 expression, which contributes to the amplification of the transcriptional response through a second wave of interferon gene expression, which includes other IFN-α genes that are not induced by the initial stage of virus infection (159). Similar to IRF-3, IRF-7 is located in the cytosol in an inactive form. Upon virus infection, kinases TBK1 and IKK□ phosphorylate IRF-7 on its serines in its C-terminal region, allowing dimerization (157, 158). The dimeric form of IRF-7, either a homodimer or heterodimer with IRF-3, induces Type I IFN gene expression (159).

Once transcribed and translated, secreted IFN-β and IFN-α bind to a cell surface heterodimeric receptor complex consisting of alpha/beta interferon receptor (IFNAR) 1 and IFNAR2 subunits. IFN-β and IFN-α act in an autocrine/paracrine fashion, and bind to its cognate receptor on the surface of the same cell or neighboring cells to induce IFN-β and IFN-α gene expression and subsequently activate the JAK-STAT pathways (160). The signal transducers and activators of transcription 1 (STAT1) and STAT2 proteins are transcriptional factors that reside in the cytosol in a latent form, which become phosphorylated by members of the Janus kinase (JAK) family, JAK-1 and TYK-2 (160). Phosphorylated STAT1 and STAT2 form heterodimers that recruit IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) complex. This heterotrimer complex is translocated into the nucleus where it binds to IFN-stimulated response element (ISRE) to induce expression of a large number of IFN-stimulated genes (ISGs), resulting in the establishment of an antiviral state. IRF-3 and IRF-7 are well known as master regulators of Type I IFN responses and are firmly integrated within the TLR/RLR dependent pathways of the innate immune response to invading viral pathogens. Since IRF-3 plays an essential role in the early induction of antiviral gene expression, many viruses have developed various mechanisms to inhibit, either directly or indirectly, the activation of IRF-3, thus blocking or limiting IFN production (156,160). It is well known that the initial virus-host interactions may significantly impact the course and/or outcome of the infection. Therefore, understanding the mechanisms by which viruses regulate the early host innate immune responses is critical to the treatment and prevention of emerging infectious diseases, such as SARS and MERS.

With respect to the role of the innate immune system in SARS-CoV infection, the fact that SARS-CoV replicates increasingly in the respiratory tract during the first ten days of the disease raises the assumption that SARS infection may cause deficiencies in the host innate immune response (5). The relationship between SARS-CoV and the IFN-α/β response is puzzling. Interestingly, in-vitro studies of SARS-CoV, contrary to other viruses, typically demonstrate that SARS-CoV is a poor inducer of type I IFN (161, 162). Furthermore, irregular IFN, Interferon Stimulated Genes (ISGs), and cytokine responses were observed in SARS patients compared to healthy individuals, providing evidence that SARS may be an innate immune-regulated disease (161,163).

Induction of the Antiviral State by Interferon

In mammals the interferon system is essential for survival because it provides an early line of defense against viral infection. This system is designed to inhibit viral replication and block the spread of virus infection in the host. In addition to their antiviral activities, IFNs also have antiproliferative, antitumor, and immunomodulatory activities, all of which have a profound effect on the physiology of the cell (154,164). There are three main IFN families: Type I (IFNα/β) and III (IFNλ) IFNs, which are most recognized for their antiviral activities, and type II (IFNγ) IFNs, which has antiviral activities but is best known for its immunomodulatory effects (164). The production of IFNs is regulated by a highly complex and coordinated sequence of signaling events

facilitated by PRRs, adaptor proteins, kinases, and transcription factors (165,166). IFNs are synthesized in host cells in response to viral infection, secreted into circulation, bind to their cognate receptors, and activate the JAK/STAT pathway to enhance the expression of hundreds of different ISGs, leading to the establishment of the antiviral state (165, 166).

ISGs are a diverse group of more than 300 genes that mediate the biological effects of IFNs (1, 164). In addition to their roles as downstream effectors of IFN, a subset of ISGs is induced in parallel with IFN by dsRNA, and this subset is thought to mediate the primary response to virus infection (1, 154). Different ISGs inhibit different steps of viral life cycles, such as viral entry, un-coating, transcription, translation, assembly and egress (165, 166). This distinctive strategy allows the IFN system to coordinate a multifaceted attack on virus replication. Studies of ISGs products' mode of action have led to important findings concerning translational control, RNA stability and editing, and protein transport and turnover (1, 164). Among the several hundreds of ISGs transcriptionally regulated by IFN, only a few have been extensively characterized. These genes encode the dsRNA-activated protein kinase (PKR), the 2',5'-oligoadenylate synthesases (2′–5′ OAS), ribonuclease L (RNase L), the mxyovirus (Mx), and ISG56. The functions of these proteins are known to be essential for the induction of an antiviral state by IFN.

PKR is constitutively expressed at low levels but not functional until activated (167). PKR is activated by the binding of viral dsRNA generated in virus-infected cells as by-products of viral replication or transcription. Binding of dsRNA to the N-terminal dsRNA binding motifs (dsRBM) of PKR relieves steric inhibition of the kinase domain; PKR dimerizes, autophosphorylates and becomes activated (168). Phosphorylated PKR can phosphorylate other proteins, but not other inactive PKR molecules (169). Therefore, autocatalytic activation of PKR is prevented. In addition to activation by viral RNAs, RNAs of cellular and synthetic origin such as IFNγ mRNA and poly I:C can activate PKR. It has been demonstrated that PKR binds to a pseudoknot structure, with sufficient double-stranded character, in IFNγ gene's 5' untranslated region (UTR), which subsequently activates PKR to then inhibit translation of the transcript via phosphorylation of eukaryotic translation-initiation factor 2α (eIF-2α) (168). Furthermore, two additional ligands, heparin and the PKR activator (PACT)—which is the only known protein activator of PKR—have been described to directly activate the kinase (1,154, 170).

Active PKR mediates translational control by phosphorylating the eIF-2α, resulting in global inhibition of protein synthesis that blocks further viral gene translation and full amplification of the viral-induced cellular stress response (1, 164). Therefore, activation of PKR causes inhibition of cellular protein synthesis and apoptosis, which is one unique strategy for IFN-induced inhibition of viral replication and spread. Besides its role as a regulator of protein translation, PKR also plays a role in cellular signaling. In response to stimuli such as poly I:C and TNFα, PKR regulates the activation of NF-κB through the phosphorylation and degradation of IκBα (1). In addition, PKR has been suggested as a serine kinase for STAT1, a modification that is required for IFN signaling

(1). The biological significance of PKR is further observed by the existence of cellular and viral regulators of PKR action. Because PKR promotes cellular apoptosis, viruses encode or induce antiapoptotic proteins such as P58IPK, a cellular protein recruited by influenza virus, to keep cells alive during virus replication (1).

The IFN-inducible 2′–5′ OASs are a family of proteins of different molecular weights encoded by multiple genes (154). Viral dsRNA can directly activate the OAS proteins. Activation of OAS does not involve a known RNA binding domain; however, it has been shown that the RNA binding site consists of a groove of positively charged amino acids formed from noncontiguous regions of OAS (171). Activated OAS uses ATP to synthesize 2′,5′-linked oligomers of adenosine (2′-5′A) molecules, yielding mainly a series of short 5′-triphosphorylated triadenylate called 2-5A [ppp5′(A2′p5′)2A] (172-173). The 2′–5′A system also plays a role in antiproliferative activities such as induction of apoptosis, senescence, and differentiation, suggesting that OAS is also activated by cellular RNAs in the absence of viral infection (174-176).

The best characterized function of 2′–5′A is activation of the endoribonuclease RNase L. The 2′–5′A molecules bind to the inactive monomers of RNase L, triggering its dimerization through their kinase-like domains and activation (177). RNase L functions to cleave viral ssRNA with specificity for sites 3′ of UpUp and UpAp sequences, and thus leads to degradation of viral RNAs (164). On the other hand, activated RNase L also degrades both cellular mRNA and rRNA in the cytoplasm of the cell, leading to damages of the host cell machinery, which is required for viral replication and can result in apoptosis. This likely contributes to the antiviral actions of RNase L. Moreover, RNase L also cleaves self mRNAs and produces small RNAs that function to activate the RLRs to induce IFN-β, thus perpetuating and amplifying IFN-β production in virus-infected cells (164).

The IFN-inducible antiviral protein MX is a key mediator of innate antiviral defenses induced in host cells. The MX potent action in early antiviral host defenses was identified by studies in genetically defined mouse strains resistant to influenza A viruses. These studies revealed that resistance was caused by a single gene, *Mx1*, localized on chromosome 16 (172,178). Subsequently, two human Mx genes were identified and shown to encode for proteins called MxA and MxB, respectively (178). Mx proteins are large GTPases in the dynamin superfamily, which self-assemble into horseshoe- and ring-shaped helices and bind to viral nucleocapids (178,179). They inhibit viruses by interfering with intracellular trafficking and activity of viral polymerases, thus blocking an early stage of the replication cycle (164). The subcellular location of Mx proteins to some extent appears to contribute to their antiviral effects on a particular virus. These proteins can be located in the cytoplasm or in the nucleus. Nuclear Mx proteins have been shown to confer resistance to viruses, such as Influenza A and Thogoto virus, which are known to replicate in the host cell nucleus (164,178). Cytoplasmic murine Mx proteins inhibit the replication of viruses that replicate in the cytoplasm, such as VSV and LaCrosse virus (LACV) (178). In contrast, some human cytoplasmic Mx proteins have a wide-range antiviral specificity against different types of viruses, regardless of their intracellular replication site. These MxA-sensitive viruses include members of the

bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, picornaviruses, reoviruses and hepatitis B virus, a DNA virus with a genomic RNA intermediate (178).

ISG56, which encodes for P56, is one of the first interferon (IFN)-inducible genes to be discovered and is the most potently induced gene among all ISGs (154). ISG56 belongs to the IFIT genes, which are grouped on human chromosome 10 and consist of 4 members including ISG56, ISG54, ISG60 and ISG58. (180, 181). Normally most cell types express very low constitutive levels of ISG56 in the absence of stimuli. However, induction of ISG56 is initiated by many stimuli, such as IFN, dsRNA and many viruses. Since ISG56 mRNAs are very abundant in virus-infected cells, they are used extensively as read-outs for studying transcriptional regulation of ISGs by IFN, PRRs, and other signaling molecules such as IRF-3 (180). The most potent inducer of ISG56 is type I IFNs (IFNα/β) and type III IFNs (IFNλs), whereas type II IFN (IFN-γ) is a much weaker inducer. Although novel insights into the functions of the IFIT-encoded proteins continuously emerge in the literature, the best described function of ISG56 and ISG54 is the inhibition of cellular translation by binding to specific subunits of eukaryotic initiation factor 3 (eIF3), presenting a mechanism of cell growth inhibition distinct from other ISGs such as PKR and OAS (180, 181). Use of this distinct strategy may possibly delay or inhibit virus replication by diminishing the overall cellular metabolism. Interestingly, a newly identified function of ISG56 protein product p56 has been reported. Direct binding of p56 to Human Papillomavirus (HPV) E1 helicase inhibits E1 helicase activity and sequesters the majority of HPV E1 in the cytoplasm, separating it from the viral genomes in the nucleus, thus inhibiting viral DNA replication (181-183).

Lastly, many IFN-pathway signaling proteins, such as RIG-I and MDA5, are themselves ISGs, thus providing an autocrine loop that augments IFN responses. Similar to RLRs, PKR and OAS proteins are classified as PRRs for the viral PAMP, double-stranded RNA (dsRNA). However, they differ from typical PRRs in that ligand binding results in direct activation of their enzymatic activity rather than initiation of a signaling cascade (142, 146,184). Therefore, PKR and OAS can function as early sensors to viral infection and can also be augmented as a secondary effect of IFN induction through TLR/RLR pathways. The importance of ISGs is further proven by the finding that several virally encoded antagonists can specifically interrupt the functions of ISGs products, thus blocking the establishment of the antiviral state.

IFNs provide a powerful immediate cellular defense against viral infection and thus are fundamental for mammals' survival. The IFN system is key to the inhibition of viral replication and viral spread, which is mediated through the action of specific ISGs. The restriction of virus replication by several of these IFN-induced proteins is associated with a multitude of physiological changes for the host cell. Although some ISGs function to confer a total disruption in the cellular translational machinery to maximize the host control of virus infection, IFNs also induce counteractive signals that limit the duration or toxicity of IFN-mediated responses to the host, in an attempt to prevent deleterious effects and facilitate cell survival in uninfected cells. Most importantly, these responses constitute a negative feedback loop to counteract the massive reorganization of cellular

metabolism and machinery triggered by IFN. Furthermore, it is important to consider that many IFN-induced antiviral proteins in virus-infected cells are directly induced independent of IFN (1). This consideration suggests that these powerful coordinated protein functions all work in concert to achieve a fully functional antiviral state and to maintain host-virus homeostasis, thus exemplifying the importance of the host innate immune system during viral infection.

Overview of IFN Antagonists

In order to successfully replicate and spread in a host, a virus must circumvent multiple cellular intracellular signaling pathways regulating a wide variety of host cell functions. The production of IFNs is an essential mechanism of the host response to viral infections. However, a main priority for most invading viral pathogens is the down-regulation of the IFN system, a powerful first-line host defense against virus infection. The strategies that viruses use to counteract the IFN system are plentiful and include the inhibition of IFN synthesis, the inhibition of IFN-mediated signaling pathways, and the disruption of the action of IFN-induced proteins with antiviral activity (160). Several different viruses contain an ever growing number of IFN-antagonistic proteins that target almost all components of the IFN system. These IFN antagonists are typically multifunctional proteins that are involved in regulating several different functions in virus-infected cells. For example, the P proteins of some negative-strand RNA viruses like rabies virus are essential components of the viral RNA polymerase but also inhibit the induction of IFN-β in virus-infected cells by targeting TBK1, thus disrupting the activation of IRF-3 (185).

It is well known that CoVs possess multiple mechanisms by which they evade the host innate immune response, and it has been suggested that this immune evasion may contribute to severe coronavirus disease, such as SARS (113,114,186,187). The observation that SARS-CoV induces low, sometimes undetectable Type I IFNs following productive infection in cell culture, and is relatively resistant to the antiviral effects of IFN signaling, suggests that the genome may encode antagonists of IFN synthesis and signaling (162,188). Described below are a few examples of SARS-CoV proteins that directly affect either IFN induction or signaling.

SARS-CoV encodes the largest number of accessory proteins that share no homology with accessory proteins from any other HCoV. Although none are essential for virus replication in cell culture (189), SARS-CoV ORF3b and ORF6 were shown to block IFN induction and IFN signaling (114). How ORF3b antagonizes IFN induction has not been well-defined; however, the mechanism by which ORF6 antagonizes the IFN signaling arm of innate immunity has been illustrated. ORF6 protein disrupts the formation of the nuclear import complex by tethering karyopherin alpha 2 and karyopherin beta 1 to the membrane. The subsequent retaining of nuclear import factors at the membrane leads to a loss of STAT1 transfer into the nucleus in response to interferon signaling, thus inhibiting the expression of genes dependent on STAT-1 activation to establish an antiviral state (113). Furthermore, it has been shown that

MERS-CoV is more sensitive to PEG-IFN treatment than SARS-CoV is (190). This profound phenotype may be attributed to the lack of a SARS-CoV ORF6 homologue.

The Nucleocapsid (N) protein of SARS-CoV, a structural component of the SARS-CoV virion, has been shown to also act as an antagonist of IFN. Kopecky-Bromberg et al demonstrated that SARS-CoV N protein was able to inhibit the induction of IFN-β promoter gene expression (114). They also showed that in response to Sendai virus infection or polyI:C the N protein is able to block an ISRE promoter (114). This finding indicates that the N protein exerts its effects on both IFN induction and IFN-mediated signaling to downregulate the IFN system. Studies of the first nonstructural protein of SARS-CoV nsp1 suggest that this protein utilizes several mechanisms to antagonize Type I IFN production and signaling (191-193). Kamitami et al observed that in human 293 cells, SARS-CoV infection suppresses IFN-β mRNA accumulation (192). Using a two-pronged mechanism, SARS-CoV nsp1 mediates the degradation of host mRNAs and inhibits the cellular translational machinery in infected cells (192,193). Furthermore, nsp1 inhibits the phosphorylation of STAT1 (192). In addition to SARS-CoV nsp1, nsp7 and nsp15 have both been shown to act as potential IFN antagonists but are not well characterized (194).

Regulation of Innate Immunity by the PLpro Domain of Coronaviruses

Viruses encode proteases that can act as multifunctional proteins that not only generate their mature proteins, which is an essential step in viral replication, but also can play specific roles in the interaction of the virus with the host innate immune response, by cleaving or disrupting the function of key host proteins important for antiviral immunity. For example, the coxsackievirus B3 (CVB3) 3C^{pro} cysteine protease cleaves the innate immune adaptor MAVS and TRIF as a strategy to disrupt Type I IFN responses (195). Furthermore, hepatitis C virus (HCV) serine protease NS3/4A and hepatitis A virus 3ABC cysteine protease both use similar mechanisms to cleave MAVS, disrupting the RLR signaling pathway, thus abolishing mitochondrial targeting of the adaptor protein and ultimately suppressing antiviral innate immune responses (196-198). For these viruses and many more, the catalytic activity of the viral proteases represents a unique viral strategy to inhibit antiviral innate immune responses.

Whereas most CoVs contain two analogous enzymes called PLP1 and PLP2, SARS-CoV utilizes one termed PLpro contained within nsp3, to process the amino-terminal end of the replicase polyprotein (3-5,120). In addition to recognizing its protease activities, Sulea et al predicted that the PLpro of SARS-CoV possesses DUB activities based on structural similarities with herpesvirus-associated ubiquitin-specific protease (HAUSP), a cellular DUB enzyme (199). Thus, SARS-CoV's predicted ability to recognize the C terminus sequence of ubiquitin, which is common to ubiquitin-like molecules, has led to the assumption that the Plpro domain may also cleave ubiquitin-like molecules such as ISG15. Several studies purified the catalytic domain of PLpro and demonstrated that PLpro proficiently removes di-ubiquitin and branched polyubiquitin chains, cleaves ubiquitin-AMC substrates, and has deISGylating activity (135,139, 200,

201). These studies were the first to characterize the multifunctional nature of coronaviral PLPs. Since then, several groups have demonstrated the DUB and deISGylating activities of HCoV-NL63, MHV, and most recently MERS-CoV papain-like proteases (137,138,202). However, it is unclear if the DUB and deISGylating activities of coronaviral PLPs play a role in the viral replication cycle.

Ubiquitination and ISGylation are post-translational modifications that are essential for a wide variety of biological processes, including regulating the innate immune responses to pathogens (203, 204). It has been shown in a cell culture system that expression of ISG15 inhibited the release of HIV-1 virions from infected cells (205), diminished alphavirus replication (206) and inhibited Influenza A virus gene expression and replication in human cells (207). Furthermore, ISG15-deficient mice are more susceptible to several human pathogens, including influenza A and B viruses, herpesviruses, Chikungunya virus (CHIKV), vaccinia virus, and Sindbis viruses (208-213). Several laboratories have highlighted proteases DUB activity-mediated innate immune evasion by many virus groups such as arteriviruses, picornaviruses, herpesviruses and CoVs (186, 214). Ubiquitination and ISGylation are important elements of the host antiviral innate immune response, and SARS-CoV PLpro has the ability to negatively regulate these fundamental processes, an ability that may contribute to SARS pathogenesis.

Several reports have identified coronaviral papain-like protease domains as negative regulators of innate immunity, specifically IRF-3-dependent Type I IFN responses. Antagonism of the IRF-3-dependent Type I IFN system by coronavirus PLPs has been most extensively studied for the SARS-CoV PLpro. In 2007, Devaraj et al were the first to report that SARS-CoV PLpro had the ability to inhibit type I IFN production. It was shown that SARS-CoV PLpro mediated antagonism of type I IFN production functions upstream of IRF-3 activation by interacting with IRF-3 and inhibiting its phosphorylation, dimerization, and nuclear translocation. SARS-CoV PLpro was also able to inhibit the activation of the IFN-β promoter in HeLa cells stimulated with SeV infection or poly(I:C) treatment (186). It was also reported that the inhibition of the IFN response was independent of the protease activity and that the PLpro had no inhibitory effect on the NF-κB pathway (186). Subsequently, Frieman et al demonstrated that the PLpro domain inhibits IRF-3-dependent induction of IFN-β (194). However, the mechanism by which this occurs is controversial. Frieman et al confirmed that PLpro inhibits the phosphorylation of IRF-3; however, in contrast to the study detailed above, the authors demonstrated that there is no direct interaction between SARS-CoV PLpro and IRF-3 (194). Mutagenesis of the active site at two different residues, which has been shown to abolish catalytic activity, affected the PLpro antagonistic activity, but at varying degrees, with some mutants maintaining the IFN antagonist ability, and others losing their antagonistic activity. Interestingly, a variation in DUB activity of the mutants was also observed, suggesting that the catalytic activity of PLpro may play a role in IFN antagonism (165,194). However, HCoV-NL63 PLP2 has been shown to antagonize type I IFN production independent of its catalytic and DUB activities (215).

Studies detailing the IFN antagonist activity of MHV PLP2 domain have demonstrated that PLP2 can deubiquitinate and inactivate IRF-3 to inhibit IFN induction (216). Furthermore, the authors show that wild-type PLP2, but not the mutant PLP2 that lacks the DUB activity, can block IFN induction, thus suggesting that the viral DUB activity may be required for PLP2-mediated IFN antagonism (216). More recently, Wang et al reported that MHV PLP2 antagonizes IRF-3 dependent signaling by targeting TBK1, a kinase that induces phosphorylation and dimerization of IRF-3, and the authors suggest that this mechanism is dependent on PLP2 DUB activity (202). Later studies have also associated the ability of CoV PLPs to inhibit type I IFN induction with their ability to antagonize stimulator of interferon genes (STING), a scaffolding molecule required for the activation of IRF-3, and this ability is mediated by catalytic-dependent and catalytic-independent activities (217, 218).

Remarkably, it was reported that deletion of the UBL domain at the amino terminus of SARS-CoV PLpro led to a loss in IFN antagonistic function for both IRF-3 and NF-κB pathways; however, the protease and DUB activity remained intact (194). However, the contribution of the UBL domain to the SARS nsp3 IFN antagonism was not observed in the study by Clementz et al (215). Instead, when using purified wild-type and Ubl mutant proteases, Mielech et al demonstrated that the Ubl domain adjacent to PLP2 of MHV altered viral protease activity and stability. Furthermore, the authors found that these mutations resulted in a decrease of virus replication and an obvious attenuation of virulence (219). The relationship between the UBL domain present alongside the PLP and the protease activity remains elusive. However, it is quite clear that the Ubl domain may play a role in the pathogenesis of CoVs, and further studies are needed to elucidate the role of coronavirus protease/DUB activity in PLP-mediated interferon antagonism.

The underlying mechanisms that lead to the difference in IFN antagonism profiles of the PLPs from different CoVs are not clearly understood. Moreover, it is not clear if the catalytic activity of the PLpro is indispensable for functions unrelated to the proteolytic processing of the replicase polyprotein. Additionally, the catalytic activity and the DUB activity both depend on the same protease active site; therefore, it is difficult to study them independently during virus infection. As such, no direct evidence has been reported linking DUB activity to the suppression of PLpro-mediated innate immune responses in virus-infected cells. It is also important to consider that a mutation of the catalytic active site of the PLpro may not affect its complete ability to interact with ubiquitin and ISG15 molecules in virus-infected cells; therefore, IRF-3-dependent signaling could still be disrupted. Most importantly, the studies detailed above revealed that SARS PLpro and other CoV PLP2 domains are Type I IFN antagonists.

Unless specified, the studies described above make use of constructs consisting of just the PLpro domain and its transmembrane domain; however, in virus-infected cells, we do not know if the PLpro is exposed to execute the IFN-antagonizing function in the context of the full-length nsp3 protein. Thus, overexpression of the PLpro domain construct may not reveal a complete profile of the PLpro IFN antagonist properties. Therefore, examining the functional role of SARS-CoV PLpro should be in the context of

the full-length nsp3, which to date has not been done. Therefore, although a wealth of knowledge has been gained from these studies, it remains elusive whether the PLpro impacts the IRF-3-dependent innate immune responses when expressed in the context of the full-length nsp3 protein, which is the case in virus-infected cells.

Statement of Purpose

Within the 21st century, two novel human CoVs have emerged: SARS-CoV and MERS-CoV, both of which cause a highly pathogenic respiratory disease in the lung and are associated with high mortality rates. On the contrary, other previously known HCoVs, such as OC43 and NL63, generally cause mild upper respiratory tract infections. The emergence of MERS-CoV nearly ten years after the SARS-CoV pandemic demonstrates the capacity of highly pathogenic CoVs to continue to spill over from zoonotic reservoirs into the human population, with the potential to become pandemics. The emergence of both SARS-CoV and MERS-CoV highlights the significance of understanding the pathogenesis of *Coronaviruses*. Over the past 10 years, much has been learned about highly pathogenic CoVs from the investigation of SARS-CoV, which aids in our efforts to combat MERS-CoV; however, gaps in our understanding remain. It is well known that the initial virus-host interactions may dramatically impact the course and/or outcome of the infection. At present, the differences in the molecular and cellular mechanisms for how human CoVs interact with the host innate immune system, resulting in less severe or fatal outcomes, are poorly understood. Several reports have shown the papain-like protease (PLP) domain of coronaviral nonstructural protein 3 (nsp3) as a potent IFN antagonist by suppressing IRF-3-dependent innate antiviral defenses. The goal of this dissertation is to understand the mechanism by which the full-length nsp3 protein of different human coronaviruses regulates IRF-3-dependent innate immune responses.

- The objective of Chapter 3 is to create an in-vitro system that can be used to characterize the function of the full-length nsp3 protein of CoVs on the antagonism of the host type I IFN responses. By investigating the function of the full-length nsp3, I hope to assess whether the full-length nsp3 protein can differentially regulate the IRF-3-dependent host innate immune responses and whether there are any differences in this ability among different coronaviruses.

- The objective of Chapter 4 is to determine whether the full-length nsp3 protein is a major contributor to HCoVs antagonism of the interferon response and whether this ability may be differentially regulated among the different HCoVs, thus modulating the IRF-3-dependent pathways differentially.

CHAPTER 2.   MATERIALS AND METHODS

Cell Lines

The Flp-In T-Rex expression system (Invitrogen) was utilized to generate isogenic, stable HeLa cell lines exhibiting tetracycline-inducible expression of HcoV-OC43-nsp3, HcoV-NL63-nsp3, MERS-CoV-nsp3, or wild type or mutant SARS-nsp3 by a *Saccharomyces cerevisiae*-derived Flp recombinase-dependent DNA homologous recombination event. Refer to chapter 3 for detailed procedures. Human embryonic kidney (HEK) 293FT cells, were grown and maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G, and 100 U/mL streptomycin in a humidified 37°C/5% CO2 incubator.

Plasmids

Conventional PCR and Quick-change site directed mutagenesis (Stratagene) (using specific primers containing desired mutations) techniques were used to construct N-terminal 2×HA-tagged and mutant forms of HCoV-OC43, HCoV-NL63, MERS-CoV, and SARS-CoV nsp3 encoding plasmids in the pcDNA5/FRT/TO backbone (Invitrogen). The primers and restriction enzymes used for construction and cloning of recombinant plasmids are listed in **Table 2.1**. The following plasmids were kind gifts from the indicated contributors; pcDNA3-FLAG TBK1 and pcDNA3-FLAG IKK (from Kate Fitzgerald) (158); pIFN-β-luc. and GFP-IRF3-5D (from Rongtuan Lin) (220). The identities of all plasmids were confirmed by DNA sequencing.

Immunoblot Analysis

Cell lysates were prepared and quantified for protein concentration and subjected to immunoprecipitation (IP) and immunoblot analysis as previously described (221, 222). However briefly, to confirm expression of proteins equivalent amounts of whole cellular extracts were prepared, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a 7.5 % or 10% polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose blotting membrane (Amersham) in transfer buffer for 1 hr. The membrane was blocked by incubation in phosphate-buffered saline (PBS) containing 3% dried milk for 1 h. The following monoclonal (mAb) and polyclonal (pAb) antibodies were utilized: rabbit anti-ISG56 pAb (generated by immunizing rabbits with a keyhole limpet hemocyanin-coupled peptide spanning amino acids 2 to 19 of human ISG56) (221), mouse anti HA mAb (12ca5 hybridoma culture supernatant), mouse anti-VSV-IN mAb (NIH), rabbit phosphorylated IRF3 pAB (cell signaling), or mouse anti-actin mAb (Sigma) in 3% milk–PBS at a dilution of 1:500, 1:50, 1:1000, and 1:5000 respectively. These incubations were done at 4°C overnight or at room temperature for 1 to 2 hours. After three 5-min washes with PBS, membranes were reacted with a peroxidase-conjugated secondary goat anti-rabbit and goat anti-

Table 2.1.   Primers used to construct the full-length NSP3 proteins.

NSP3 protein	Primer name	Sequence (5'-3')
OC43-NSP3-N	OC43BamHINSP3-F OC43NotINSP3N-R	cgc**ggatcc**atgggaaggcgtgttacatttaagg ctgac**gcggccgc**ttacaaggctttaatttgagcaac
OC43-NSP3-C	OC43BamHINSP3C-F OC43XHoINSP3-R	cgc**ggatcc**atggagtgtactggaggcatagata cttca**ctcgag**tagttaacctcctttaagactaaagggtgta
NL63-NSP3-N	NL63AgeINSP3-F NL63NotINSP3N-R	gc**accggt**atgggtaaaatatctttttctgatgat cttca**gcggccgc**ttaatcatttatcgatggtacaacaca
NL63-NSP3-C	NL63 AgeINSP3C-F NL63XHoINSP3-R	cgctt**accggt**atggtaccatcgataaatgatctttct cttca**ctcgag**ttaagcaccctgttttgcaactatac
MERS-NSP3-N	MERSBamHINSP3-F MERSNheINSP3N-R	cgc**ggatcc**atggcacctgtaaaaaaagtagcc ctgac**gctagc**ttagtctgtacacacaaaaacagtaaaacc
MERS -NSP3-C1	MERSBamHINSP3C1-F MERSXHoINSP3C1-R	cgc**ggatcc**atggtgtgtacagacaactctgctaac cttca**ctcgag**ttaagagattcctaggtaagctctaac
MERS -NSP3-C2	MERSBamHINSP3C2-F MERSXHoINSP3C2-R	cgc**ggatcc**atggcttacctaggaatctcttctgct cttca**ctcgag**ttaaccaccaacaattttgttagcag
SCoV-NSP3-N	SCoVBamHINSP3-F SCoVNotINSP3N-R	cttca**ggatcc**atggcaccaattaaaggtgtaaccttg cttca**gcggccgc**ttaatcaaagctagcatttggtaatggttg
SCoV-NSP3-C	SCoVBamHINSP3C-F SCoVXHoINSP3-R	cttca**ggatcc**atgccaaatgctagctttgataatttcaaac cttca**ctcgag**ttaaccacccttgagtgagattttagt
SCoV-delPLP-N	SCoVdelPLPNSP3-F SCoVdelPLPNSP3-R	ctcgatggagttacttacaca**gag** **ctc**acgcagggataagagact

mouse immunoglobulin G pAbs (Southern Biotech) at a dilution of 1:8000 for 1hr at room temperature. After two 5 min washes and one 10 wash, protein bands were then visualized with the enhanced chemiluminescence (ECL) detection system as recommended by the manufacturer (Millipore), followed by exposure to Kodak Biomax film

Sendai Virus (SeV) Infection, and Poly (I-C) Treatment

Where indicated, cells were infected with 100 hemagglutinin units (HAU)/ml of SeV (Cantell strain, Charles River Laboratories) for 16 h prior to cell lysis for luciferase reporter assay and/or immunoblot analysis as described previously (221-223). For poly (I-C) (Sigma) treatment, poly (I-C) (Sigma) was added directly to the culture medium at 25μg/ml (M-pIC) and loaded onto the cells for the indicated time period.

Transfection and IFN-β Luciferase Reporter Gene Assay

Luciferase assays were performed in 48-well plates (Greiner Bio-one) seeded with 20000 cells per well in triplicates, cells were then un-induced or induced with tetracycline 2ug/ml for 48hrs before transfection. Cells were then transfected in triplicate with the IFNβ-Luc (IFNB-pGL3) reporter plasmid (80 ng), and pRL-TK (20ng) (internal control to normalize the activity of the IFNβ-Luc reporter plasmid transfection efficiency) (Promega), using Lipofectamine 3000 transfection reagents, as per the manufacturer's instructions (220). Twenty four hours later, transfected cells were mock-treated, treated with poly (I-C) (Sigma) for 10 h, or infected with SeV (Cantell strain, Charles River Laboratories) for 16 h before cell lysis. IFN-β promoter activities were determined by assaying for both firefly luciferase and renilla activities. Data were expressed as mean relative luciferase activity (luciferase activity divided by renilla luciferase activity) with standard deviation from a representative experiment carried out in triplicate. A minimum of three independent experiments were performed to confirm the results of each experiment.

Vesicular Stomatitis Virus Luciferase Reporter Gene Assay

VSV-Luc is a recombinant firefly luciferase-encoding vesicular stomatitis virus, in which in infected cells replication can be monitored by assaying for luciferase activity (224). The VSV luciferase assay were performed in 24 well plates (Greiner Bio-one) seeded with 40,000 cells per well in triplicates, cells were then un-induced or induced with tetracycline 2ug/ml for 48hrs. Where indicated, cells were mock-treated or treated with poly (I-C) (GE Health) or transfected with 2μg of HCV RNA replicon (HCV genotype 2a strain JFH1-1581) using Lipofectamine 3000 reagents (Invitrogen) per the manufacturer's instructions for 8-10 hrs. Cells were then infected with rVSV-Luc. at an MOI of 0.1 and virus was removed after 1hr. At 6-8 hours post infection, luminescence was measured using Steady-Glo firefly luciferase reagent (Promega) according to the

manufacturer's instructions. The luminescence values were recorded on a Glomax 20/20 Luminometer (Promega)

CHAPTER 3.   GENERATION OF STABLE CELL LINES INDUCIBLY EXPRESSING HUMAN CORONAVIRUSES (COV) NONSTRUCTURAL PROTEIN 3 (NSP3)

Introduction

There are many viruses that cause severe diseases for which there are neither vaccines nor effective antiviral therapies. As a result, millions of people lack treatment and preventive measures for these virally induced diseases that may lead to fatal outcomes. Thus, many viral diseases continue to be a challenging global health issue. Severe acute respiratory syndrome (SARS), the first epidemic of the 21st century, was caused by a novel human coronavirus (HCoV), referred to as SARS-associated coronavirus (SARS-CoV). In humans, SARS outbreak was the first paradigm of serious illness caused by a CoV (1, 5). Moreover, the SARS-CoV epidemic serves as an example of the quickness of virus spread expedited by air travel, mass transit and increased population density nowadays. Unfortunately, there are no approved vaccines or therapies for humans to control SARS-CoV, or any of the HCoVs known to date. In order to design successful treatment and prevention methods to virally induced diseases, an in-depth understanding of viral components and their contribution to viral pathogenesis is essential.

Human coronaviruses were initially considered to be agents of the common cold and caused little mortality (1, 12-15). HCoV-OC43 and HCoV-229E were the first two HCoVs identified. These two are responsible for about 30% of common colds worldwide although the molecular basis of disease was not well studied. However, the SARS-CoV epidemic of 2003 demonstrated the ability of a novel CoV to quickly to spread globally in immunologically naïve human populations, causing over 8000 cases and a ~10 % mortality rate in 29 countries (5, 21). Evidence suggests that SARS-CoV most likely evolved from viruses circulating within Chinese horseshoe bats, which are believed to be the natural animal reservoirs (31-33). The SARS outbreak sparked a keen interest in the disease pathogenesis of CoVs and led to an intense search for additional HCoVs that could cause severe disease in humans. Within the following couple of years, HCoV-NL63 and HCoV-HKU1, two new HCoVs causing mild disease in humans, were identified (22, 23). Most recently, another novel highly pathogenic CoV, which can cause severe disease in humans, emerged suddenly in the Middle East region and was designated MERS-CoV (24-26). Likewise, MERS-CoV emerged from a zoonotic reservoir to infect humans (34-37). To date, there are 6 known HCoVs. There is an obvious difference in the severity of the disease outcome among HCoVs. Four of these are endemic in humans and are mainly associated with mild respiratory illnesses whereas the other two CoVs present as emerging infections causing a severe respiratory syndrome leading to fatality, thus highlighting the capacity of severe disease potential of the CoV family (1, 5,12-15, 21, 24-26). Insight into how CoVs regulate the host cell is critical for a full understanding of the molecular mechanisms that contribute to the severe disease state caused by these viruses.

Coronaviruses, a genus belonging to the *Coronaviridae* family, are positive-strand enveloped RNA viruses that can infect a variety of animal hosts, including humans (1, 2, 5). They have an incredibly large single-stranded genome, the largest of all RNA viruses known to date (2). Coronaviruses encode large replicase polyproteins that are processed by two virally encoded proteases, i.e., the papain-like protease (PLP) and a 3C-like protease (3CLpro), to generate 15 or 16 nonstructural proteins that are involved in viral replication (1-6). For these viruses, the PLP is essential for processing the amino-terminal end of the replicase polyproteins (1-6, 118, 120). All human coronaviruses, with the exception of SARS-CoV and MERS-CoV, contain two PLPs to process the amino-terminal portion. It is well known that when two PLPs are encoded within the coronavirus replicase polyprotein, PLP2 has similar characteristics to SARS-CoV PLpro (120, 132). The investigation of the PLP of SARS-CoV has illustrated the multi-functionality of coronaviral PLPs. Since PLPs are essential for coronaviral replication, they are attractive targets for antiviral treatments. The PLP domain is located within the largest replicase subunit, nsp3. Nsp3 is a multi-domain protein, essential in the formation of double membrane vesicles (DMV), a hallmark of coronavirus replication (122-125). Earlier reports demonstrated that the PLP of nonstructural protein 3 (nsp3), functions not only as a viral protease, but also possesses deubiquitinating (DUB) and deISGylating activities (134-139, 199, 201), which are suggested to participate in regulation of the host innate immune response to viral infection.

Subsequent reports have shown the papain-like protease (PLP) domain of coronaviral nonstructural protein 3 (nsp3) as a potent IFN antagonist by suppressing IRF-3-dependent innate antiviral defenses. Devaraj et al first reported that SARS-CoV PLP inhibits IRF3 activation (186), which was corroborated by reports by other groups (194, 202, 216). Of note, studies investigating the role of the PLpro domain in regulating the host innate immune defenses have mainly relied on the use of transient transfection of mammalian cell lines with plasmids encoding truncated nsp3 PLP domain constructs. It is likely that this is due to the extremely large size of the nsp3 protein, which made it very difficult to clone and express in mammalian cells.. Although transient transfection provides a faster way of expression of a protein, the efficiency of transfection typically decrease with very large plasmids, making it difficult to achieve comparable expression of proteins among each experiment. Stable and regulated gene expression is a very useful tool to study further and characterize the function of a gene product. Since the 1990's, tetracycline-regulated expression systems have been widely used for inducible protein expression in cell culture (225-227). The construction of stable inducible cell culture systems may greatly facilitate the analysis of the function of genes and gene products.

More importantly, it has not been shown whether the CoV PLP domain impacts IRF-3 signaling when expressed in the context of the full-length nsp3 protein, which may lead to a better understanding of the PLP-mediated function of the nsp3 protein in virus-infected cells. Thus, it is helpful to establish in-vitro cell systems with stable and comparable nsp3 expression, for the study of nsp3 interactions with the host innate immune responses. Understanding if the nsp3 protein is a bona fide contributor to human CoVs antagonism of the host innate immune response, and if this ability may be differentially regulated among different HCoVs, will provide insight into essential

virulence factors contributing to CoVs disease outcomes and pathogenesis. To understand better the functional role of the nsp3 protein of different HCoVs in regulating IRF-3-dependent innate immune responses, I used an Flp-In-T-Rex expression system successfully to create stable cell lines that express, in a tetracycline-regulated fashion, the full-length nsp3 protein of HCoVs OC43, NL63, MERS-CoV, and SARS-CoV respectively.

Materials and Methods

Construction of CoV-nsp3 Expression Plasmids

Primers specific for the N and C terminal regions of the nsp3 proteins were used to construct the plasmid vectors encoding full length nsp3 protein (HCoV-OC43, HCoV-NL63, MERS-CoV, and SARS-CoV, respectively) and the PLP-deletion mutant nsp3 protein of SARS-CoV (Table 2.1). Briefly, the N and C terminal fragments of nsp3s were amplified by PCR from corresponding nsp3 cDNAs. The cDNA source for HCoV-OC43 nsp3 was total RNA extracted from monkey kidney epithelial cells (BSC-1) infected with HCoV-OC43 (ATCC VR-1558). HCoV-NL63 (Amsterdam I) RNA from B.E.I. resources was as the starting material for HCoV-NL63 cDNA and MERS-CoV cDNA was kindly provided by Dr. Heinrich Feldmann's lab at the National Institute of Health (NIH) Rocky Mountain Laboratories. The N and C terminal fragments of SARS-CoV nsp3 cDNA was amplified from the full-length nsp3 cDNA of SARS-CoV nsp3 (a gift from Dr. Marc Wathelet, Lovelace Respiratory Research Institute) (191). The PCR products was digested with appropriate restriction enzymes, purified after gel electrophoresis, and ligated into pcDNA5/FRT/TO (Invitrogen), in which nsp3 was fused to an N-terminal 2XHA epitope tag. Subsequently, the N- and C-fragments were joined together to construct the full length nsp3 protein-encoding plasmids, also in the pcDNA5/FRT/TO backbone. Constructs were digested with restriction enzymes to verify positive recombinant plasmids **Figure 3.1**. Subsequently, constructs were sequenced to verify incorporation and identity of nsp3 cDNAs.

Cell Culture and Generation of Stable Cell Lines

HeLa Flp-In T-Rex cells (a gift from Stephen Taylor at University of Machester) (228) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G, 100 U/mL streptomycin, and 2 µg/mL Blasticidin. The cells were co-transfected with the pOG44 vector, which expresses the Flp recombinase under control of the human CMV promoter, and individual CoV nsp3 encoding plasmids, i.e., pcDNA5/FRT/TO/HA-HCoV-OC43-nsp3, pcDNA5/FRT/TO/HA-HCoV-NL63- nsp3, pcDNA5/FRT/TO/HA-MERS-CoV- nsp3, pcDNA5/FRT/TO/HA-SCoV- nsp3 and pcDNA5/FRT/TO/HA-SCoV-nsp3-delPLP, respectively. The pcDNA5/FRT/TO-derived expression plasmids contains a FRT site linked to the hygromycin resistance gene for Flp recombinase-mediated integration and

selection of stable cell lines expressing individual CoV nsp3 protein under control of a tetracycline-regulated CMV/TetO2 promoter (225, 229). The pOG44 and the pcDNA5/FRT/TO/HA-CoV-nsp3 expression constructs were co-transfected in a 9:1 ratio using Lipofectamine 3000 reagent (Invitrogen) as described by the manufacturer. Two days after transfection the cells were trypsinized and transferred to 10-cm dishes. After cells had attached, the growth media was replaced with a selection media containing 200 µg/mL Hygromycin B and 2 µg/mL Blasticidin (Invitrogen). The selection media was changed every 3 to 4 days until colonies emerged and control cells (untransfected HeLa-Flp In T-Rex cells) were all dead. Initial experiments were performed using pooled Hygromycin B resistant colonies, which by nature of the Flp-In system were isogenic. In selected experiments involving HeLa-Fit-HCoV OC43-nsp3, HeLa-Fit-HCoV NL63-nsp3, and HeLa-Fit-MERS CoV-nsp3 stable expressing cells were also performed using individual Hygromycin B resistant clones. To induce nsp3, cells were exposed to 2 µg/ml of tetracycline, harvested at 48hrs post Tet induction and lysed. Western blot analysis was performed to demonstrate inducible expression of nsp3.

Western Blot Analysis

To confirm expression of nsp3 proteins, equivalent amounts of whole cellular extracts were prepared, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in a 7.5 % or 10% polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose blotting membrane (Amersham) in transfer buffer for 1 hr. The membrane was blocked by incubation in phosphate-buffered saline (PBS) containing 3% dried milk for 1 h. NSP3 were detected via their HA-epitope tags with a monoclonal antibody (mAb) against the HA tag (Invivogen) or a mouse anti-HA mAb (clone 12ca5 hybridoma culture supernatant) in 3% milk–PBS at a dilution of 1:2000 and 1:50, respectively. Expression of SARS-CoV nsp3 protein was also confirmed by immunoblotting with a rabbit pAb against nsp3 (Rockland). ISG56 was detected via rabbit anti-ISG56 pAb (221) in 3% milk–PBS at a dilution of 1:500. These incubations were done at 4°C overnight or at room temperature for 1 to 2 hours. After three 5-min washes with PBS, membranes were reacted with a peroxidase-conjugated secondary goat anti-mouse or secondary goat anti-rabbit immunoglobulin G pAbs (Southern Biotech) at a dilution of 1:8000 for 1 hr at room temperature. After two 5 min washes and one 10 wash, protein bands were then visualized with the enhanced chemiluminescence (ECL) detection system as recommended by the manufacturer (Millipore), followed by exposure to Kodak Biomax film.

Sendai Virus (SeV) Infection

Where indicated, cells were infected with 100 hemagglutinin units (HAU)/ml of SeV (Cantell strain, Charles River Laboratories) for 16 h prior to cell lysis for immunoblot analysis.

Results

Identification of Recombinant pcDNA5/FRT/TO/HA-CoV Nsp3 Plasmids by Restriction Enzyme Analysis

In order to express the full-length nsp3 protein of HCoVs OC43, NL63, MERS-CoV and SARS-CoV, I first sub-cloned each nsp3 fragment into the multiple cloning sites of the mammalian expression plasmid pcDNA5/FRT/TO/HA. The sizes of pcDNA5/FRT/TO/HA, and the cDNA fragment of OC43- nsp3, NL63- nsp3, MERS-nsp3, SARS- nsp3 and SARS-nsp3-delPLpro are 5.2Kb, 5.7Kb, 4.7Kb, 5.7Kb, 5.8Kb, and 4.8Kb, respectively. After the completion of standard molecular biology techniques to construct the recombinant plasmids, positive transformants were analyzed by restriction digestion of miniprep DNA. Due to the size similarities of the mammalian expression vector and CoV-nsp3 cDNA fragments, to identify successful recombinant clones, recombinant pcDNA5/FRT/TO/HA-CoV-nsp3 plasmids were digested with multiple restriction enzymes to separate the expression vector and CoV-nsp3 fragments.

Nine pcDNA5/FRT/TO/HA-OC43-nsp3 minipreps were digested with BamHI, BsrGI, and XhoI. Fragments of 5.2Kb, 3.6Kb, and 2.1Kb were generated by each miniprep, suggesting all nine minipreps were positive clones **Figure 3.1A**. Eight pcDNA5/FRT/TO/HA-NL63-nsp3 plasmids were cleaved with AgeI, ClaI, and XhoI. Fragments of 5.2Kb, 2.8Kb, and 1.9Kb were generated by five of the eight minipreps **Figure 3.1B**. Six pcDNA5/FRT/TO/HA-MERS-nsp3 plasmids were cleaved with AvrII, BamHI, and XhoI. Fragments of 5.2Kb, 4.1Kb, and 1.6Kb were generated by four of the minipreps **Figure 3.1C**. Two restriction enzyme digestion reactions were used to verify pcDNA5/FRT/TO/HA-SARS- nsp3 plasmids. Two plasmids were digested with PmeI, generating fragments of 5.9Kb and 5.0Kb and EcoRI and SnaBI generating fragments of 4.2Kb, 3.8Kb and 2.8Kb, suggesting that both were positive clones **Figure 3.1D**.

To demonstrate the difference in fragment size, three pcDNA5/FRT/TO/HA-SARS-nsp3-delPLP plasmids along with two pcDNA5/FRT/TO/HA-SARS-nsp3 wt mini-prep plasmids, were cleaved with BamHI, NheI and XhoI. Fragments of 5.2Kb, 2.3Kb, and 2.5Kb were generated, confirming lane 6 to be a positive mini-prep for pcDNA5/FRT/TO/HA-SARS-nsp3-delPLP **Figure 3.1E**. Restriction enzyme mapping showed that the full length of each CoV-nsp3 and deletion mutant of SARS-nsp3 product had been successfully cloned into the specified restriction sites in the pcDNA5/FRT/TO/HA vector, and the sizes of the recombinant plasmids were consistent with expectation. Sequence analysis confirmed the sequence and orientation of the inserted CoV-nsp3 cDNAs in the pcDNA5/FRT/TO/HA vector.

Transient Expression of CoVs-Nsp3 Recombinant Plasmids

In an effort to establish stable Tet-regulated inducible cell lines, I first determined if the positive clones of each CoV-nsp3 plasmid allowed expression of the full-length or
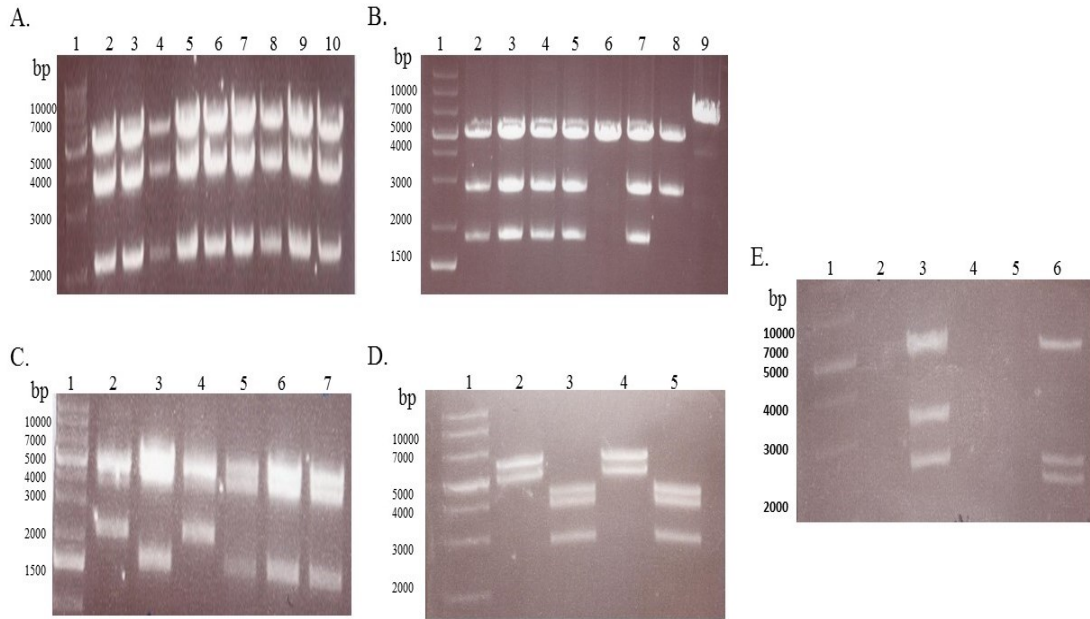
Figure 3.1.   Restriction enzyme digestions of recombinant pcDNA5/FRT/TO/HA-CoV-nsp3 expression plasmids

(A). Lane 1: 1Kb marker, Lane 2-10: pcDNA5/FRT/TO/HA-OC43-nsp3 minipreps, cleaved with BamHI, BSRGI, and XhoI, fragments of 5.2Kb, 3.6Kb, and 2.1Kb indicate positive clones. (B) Lane 1: 1Kb marker, Lane 2-9: pcDNA5/FRT/TO/HA-NL63-nsp3 minipreps, cleaved with AgeI, ClaI, and XhoI. Fragments of 5.2Kb, 2.8Kb, and 1.9Kb indicate positive clones. (C) Lane 1: 1Kb marker, Lane 2-7: pcDNA5/FRT/TO/HA-MERS-nsp3 minipreps, cleaved with AvrII, BamHI, and XhoI. Fragments of 5.2Kb, 4.1Kb, and 1.6Kb indicate positive clones. (D) Lane 1: 1Kb marker, Lane 2-5: pcDNA5/FRT/TO/HA-SARS-nsp3 minipreps, cleaved with PmeI, fragments of 5.9Kb and 5.0Kb and ECoRI and SnaBI generating fragments of 4.2Kb, 3.8Kb and 2.8Kb indicate positive clones. (E) Lane 1: 1Kb marker, Lane 2-3: pcDNA5/FRT/TO/HA-SARS-nsp3 wild-type minipreps, Lane 4-6: pcDNA5/FRT/TO/HA-SARS-nsp3-delPLpro minipreps, cleaved with BamHI, NheI and XhoI, fragments of 5.2Kb, 2.3Kb, and 2.5Kb indicate positive clones.

mutant nsp3 protein in transiently-transfected cells. The expression plasmid encoding viral nsp3 is N-terminally tagged with two copies of HA tag in the pcDNA5/FRT/TO backbone (Invitrogen); therefore, each CoV-nsp3 was detected by using an anti-HA antibody. After each construct was developed, I assayed for transient expression of the CoV-nsp3 protein. HEK293FT cells were transfected with two micrograms of each recombinant plasmid DNA, 48 hrs after transfection cells were assayed for expression. Using a monoclonal anti-HA antibody Western Blot analysis confirmed that recombinant plasmids gave rise to expression of the full-length OC43-nsp3, NL63-nsp3, MERS-nsp3, and SARS-nsp3 wt and mutant proteins, respectively **Figure 3.2A-3.2E**. The molecular weight of each nsp3 proteins are 212 KDa, 173 KDa, 209 KDa, 213 KDa, and 178 KDa respectively. The molecular weight of each nsp3 protein was consistent with the expected size.

Establishment Stable Cell Lines Using the Flp-In T-Rex System

A system that allows stringent inducible regulation of gene expression in mammalian cells is a valuable tool for the functional characterization of genes. The most widely used inducible protein expression systems are those regulated by tetracycline (Tet) and its derivatives. In 1992, Gossen et al first described a Tet promoter activating system for regulating individual genes in mammalian cells (225). Since then, the tetracycline-regulated gene expression system has gained wide acceptance and has been proven to work in both cell lines and mouse models; nonetheless, this system has undergone several modifications (227, 230-233). The Flp-In-T-Rex system (Invitrogen) is designed to generate cell lines that stably express proteins of interest in an isogenic and inducible fashion. This unique feature is due to the fact that the gene of interest (GOI) is integrated into the chromosome at a specific site, thus eliminating the possible variation in expression levels or patterns that may be due to random integration into the chromosome. Furthermore, cells can be grown without expression of the protein of interest until such time as it is needed. The ability to induce expression by addition of Tet to the culture medium has several advantages over either transient transfection or the generation of constitutively expressing stable cell lines. Thus, I used the Flp-In-T-Rex expression system successfully to generate stable cell lines that express, in a tetracycline-regulated fashion, the full-length nsp3 protein of HCoVs OC43, NL63, MERS-CoV and SARS-CoV, respectively.

The Flp-In T-Rex-293 system cell line, based upon a derivative of human 293 embryonic kidney fibroblasts, is widely used by investigators. However, owing to their physiological properties, in particular their intact innate immune signaling pathways, we selected the Flp-In T-Rex-HeLa system based upon HeLa cells. The generation of the HeLa-Flp-In-CoV-nsp3 cell lines requires that the parental cell lines HeLa-Flp-In-T-Rex cells (234) are co-transfected with two plasmids, one containing the gene of interest expressing the tetracycline repressor, and the other containing an Flp Recombination Target (FRT) site. After verifying transient expression of the CoV-nsp3 constructs, each clone that expressed the highest level of CoV-nsp3 protein, based on Western blot analysis, was chosen to co-transfect with the pOG44 plasmid, an Flp recombinase
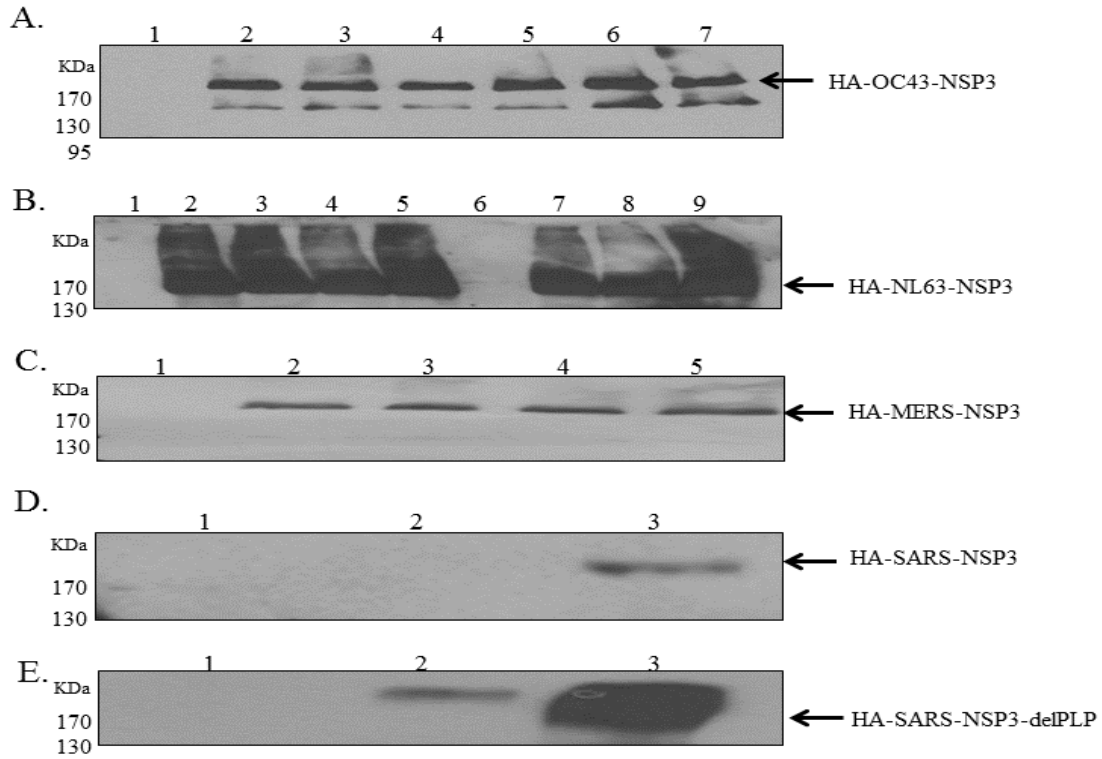
Figure 3.2.   Transient expression of recombinant pcDNA5/FRT/TO/HA-CoV-nsp3 expression plasmids.

Unless noted, HEK293FT cells were transfected with 2 μg of pcDNA5/FRT/TO/HA-CoV-nsp3 mini-preps. 48 hrs post transfection cell lysates were harvested, and expression of each CoV-nsp3 protein was detected using a monoclonal anti-HA antibody, analyzed by Western blotting (A) Lane 1: Mock, Lane 2-7:pcDNA5/FRT/TO/HA-OC43-nsp3 (B) Lane 1: Mock, Lane 2-9:pcDNA5/FRT/TO/HA-NL63-nsp3 (C) Lane 1: Mock, Lane 2-5: pcDNA5/FRT/TO/HA-MERS-nsp3 (D) Lane 1: Mock, Lane 2-3: pcDNA5/FRT/TO/HA-SARS-nsp3 (E) HEK293FT cells expressing 2.5 μg of DNA Lane 1: Mock, Lane 2: pcDNA5/FRT/TO/HA-SARS-nsp3 Lane 3: pcDNA5/FRT/TO/HA-SARS-nsp3-delPLP. Mock: No transfection

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expression plasmid, into HeLa-Flp-In-T-Rex cells. Upon integration of the recombinant plasmid into the FRT site, the cells were rendered hygromycin resistant, allowing selection of the required integrants with hygromycin.   For each cell line, after 2-3 weeks of the selection process, the polyclonal population of cells was pooled, expanded and using a monoclonal anti-HA antibody screened for tetracycline-regulated expression of the nsp3 protein **Figure 3.3A-3.3E**. Established cell lines were generated and characterized independently and designated HeLa-Fit-OC43-nsp3, HeLa-Fit-NL63-nsp3, HeLa-Fit-MERS-nsp3, HeLa-Fit-SCoV-nsp3, and HeLa-Fit-SCoV-nsp3-delPLpro, respectively. In addition, it was observed that the overall growth kinetics of each nsp3-expressing cell line was similar to that of the parental HeLa-Flp-In T-Rex cells, indicating that the CoV-nsp3 expression has no demonstrable effect on cell growth (data not shown). These observations supports their potential use as a tool to study the function of the full-length CoV-nsp3 proteins.

Characterization of Human Coronaviruses Full-Length Nsp3 Inducible Cell Lines

Each cell line was analyzed by immunofluorescence (IFA) staining, using an anti-HA monoclonal antibody, to further examine CoV-nsp3 expression levels. In the absence of tetracycline, each cell line exhibited no detectable nsp3 protein. Upon addition of tetracycline, each cell line demonstrated nsp3 expression, suggesting that tetracycline exerted a tight control over the expression of CoV-nsp3 (data not shown). As expected, I observed a cytoplasmic localization pattern for each CoV-nsp3 protein (data not shown). However, once all cell lines were generated and examined alongside each other, there was an obvious difference in the signal intensity and percentage of cells that expressed each CoV-nsp3 (data not shown). The percentage of cells expressing SARS-nsp3 was far greater in number than was the percentage of cells expressing HCoV-OC43, HCoV-NL63, and MERS-CoV nsp3 proteins (data not shown). Although each cell line was generated in the same manner, these results suggest that the CoV-nsp3 stable expressing cell lines are not expressing at comparable levels.

In an effort to establish stable cell lines with comparable expression, new Tet-inducible MERS-CoV, HCoV-OC43, and HCoV-NL63-nsp3 stable expressing cell lines were produced by selecting and expanding individual clones and pooled populations, as indicated previously. Unexpectedly, upon the addition of Tet for the pooled population, HeLa-Fit-OC43-nsp3 and HeLa-Fit-NL63-nsp3 stable cells still demonstrated a low percentage of cells expressing nsp3 (data not shown). Furthermore, in the presence of Tet, stable cells generated from expanding individual clones did not result in an increase in HCoV-OC43 and HCoV-NL63 nsp3 expression (data not shown). However, selecting individual colonies to generate HeLa-Fit-MERS-nsp3 stable cells revealed promising results. Clone 1 of HeLa-Fit-MERS-nsp3 stable cells demonstrated higher expression of MERS-nsp3 in the presence of Tet, when compared to the pooled HeLa-Fit-MERS-nsp3 stable cells (data not shown).

Ideally, regulation of gene expression can be controlled by adding different concentrations of the inducers (234). Therefore, while the expression of HeLa-Fit-MERS-
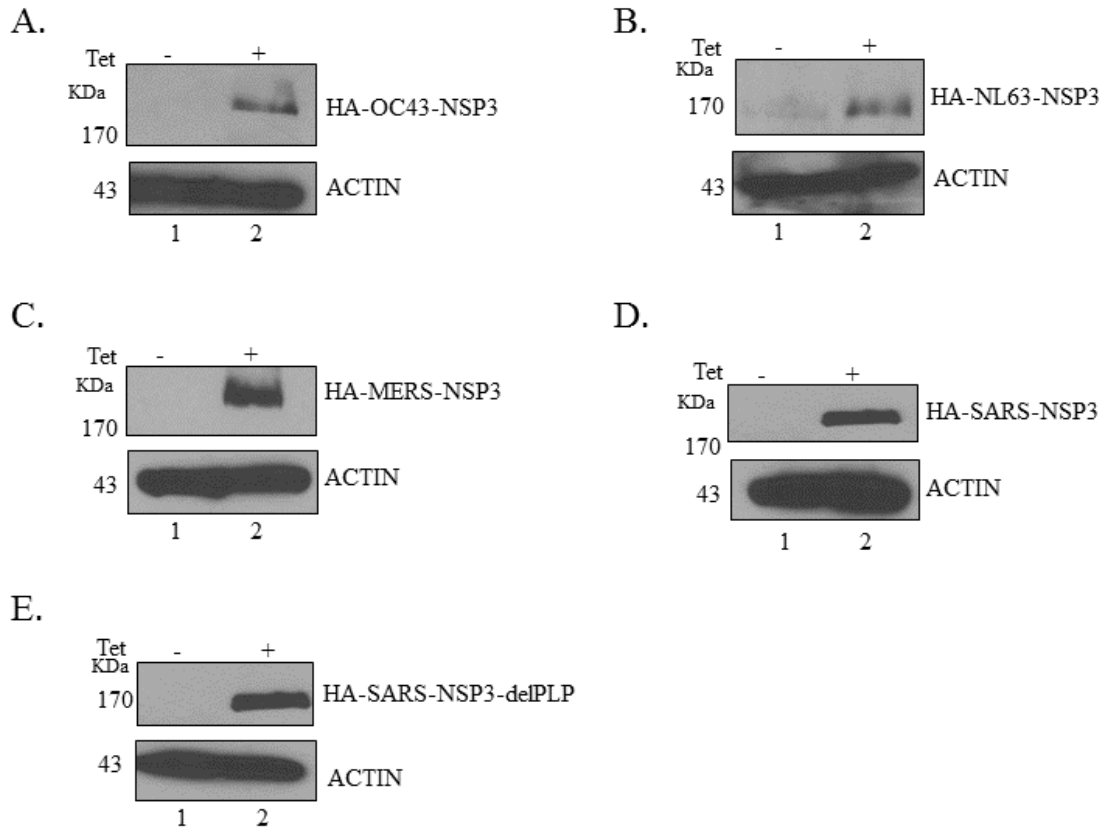
Figure 3.3. **Tet-inducible expression of nsp3 in stable cell lines.**

Cells were grown for 48 hrs in the absence (Tet-) (lane 1) or presence (Tet+) (lane 2) of
2µg/ml Tet treatment. Using an anti-HA antibody, the expression of CoV-nsp3 protein
was analyzed by Western blotting. (A) HeLa-Fit-OC43-nsp3 cells (B) HeLa-Fit-NL63-
nsp3 cells (C) HeLa-Fit-MERS-nsp3 cells (D) HeLa-Fit-SCoV-nsp3 cells (E) HeLa-Fit-
SCoV-nsp3-delPLpro cells. Beta actin was used as a loading control.

CoV-nsp3 stable cells was not comparable to that of HeLa-Fit-ScoV-nsp3 cells, I investigated dose-dependent inducibility of CoV-nsp3 expression between the two cell lines. Since Tet-inducible expression of HeLa-Fit-ScoV-nsp3 cells resulted in robust expression of SARS-nsp3, to generate similar levels of expression, concentration dependency was examined by inducing SARS-nsp3 with different concentrations of Tet (0.1-1µg/ml) for 48 hrs. SARS-nsp3 expression levels that were induced at low concentrations of Tet (0.2-1µg ug/mL) were still higher than MERS-nsp3 expression levels induced at 2µg/ml of Tet, a concentration that has proven to be optimal in the use of the Tet system (data not shown). These findings suggest that for the Tet concentration range tested, there was not a concentration-dependent decrease in the amount of SARS-nsp3 protein induced.

The nsp3 protein is not well conserved between coronaviruses (128). Thus, it must be considered that the CoV-nsp3 proteins are being degraded or may have different turnover rates. To examine this aspect, I assayed for transient expression of each CoV-nsp3 protein, using the CoV-nsp3 expression construct used to generate the stable expressing cell lines. HEK293T cells were transfected with two micrograms of each recombinant plasmid, 48 hrs after transfection cells were assayed for expression of HA-nsp3. Using a monoclonal anti-HA antibody Western Blot analysis demonstrated that the transient CoV-nsp3 protein expression levels were obviously different **Figure 3.4A**. At a short exposure time, HCoV-OC43, MERS-CoV and SARS-CoV-nsp3 protein expression was observed **Figure 3.4A**. In contrast, HCoV-NL63-nsp3 protein expression was only observed after prolonged exposure of the Western blot **Figure 3.4B**. Although gene expression is controlled at many levels, it is possible that there are differences in the CoV-nsp3 mRNA transcript levels. It is also possible that the codons for each CoV nsp3 differ in their efficiency in expression in human cells. Until comparable expression can be demonstrated, HeLa-Fit-OC43-nsp3, HeLa-Fit-NL63-nsp3, and HeLa-Fit-MERS-nsp3 stable cells will not be utilized further to characterize the role of these CoV nsp3s in regulation of the host innate immune response.

Furthermore, comparable expression was not achieved when 293-Fit-T-Rex, a derivative of human embryonic kidney 293 cells, was used as the founder line to generate SARS-CoV-, MERS-CoV-, HCoV-OC43-, and HCoV-NL63-nsp3 Tet inducible cell lines (data not shown). Thus my subsequent studies were focused on SARS-CoV nsp3. Importantly, HeLa-Fit-derived, Tet-regulated stable cells allowed comparable expression of WT and delPLP nsp3 of SARS-CoV **Figure 3.5**. The observation that other CoV-nsp3 stable cells failed to express nsp3 at levels comparable to SARS-CoV nsp3, suggesting that SARS-CoV codon is optimal for expression in human cells. Therefore, we used HeLa-Fit-ScoV-nsp3 cells to gain better insight into the role of nsp3 in the regulation of host antiviral innate immune responses

Discussion

The inducible expression of protein of interest in mammalian cells provides an invaluable opportunity to examine protein functions. The Tet-regulated expression
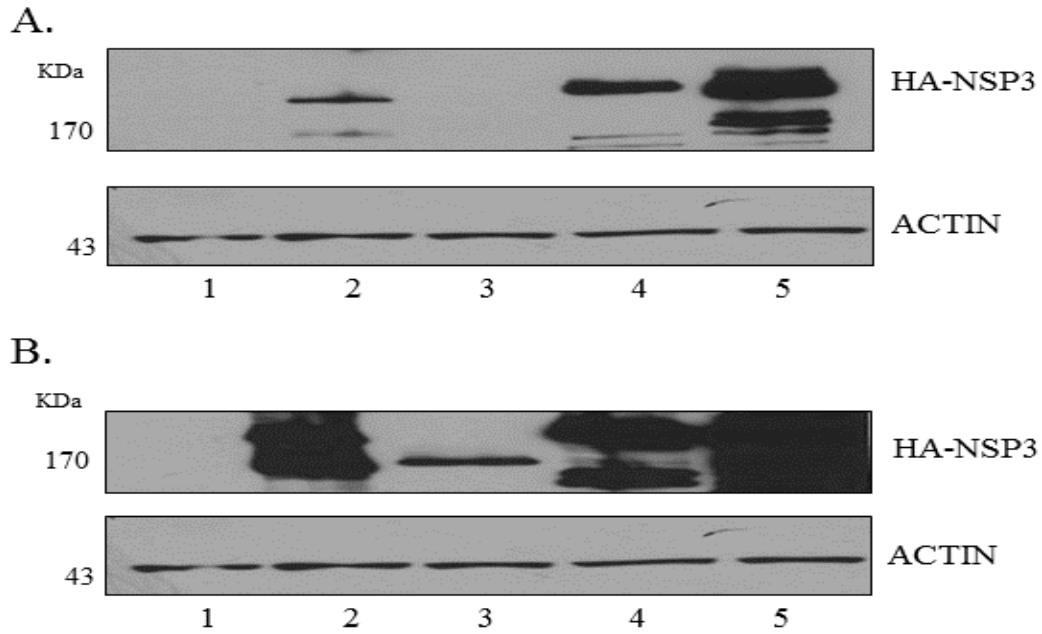
Figure 3.4. **Transient expression of recombinant pcDNA5/FRT/TO/HA-CoV-nsp3 expression plasmids used to generate Tet inducible stable cells.**

(A) Short exposure of HEK293T cells were transfected with 2 µg of pcDNA5/FRT/TO/HA-CoV-nsp3 mini-preps. 48 hrs post transfection cell lysates were harvested, and expression of each CoV-nsp3 protein was detected using a monoclonal anti-HA antibody, analyzed by Western blotting. Lane 1: Mock, Lane 2:pcDNA5/FRT/TO/HA-OC43-nsp3, Lane 3: pcDNA5/FRT/TO/HA-NL63-nsp3, Lane 4: pcDNA5/FRT/TO/HA-MERS-nsp3, Lane 5 pcDNA5/FRT/TO/HA-SARS-nsp3 Mock: No transfection. Actin was used as loading control. (B) Long exposure of HEK293T cells were transfected with 2 µg of pcDNA5/FRT/TO/HA-CoV-nsp3 mini-preps. 48 hrs post transfection cell lysates were harvested, and expression of each CoV-nsp3 protein was detected using a monoclonal anti-HA antibody, analyzed by Western blotting. Lane 1: Mock, Lane 2:pcDNA5/FRT/TO/HA-OC43-nsp3, Lane 3: pcDNA5/FRT/TO/HA-NL63-nsp3, Lane 4: pcDNA5/FRT/TO/HA-MERS-nsp3, Lane 5 pcDNA5/FRT/TO/HA-SARS-nsp3 Mock: No transfection. Actin was used as loading control.
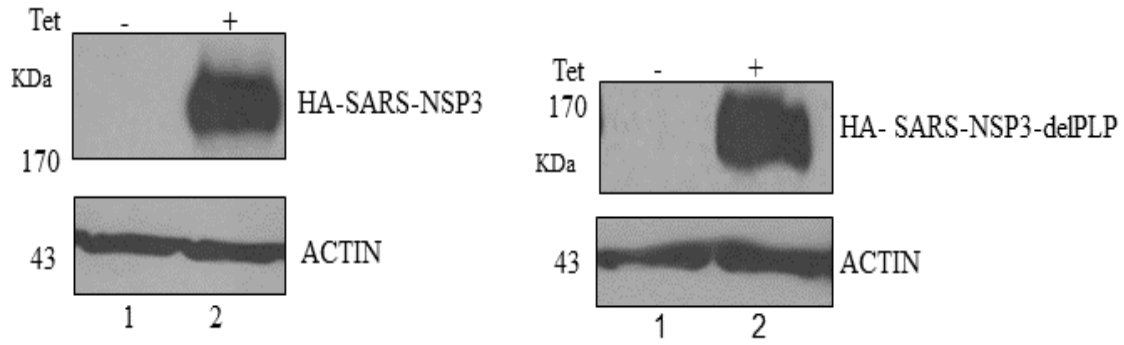
Figure 3.5.   Tet-inducible expression of WT and delPLP mutant of SARS-CoV nsp3 in HeLa-Fit derived cells.

HeLa-Fit-SARS-nsp3 and HeLa-Fit-SARS-nsp3-delPLP stable cells were grown for 48 h in the absence (Tet-) (lanes 1) or presence (Tet+) (lanes 2) of 2 µg/ml of Tet. Using mouse anti HA mAb (12CA5 hybridoma culture supernatant) inducible expression of SARS-nsp3 and SARS-nsp3-delPLP was analyzed by western blotting.

system developed by Gossen and Bujard (225) has been widely used to control inducible expression of proteins in cultured mammalian cells. Tet inducible systems are divided into two classes, the Tet-On system in which gene expression is induced in the presence of tetracycline, and the Tet-Off system where expression of a gene is turned off by Tet (234). Although there are two variants of the Tet based system, we decided to use the Tet-on system due to its advantage of not exposing the cells to Tet for prolonged periods of time under non-inducing conditions. While possessing many unique attributes, the Tet-regulated gene expression system has undergone many modifications to tighten the control of gene expression.

The Flp-In T-Rex system (Invitrogen) uses a genomic FRT site for integration of a gene-of-interest (GOI) by FLP recombinase and is based on the Tet-repressor (TetR) that inhibits, via two tetracycline operator (tetO) sequences immediately downstream of the CMV promoter, expression of the GOI (229). The incorporation of FLP recombinase-mediated integration into a pre-integrated FRT site is a useful addition to the Tet-regulated expression system. It is suggested that this will routinely generate highly reproducible stable transgenic cell lines in which protein expression is induced and comparable across a population of cells. To achieve highly reproducible results, site-specific integration of one single gene copy is important. Therefore, to elucidate the functional role of various CoV-nsp3 proteins on the antagonism of the host antiviral innate immune responses, I chose the well-characterized Flp-In-T-Rex expression system to generate stable cell lines that express, in a Tet-regulated fashion, the full-length nsp3 protein of HCoVs OC43, NL63, MERS-CoV and SARS-CoV, respectively.

In the present study, the full-length cDNA of nsp3 from SARS-CoV, MERS-CoV, HCoV-OC43, and HCoV-NL63 was cloned into a mammalian expression vector, and positive clones were identified by restriction enzyme digestion of mini-preps **Figure 3.1A-3.1E**. Subsequently, Western-blot analysis confirmed that the recombinant plasmids enabled expression of the full-length OC43-nsp3, NL63-nsp3, MERS-nsp3, and SARS-nsp3 proteins, in transient transfection experiments **Figure 3.2A-3.2E**. The Flp-In-T-Rex stable cell lines were utilized to ensure comparable expression amongst each CoV-nsp3 Tet inducible cell line, thus allowing the investigation of CoV-nsp3 effects in physiologically relevant conditions. However, my findings suggest that there are considerable variations between stable cells expressing different CoV-nsp3 proteins (data not shown). In an attempt to understand the underlying reason, I assayed transient expression of all four CoV-nsp3 proteins from the CoV-nsp3 expression constructs used to generate the stable expressing cells **Figure 3.4**. It is possible that the mRNA levels of each CoV-nsp3 proteins are different, or that the codons of different CoV nsp3s differ in their efficiency for expression in human cells. While various approaches were taken to enhance the diminished expression of MERS-CoV-, HCoV-OC43-, and HCoV-NL63-nsp3 proteins, I show in this study that comparable expression of nsp3 among all four CoV-nsp3 stable cell lines could not be achieved (data not shown).

A good way to study the function of a viral protein is to see what happens in the host cell when the protein is present. For this, using a cell-culture system that stably expresses the viral protein is advantageous. However, research conducted while

characterizing CoV-nsp3 stable expressing cell lines demonstrates that the generation of stable Tet-inducible cell culture systems that express large viral proteins in mammalian cells can be very complex. Ultimately, with these kind of studies, many biological aspects must be considered in the development of these cell-culture systems. One may argue that the amount of mRNA transcribed does not necessarily equal the amount of protein produced. However, there are instances in which variations in mRNA levels strongly correlate with change in protein levels. It is possible that the expression of CoV-nsp3 genes is tightly controlled at the levels upstream of translation. Therefore, it would be important for this project to measure each CoV-nsp3 gene product. Quantifying the level of mRNA transcripts will indicate at what magnitude each CoV-nsp3 gene is transcribed. To visualize variances in the abundance of mRNA transcripts produced by the different CoV-nsp3 stable cells or at different times, I could use the RT-qPCR method. In this technique, reverse transcription is followed by quantitative PCR. This will be an important way to determine if there are differences in the transcriptional regulation of the CoV-nsp3 proteins, thus causing differences in CoV-nsp3 protein production.

Interestingly, Western blot analysis demonstrates that SARS-CoV-nsp3 protein is expressed robustly in either stable **Figure 3.3D** or transient **Figure 3.4 lane 5** expression settings. This observation strongly suggests that the SARS-CoV codon is optimal for expression in human cells. Notably, this is not a cell type-specific phenomenon, as 293-Fit-SARS-nsp3 stable cells also demonstrated robust, inducible expression of SARS-nsp3. With adjusting the codon usage within HCoV-OC43-, HCoV-NL63-, and MERS-CoV- nsp3 genes to the codons most commonly used by human cells, expression of these CoV nsp3s may be achieved (235). Several studies have demonstrated that codon optimization can strongly enhance protein expression from several of human immunodeficiency virus type-1 (HIV-1) genes in human cells (235-236). Even though codon optimization suggests a primary effect on the translation of the protein, it has been suggested that codon optimization could also lead to higher levels of mRNA accumulation (237). Therefore, codon optimization is one approach that may be used to increase CoV-nsp3 expression levels.

Although, comparable expression of different CoV-nsp3s was not achieved, the generation of stable cell lines has many advantages over transient gene expression systems, such as a more uniform gene expression within a cell population, the elimination of the need for multiple transfections during experiments, and provision of a system for long-term experiments (238). The Flp-In T-Rex system enables the targeted insertion of exogenous GOI in a site-specific manner in all transfected cells, thus eliminating the potential influence of random genomic insertion and ensuring homogenous levels of the GOI expression, allowing for comparisons to be made. There has been much success in utilizing this system to induce expression of mammalian proteins to investigate factors that control cell proliferation and to reproducibly identify target genes of transcription factors (239)**.** Our laboratory previously used this system to demonstrate that TRIM56, an IFN- and virus-inducible E3 ubiquitin ligase, is a positive regulator of the TLR3 antiviral signaling pathway (223). More recently, we used this system to determine the molecular basis of the versatility and specificity of TRIM56's antiviral activities against positive-strand RNA viruses (240). Moreover, using the Tet-Off version of the Tet-regulated

expression system, work from this lab first identified the PLpro of SARS-CoV as a potent IFN antagonist, which disrupts IRF-3-dependent IFN induction by interacting with IRF-3, and inhibiting its phosphorylation and nuclear translocation (186).

In future studies, to provide a more genetically homogenous and clonal population, individual CoV-nsp3 clones allowing higher expression levels may be selected through limiting dilution. Moreover, it may be useful to tag CoV-nsp3 with a fluorescent marker (e.g., GFP) and sort high expressers by flow cytometry. In conclusion, I have generated a tetracycline-inducible gene expression model system in HeLa cells that stably express SARS-CoV-nsp3 **Figure 3.3D**. I have demonstrated that HeLa-Fit-ScoV-nsp3 cells express SARS-CoV-nsp3 robustly and the expression is comparable to that of a mutant nsp3 lacking the PLP domain in the HeLa-Fit-ScoV-nsp3-delPLP cell line **Figure 3.5**. Such inducible cell lines can serve as a valuable in-vitro model system for studying the function of SARS-CoV proteins, which will provide insight into SARS pathogenesis.

CHAPTER 4.   REGULATION OF IRF-3-DEPENDENT INNATE IMMUNE SIGNALING PATHWAY BY THE PLPRO DOMAIN OF NONSTRUCTURAL PROTEIN (NSP3) OF SEVERE ACUTE RESPIRATORY SYNDROME (SARS) CORONAVIRUS (COV)

Introduction

The innate immune response constitutes the first line of defense upon viral infection. A hallmark of the host innate immune response to invading viral pathogens is the rapid induction of type I IFNs (IFNα and IFNβ) (155). Type I IFNs are potent antiviral cytokines that function in an autocrine/paracrine fashion to induce the expression of hundreds of IFN-stimulated genes (ISGs) in the host early after virus exposure, thereby establishing an antiviral state that inhibits viral replication and viral spread (147,154-155). Because of these essential antiviral properties, type I IFNs are considered master regulators of antiviral immunity. In addition to direct inhibition of viral replication and spread, another level of IFN action is to shape the adaptive and acquired immune responses. The induction of type I IFNs begins by cellular pattern-recognition receptors (PRRs) detection of viral molecular signatures called pathogen-associated molecular patterns (PAMPs) (241). Toll-like receptors (TLRs) and retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) are two important classes of PRRs that have been shown to be involved in the recognition of virus-specific components, such as viral surface glycoproteins, intracellular viral proteins, and viral nucleic acids (140, 146, 147, 241). Of these, double-stranded RNA (dsRNA) is a prominent viral PAMP produced by many RNA viruses as viral replication intermediates, and dsRNA is sensed by RIG-I, MDA5, and TLR3 to trigger IFNβ induction (147-150, 241).

Upon engaging viral dsRNAs, RIG-I, MDA5, and TLR3 recruit their cognate adaptor proteins MAVS and TRIF respectively, triggering intracellular signaling pathways converging on a common TRAF3 adapter complex, which then activates two protein kinases TBK1 and IKKε. These kinases function to activate three distinct families of transcription factors, including NF-κB, ATF2/c-Jun and IRF-3 (146,147,154,158). Assembly of these three transcription factors to the positive regulatory domains (PRD) in the IFNβ promoter region induces transcription of the IFNβ gene. Of these three transcription factors, IRF-3 is the main regulator in the initial induction of IFNβ gene expression induced by viruses. IRF-3 is a constitutively expressed protein that resides in an inactivated state in the cytosol. Upon virus infection, activated TBK1 and IKKε directly phosphorylate serine 385 and 386 residues on the carboxyl-terminal (c-terminal) region of IRF-3 (242). Furthermore, IRF-3 has been shown to undergo virus-induced phosphorylation on a serine-threonine cluster in the C-terminal region, at amino acids (aa) 396 to 405 (220). Phosphorylated IRF-3 forms homo-dimers, translocates into the nucleus and binds to its target DNA sequence on the IFNβ promoter, resulting in the induction of IFN-β gene expression (156-158). Consequently, for every host defense, there is a viral offense, and during coevolution with their hosts, viruses have evolved and adapted strategies to exploit and modulate the cellular IFN responses by encoding viral factors that disrupt host innate immune

signaling pathways. The interplay between a virus and its host early in infection largely defines disease pathogenesis, thus defining the mechanisms by which viruses modulate the host innate immune response, and may provide valuable knowledge that can facilitate the development of vaccines and antiviral therapeutics.

SARS-CoV emerged from a zoonotic reservoir in late 2002 to cause the first epidemic of the millennium. First identified in Guangdong Province, China, SARS-CoV is the causative agent of the highly contagious viral respiratory disease known as Severe Acute Respiratory Syndrome (SARS) (5). Over a small number of months, this novel viral disease became a worldwide threat, spreading to more than two dozen countries across 5 continents, infecting 8098 people of all ages, with the elderly more severely affected. The World Health Organization (WHO) reported that of those 8098 people who became ill, 774 died (http://www.who.int/csr/sars/country/table2003_09_23/en/index.html), 68). In humans in whom the outcome was fatal, SARS disease was associated with continued uncontrolled viral replication and an aberrant inflammatory response in the lung, suggesting that SARS-CoV evades and/or modulates host innate antiviral immune responses (68). The SARS epidemic has sparked a keen interest among the scientific community to focus its attention on the capacity of coronaviruses to counteract or evade the host innate immune system and on the need to develop preventive strategies to protect against SARS-CoV should the virus re-emerge. In 2012, another highly pathogenic coronavirus emerged to cause severe disease in humans, named MERS-CoV, which has a mortality rate of 38% (24). To date, there are no FDA-approved antiviral therapeutics or vaccines for any of the human coronaviruses. Although there have not been any reported SARS cases in humans since 2004, the emergence of MERS-CoV nearly ten years later illustrates that highly pathogenic coronaviruses will likely continue to emerge from zoonotic sources to cause severe disease in the human population. Furthermore, such emergence highlights the need for the discovery of antiviral drugs and/or vaccines against infections caused by coronaviruses.

SARS-CoV genome is a large, nonsegmented, positive-stranded, 5'-capped, 3'-polyadenylated RNA molecule that can function as messenger RNAs (mRNAs) (1). Fourteen functional open-reading frames have been identified in the genome of SARS-CoV (4,116). As with all coronaviruses, genomic RNA is released in the cell cytoplasm after infection and translated into two large replicase polyproteins, called pp1a and pp1ab (1, 5, 118). The replicase polyproteins are autoproteolytically cleaved by two viral cysteine proteases, the papain-like protease (PLpro) and a 3C-like protease (3CLpro), to generate 16 nonstructural proteins (nsp 1 to 16). The nsps, also referred to as the replicase proteins, function to assemble with host cell membranes to generate a complex network of double membrane vesicles (DMV), essential for viral RNA replication (3, 118, 120-122). For these viruses, the PLpro is essential for processing the amino-terminal end of the replicase polyproteins at 3 junctions, through the recognition of LXGG motif, releasing nsp1-nsp3 mature proteins from the viral polyprotein, respectively (120, 133). Since they are critical for SARS-CoV replication, they are attractive targets for antiviral treatments.

The PLpro domain resides within nsp3, which is the largest replicase subunit. Nsp3 is a multifunctional, multidomain protein; however, the function of many of the domains identified within SARS-CoV nsp3 is not well known. Nevertheless, the investigation of the papain-like protease of SARS-CoV has illustrated the multi-functionality of coronaviral PLPs. Several reports have demonstrated that the PLpro domain of nsp3 functions not only as a viral protease, but also possesses deubiquitinating (DUB) and deISGylating activities (135, 138-139, 199, 201). The host cell uses both Ub and ISG15 as unique signaling components to promote the antiviral innate immune responses during viral infection. The fact that SARS-CoV PLpro can cleave and disrupt important host cell innate immune elements illustrates the multifunctional nature of this protease and suggests that it is a major virulence factor.

SARS-CoV generally does not induce type I IFN in infected cells in culture, which suggests that SARS-CoV evades or suppresses the induction of type I IFN (162). We previously demonstrated that PLpro domain of SARS-CoV nsp3 is a potent IFN antagonist, which acts to suppress IRF-3-dependent type I IFN induction by interacting with IRF-3 and inhibiting its phosphorylation, dimerization, and nuclear translocation (186). Furthermore, we showed that the inhibition of the IFN response was independent of the protease activity. Although the mechanisms of actions are different, other groups have demonstrated a similar phenomenon that SARS-CoV PLpro can inhibit IRF-3-dependent type I IFN induction (194). Most of the in-vitro studies examining the functional role of the PLpro domain have mainly used truncated nsp3 PLpro domain constructs. On the contrary, Wathelet et al transiently expressed SARS-CoV full-length nsp3 protein in HEK293T cells and demonstrated that nsp3 expression could inhibit virus-induced type I IFN induction (191). However, the authors did not dissect the antagonistic properties of nsp3 and determine what domain was responsible for this ability. To our knowledge, it has not been shown whether expression of SARS-CoV PLpro impacts IRF-3 signaling when stably expressed in the context of the full-length nsp3 protein, which is the case in virus-infected cells.

The objective of this study was to investigate the effects of SARS-nsp3 expression on the IRF-3 innate immune signaling pathway. Examining the function of PLpro on induction of the type I IFN responses to viruses, in the context of the full-length nsp3 protein, is essential to the understanding of SARS pathogenesis. In this present study, I provide evidence that SARS-nsp3 contributes to SARS-CoV antagonism of IRF-3-dependent host innate immune responses and that this ability is dependent on the PLpro domain.

Inducible Expression of SARS-CoV Nsp3 Inhibits IRF-3-Dependent Antiviral Gene Responses.

To guard against virus infection, mammalian cells employ a complex antiviral defense program characterized by the rapid induction of immediate early antiviral genes such as (IFN-α/β), ISG15 and ISG56 (160). We have previously reported that the PLpro domain inhibits the activation of the IFN-β promoter following engagement of TLR3 or RIG-I pathways (186). As mentioned above, the PLpro domain is a catalytically active domain within nsp3. Therefore, I investigated whether SARS-nsp3 behaves in a similar fashion and can regulate the induction of IRF-3-dependent type I IFN responses. To examine the effect of SARS-nsp3 in the antagonism of type I IFN responses, I used the Flp-In-T-Rex expression system to generate HeLa cells that stably express SARS-nsp3, in a tetracycline-regulated fashion named HeLa-Fit-ScoV-nsp3. The SARS-nsp3 protein is tagged with a 2xHA-epitope at the N-terminus, to facilitate its detection. This system is unique as it allows for the generation of stable, inducible cell lines using site-specific recombination and integration mediated by *Saccharomyces cerevisiae*-derived Flp recombinase (229), thus obliterating the sometimes difficult transient expression dynamics, where the efficiency of transfection decreases with very large expression plasmids. Furthermore, some cell lines may be refractory to transfection.

HeLa-Fit-ScoV-nsp3 cells in the absence or presence of Tet, to repress or induce expression of SARS-nsp3, were cotransfected with a plasmid expressing the firefly luciferase reporter gene under control of the human IFNβ promoter and a constitutively expressed Renilla luciferase-encoding plasmid. The Renilla luciferase activity serves as an internal control to normalize the transfection efficiency of the IFNβ reporter plasmid. After induction of the IFN pathway by SeV infection, a known potent inducer of IRF-3-dependent gene expression via RIG-I pathway, firefly luciferase activity was measured and normalized relative to Renilla luciferase expression. As expected, the IFN-β promoter is activated through TLR3 (data not shown) or RIG-I pathways (**Figure 4.1**); however, in the presence of SARS-nsp3, activation of the virally induced IFNβ promoter is significantly reduced **Figure 4.1**. SARS-nsp3 expression did not affect SeV infection, since SeV protein expression detected shows comparable levels in Tet-induced and uninduced HeLa-Fit-ScoV-nsp3 cells.These data suggest that SARS-nsp3 inhibits IRF-3-dependent transcription of IFN-β. I therefore investigated the ability of SARS-nsp3 to prevent the induction of an additional well-characterized IRF-3-dependent target gene, ISG56, and determined if this ability is dependent on the PLpro domain.

Inverse PCR mutagenesis was employed to generate a SARS-nsp3 PLpro domain deletion construct. Using the strategy that I implemented to generate the HeLa-Fit-ScoV-nsp3 cell line, I created HeLa cells that stably express HA-SARS-nsp3-delPLpro, in a tetracycline-regulated fashion designated HeLa-Fit-ScoV-nsp3-delPLpro. These cells were used in combination with HeLa-Fit-ScoV-nsp3 cells to evaluate SARS-nsp3's inhibitory effects on virus-induced ISG56 expression and to dissect the PLpro domain's
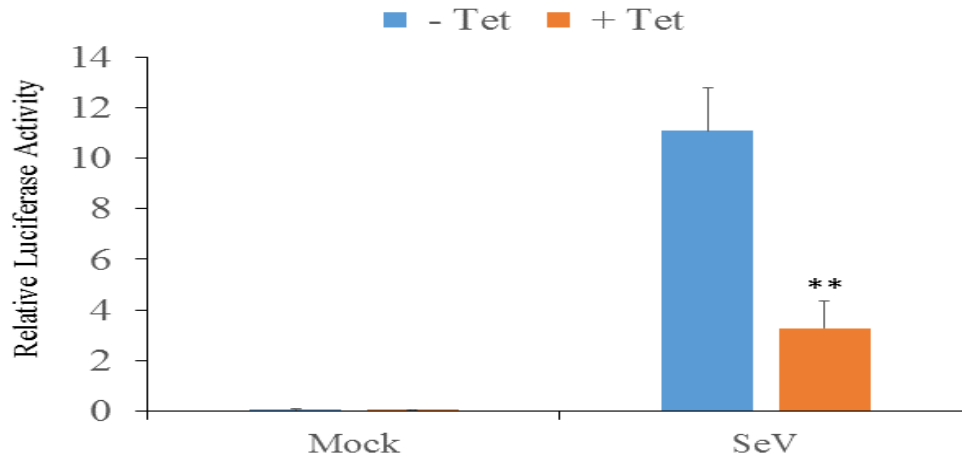
Figure 4.1. **Inducible expression of SARS-CoV nsp3 inhibits virus-dependent activation of the IFNβ promoter.**

Activation of IFNβ promotor, HeLa-Fit-ScoV-nsp3 cells were grown for 48 h in the absence (-Tet) (blue bars) or presence (+Tet) (red bars) of 2ug/ml Tet treatment to repress or de-repress HA-SARS-nsp3 expression (Performed in triplicates). Subsequently, cells were co-transfected with pIFNβ-luc (80ng/well) and pRL-TK (20ng/well). Cells were then mock infected or infected with 100 HAU of SeV for 16 hrs prior to cell lysis and assayed for relative luciferase activity as a readout of IFN-β activity. The error bars represent standard deviation.

role in the antagonism of IRF-3-dependent type I IFN responses in the context of the full-length nsp3 protein. In the absence of tetracycline in the culture medium, both cell lines exhibited no detectable exogenous protein expression **Figure 4.2** (odd numbered lanes).

However, upon the addition of tetracycline into the culture medium, both cell lines demonstrated robust expression levels of HA-SARS-nsp3 and HA-SARS-nsp3-delPLpro respectively **Figure 4.2** (even- numbered lanes). This finding suggests that expression of the wild-type (wt) and delPLpro mutant of SARS-nsp3 is tightly regulated by tetracycline and that their expression levels are comparable. Consistent with data obtained from the IFN-β promoter assay **Figure 4.1**, I found that inducible expression of wt HA-SARS-nsp3 greatly inhibited the SeV induction of endogenous ISG56 expression, but in the presence of HA-SARS-nsp3-delPLpro mutant, this ability is lost **Figure 4.2**.

To further verify my observations, I used Image J software to quantify ISG56 expression. The value of ISG56 expression is a measure of the relative intensity of each band compared to the standard (control cells -Tet), having a relative density value of 100. In the presence of HA-SARS-nsp3, ISG56 band intensity was decreased by 52%, illustrating the profound inhibitory effect that HA-SARS-nsp3 has on IRF-3-dependent gene expression **Figure 4.2** (left panel). Interestingly, there is a slight increase in ISG56 band intensity in the presence of HA-SARS-nsp3-delPLpro **Figure 4.2** (right panel), suggesting that SARS-nsp3 PLpro domain plays a key role in SARS-CoV escape from host innate immune responses. These findings demonstrate that SARS-nsp3 has the ability to efficiently disrupt virus-induced expression of IRF-3-dependent antiviral genes and that this ability is dependent on the PLpro domain.

SARS-CoV Nsp3 Does Not Inhibit the JAK/STAT1 Pathway

Our data show that HA-SARS-nsp3 can inhibit the induction of type I IFN responses; therefore, I also examined whether HA-SARS-nsp3 could inhibit type I IFN signaling downstream of the IFN receptors. The JAK/STAT pathway is a well-established IFN-dependent pathway that is triggered by the secretion of type I IFNs. Activation of this pathway consists of the nuclear translocation and initiation of gene transcription by STATs, which have been activated in response to JAK-mediated phosphorylation (160). To determine the effects that HA-SARS-nsp3 has on IFN signaling, I examined the IFN-induced phosphorylation of Stat1, a key molecule that is indispensable for the JAK/STAT pathway activity. HeLa-Fit-ScoV-nsp3 cells in the absence or presence of Tet, to repress or induce expression of HA-SARS-nsp3, were treated with IFN-α for 1 hr. Treatment with IFN-α induced the phosphorylation of STAT1; however, in the presence of HA-SARS-nsp3, pSTAT1 protein expression was not inhibited **Figure 4.3**. These data suggest that SARS-nsp3 does not block IFN-dependent signaling.
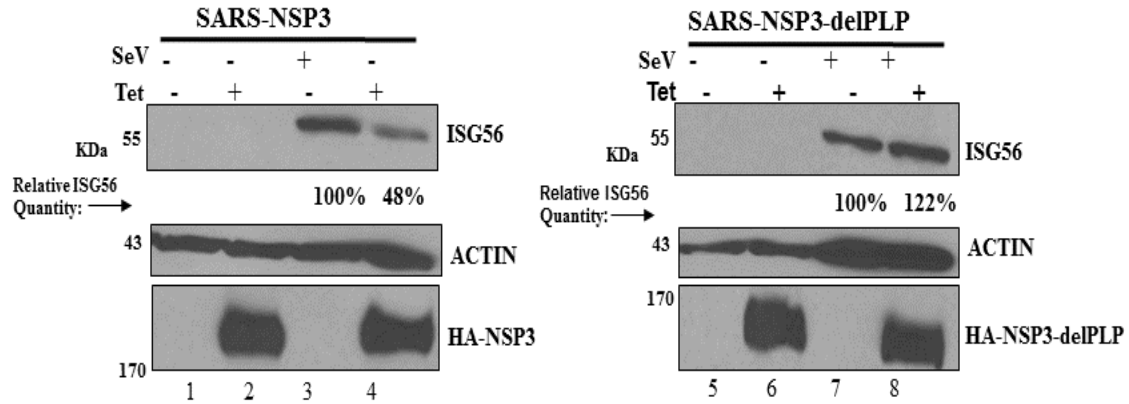
Figure 4.2.   SARS-nsp3 ability to inhibit virus-induced ISG56 expression is dependent on the PLpro domain.

Immunoblot analysis of HA-SARS-nsp3, HA-SARS-nsp3-delPLP, Actin, and ISG56 expression in HeLa-Fit-ScoV-nsp3 and HeLa-Fit-ScoV-nsp3-delPLP cells that were mock infected (lanes 1:2 and 5:6) or challenged with SeV (100HAU; lanes 3:4 and 7:8) for 16 hrs in the absence (-Tet, odd-numbered lanes) or presence (+Tet, even-numbered lanes) of 2ug/mL of Tet treatment for HA-SARS-nsp3 and HA-SARS-nsp3-delPLP inducible expression.

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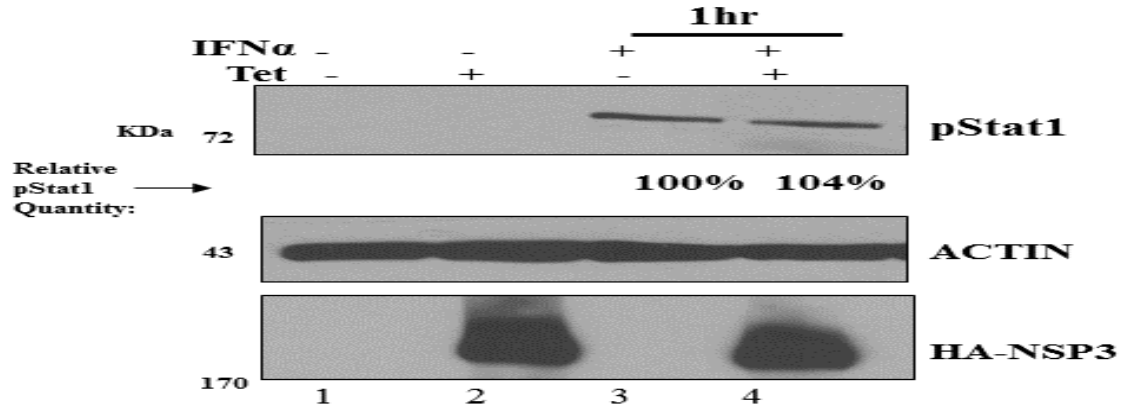
Figure 4.3.   Inducible expression of SARS-Nsp3 does not inhibit IFN-induced Stat1 Phosphorylation.

Immunoblot analysis of HA-SARS-nsp3, Actin, and pStat1 expression in HeLa-Fit-ScoV-nsp3 cells that were mock treated (lanes 1 and 2) or treated with IFNα (200 units; lanes 3 and 4) for 1 hour and (lanes 5 and 6) for 2 hrs in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 2ug/mL of Tet treatment for HA-SARS-nsp3 inducible expression.

SARS-CoV Nsp3 Has the Ability to Compromise the TLR3 and RIG-I Stimulated Antiviral Response.

Vesicular Stomatitis Virus (VSV) replication is known to be highly sensitive to the antiviral action of type I IFNs; thus, it is a useful tool to assay for antiviral activities. To investigate SARS-nsp3-mediated inhibition of type I IFN induction, I studied the effects of HA-SARS-nsp3 expression on the establishment of an antiviral state induced by TLR3 and RIG-I ligands. It is well known that the TLR3 signaling pathway can be stimulated by a synthetic dsRNA analog, polyriboinosinic:polyribocytidylic acid (poly I:C) (243). As with viral dsRNAs, engagement of TLR3 with poly (I-C) activates the TRIF-dependent pathway, culminating in the induction of type I IFNs, proinflammatory cytokines, and chemokines, mediated by NF-κB and IRF-3 activation (222). Therefore, HeLa-Fit-ScoV-nsp3 cells were left uninduced or induced with Tet, to repress or turn on expression of HA-SARS-nsp3. 48 hrs later cells were either mock treated or treated with poly (I-C) in the culture medium, to confer an antiviral state. Afterwards, cells were either mock infected or infected with an engineered firefly luciferase reporter-expressing, recombinant strain of vesicular stomatitis virus (VSV-Luc). Infected cells express the firefly luciferase reporter protein; thus, measurement of firefly luciferase activity is a readout for VSV replication. In the absence or presence of Tet, VSV-Luc replicated to similar efficiencies in mock-treated HeLa-Fit-ScoV-nsp3 cells, as determined by the VSV-encoded luciferase activity **Figure 4.4A.** Stimulation by poly (I-C) in the media produced an antiviral state in HeLa-Fit-ScoV-nsp3 cells in the absence of Tet, resulting in a significant reduction of VSV-luc replication, when compared to unstimulated cells **Figure 4.4A**. However, in the presence of Tet (HA-SARS-nsp3 expression induced), the poly (I-C)-induced antiviral state was compromised **Figure 4.4A,** suggesting that SARS-nsp3 impairs the poly (I-C)-established antiviral state via TLR3.

Using tranfection of a hepatitis C virus (HCV) RNA Replicon RNA, a RIG-I ligand, I investigated the ability of SARS nsp3 to disrupt the RIG-I-mediated induction of the antiviral state. Stimulation with the HCV RNA replicon induces an antiviral state in HeLa-Fit-ScoV-nsp3 cells in the absence of Tet, resulting in a significant reduction of VSV-luc replication, when compared to unstimulated cells **Figure 4.4B**. However, in the presence of SARS-nsp3, the RIG-I-induced antiviral state is compromised, as demonstrated by the significant increase in VSV replication when compared to control cells **Figure 4.4B**. To further prove the PLpro domain is responsible for SARS-nsp3-mediated IFN antagonistic ability, I examined if inducible expression of SARS nsp3-delPLpro mutant had the ability to disrupt the poly (I-C)-induced antiviral state. While wt SARS nsp3 maintains the ability to significantly compromise the poly (I-C)-induced antiviral state; SARS nsp3-delPLpro lost such ability.

VSV contains a ribonucleoprotein (RNP) complex that consists of the viral genome RNA enwrapped by the nucleoprotein (NP), which serves as a template for mRNA transcription as well as genome replication (1). Thus, to further investigate SARS-nsp3 inhibition of IFN induction, I tested the effects of SARS-nsp3 on VSV NP expression. The VSV NP is expressed robustly in the absence or presence of SARS-nsp3 **Figure 4.5** (lanes 3 and 7). However, upon stimulation with HCV RNA in the absence of
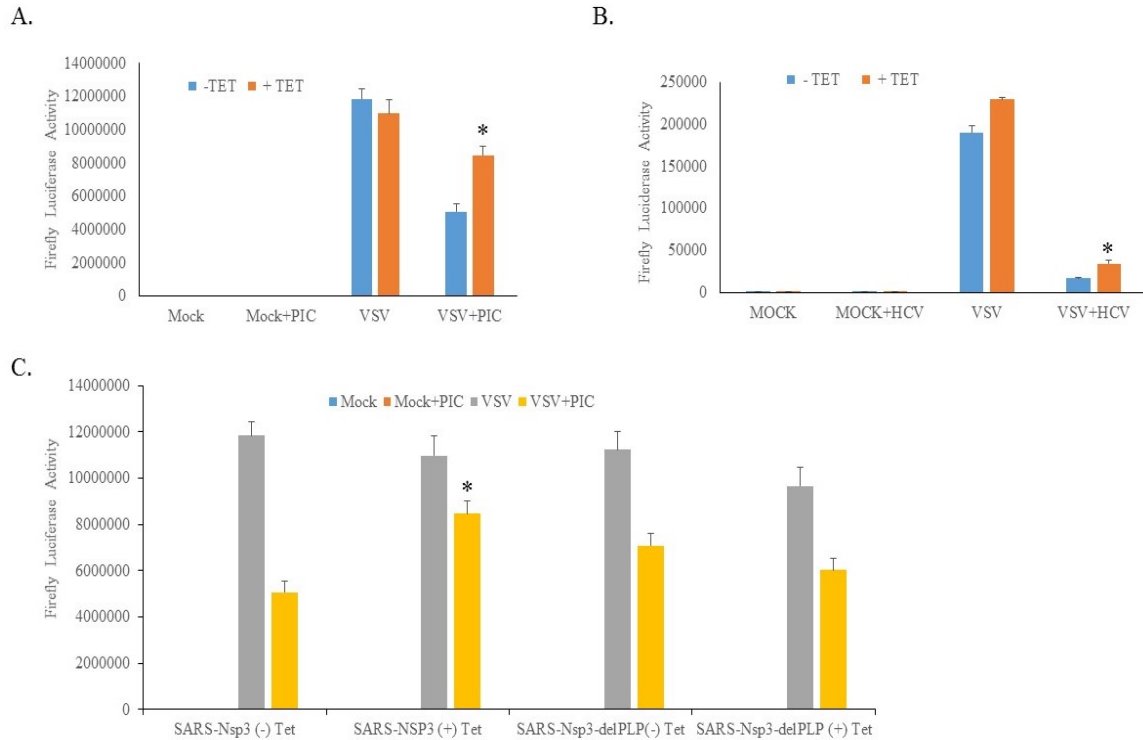
Figure 4.4.   Inducible expression of SARS-nsp3 compromises TLR3 and RIG-I mediated antiviral state.

(A) Cells grown in the absence (-Tet) or presence (Tet+) of 2ug/ml of Tet treatment for inducible HA-SARS-nsp3 expression were mock stimulated or stimulated with poly (I-C) (25ug/mL), followed by infection with VSV-Luc (MOI=0.1). At 6-8 h post infection, cells were lysed for firefly luciferase assay. (B) HeLa-Fit-ScoV-nsp3 cells grown in the absence (-Tet) or presence (Tet+) of 2ug/ml of Tet treatment for inducible HA-SARS-nsp3 expression were mock transfected or transfected with HCV replicon RNA (2ug/mL), followed by infection with VSV-Luc (MOI=0.1). At 6-8 h post infection, cells were lysed for firefly luciferase assay. (C) Cells grown in the absence (-Tet) or presence (Tet+) of 2ug/ml of Tet treatment for inducible HA-SARS-nsp3 and HA-SARS-nsp3-delPLP expression were mock stimulated or stimulated with poly (I-C) (25ug/mL), followed by infection with VSV-Luc (MOI=0.1). At 6-8 h post infection, cells were lysed for firefly luciferase assay.

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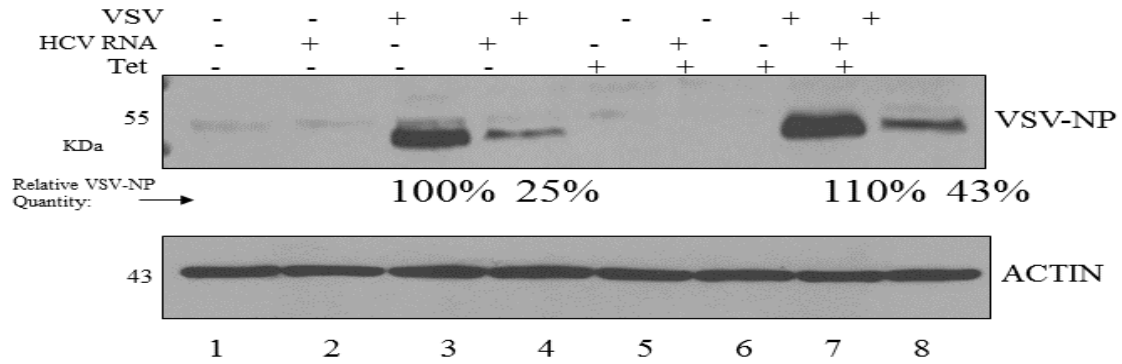
Figure 4.5. **Inducible expression of SARS-nsp3 compromises RIG-I mediated antiviral state.**

Immunoblot analysis of VSV-NP and Actin expression in HeLa-Fit-ScoV-nsp3 cells. Cells grown in the absence (-Tet) or presence (Tet+) of 2µg/ml of Tet treatment. Cells were mock transfected or transfected with HCV replicon RNA (2µg/mL), followed by infection with VSV-Luc (MOI=0.1). At 6-8 h post infection, cells were lysed for immunoblot analysis.

Tet, VSV NP expression is greatly diminished **Figure 4.5 (**lane 4), when compared to unstimulated cells **Figure 4.5** (lane 3), suggesting that a RIG-I-mediated antiviral state is produced. In comparison, upon turning on HA-SARS-nsp3 expression, VSV NP expression is increased **Figure 4.5** (lane 8) when compared to control cells, suggesting that SARS nsp3 interferes with RIG-I- stimulated antiviral responses, permitting an increase in VSV replication. These findings further prove that SARS-nsp3 has the ability to compromise the TLR3- and RIG I-mediated antiviral response, and that this ability is mediated by the PLpro domain.

SARS-CoV Nsp3 Level of Blockade

IRF-3 plays a pivotal role in the transcriptional regulation of the IFN-β promoter and subsequent ISG expression (220, 242). To further establish which step of the IRF-3 signaling pathway leading to IRF-3 activation is targeted by SARS-nsp3, I determined the effect of HA-SARS-nsp3 on the expression of ISG56, following overexpression of signaling proteins known to participate in RIG-I/MDA5 and TLR3 pathways upstream of IRF-3. I found that HA-SARS-nsp3 strongly diminished the expression of ISG56 by overexpression of TBK1 or IKKε **Figure 4.6A**. In contrast, SARS-nsp3 expression had no effect on ISG56 expression in cells overexpressing the constitutively active, phospho-mimetic IRF-3 mutant, IRF-3-5D **Figure 4.6A**. To confirm the observations presented above, image J was used to quantify the intensity of ISG56 bands. The intensity values of ISG56 bands for HeLa-Fit-ScoV-nsp3 cells in the absence of Tet were set to 100 **Figure 4.6A** (odd-numbered lanes), and the values for ISG56 bands in the presence of Tet are shown relative to those in the absence of Tet **Figure 4.6A** (even-numbered lanes). In HeLa-Fit-ScoV-nsp3 cells overexpressing the empty vector, ISG56 band intensity in the presence of SARS-nsp3 is decreased by 97% as compared with –Tet cells (no detectable nsp3 expression), thus demonstrating the ability of SARS-nsp3 to inhibit IRF-3-dependent ISG56 expression.

Overexpression of the kinases should constitutively drive up IRF-3 activation and subsequent ISG56 expression; however, in the presence of SARS-nsp3, ISG56 band intensity is decreased by 67% and 82% respectively **Figure 4.6A** (lane 6 and 8). In contrast, in HeLa-Fit-ScoV-nsp3 cells overexpressing IRF-3-5D, ISG56 band intensity increased in the presence of SARS-nsp3 **Figure 4.6A** (lane 10), suggesting that at this level SARS-nsp3's ability to impair the host innate immune responses is lost. The ability of SARS-nsp3 to inhibit TBK1- and IKKε-dependent responses indicates that SARS-nsp3 acts downstreaml of these signaling molecules to prevent IRF-3 activation. In contrast, SARS-nsp3 was unable to inhibit ISG56 expression by overexpression of IRF-3-5D, suggesting that SARS-nsp3 functions to prevent the activation of IRF-3, but is unable to disrupt IRF-3 function once it is activated by phosphorylation. Therefore, I conclude from these experiments that HA-SARS-nsp3 disrupts IRF-3-mediated signaling by acting at a level that is downstream of the IRF-3 kinases and upstream of phosphorylated IRF-3.

The active site of SARS-CoV PLpro consists of a canonical Cys1651-His1812-Asp1826 catalytic triad (136). Mutation of any of the three sites is known to abolish the
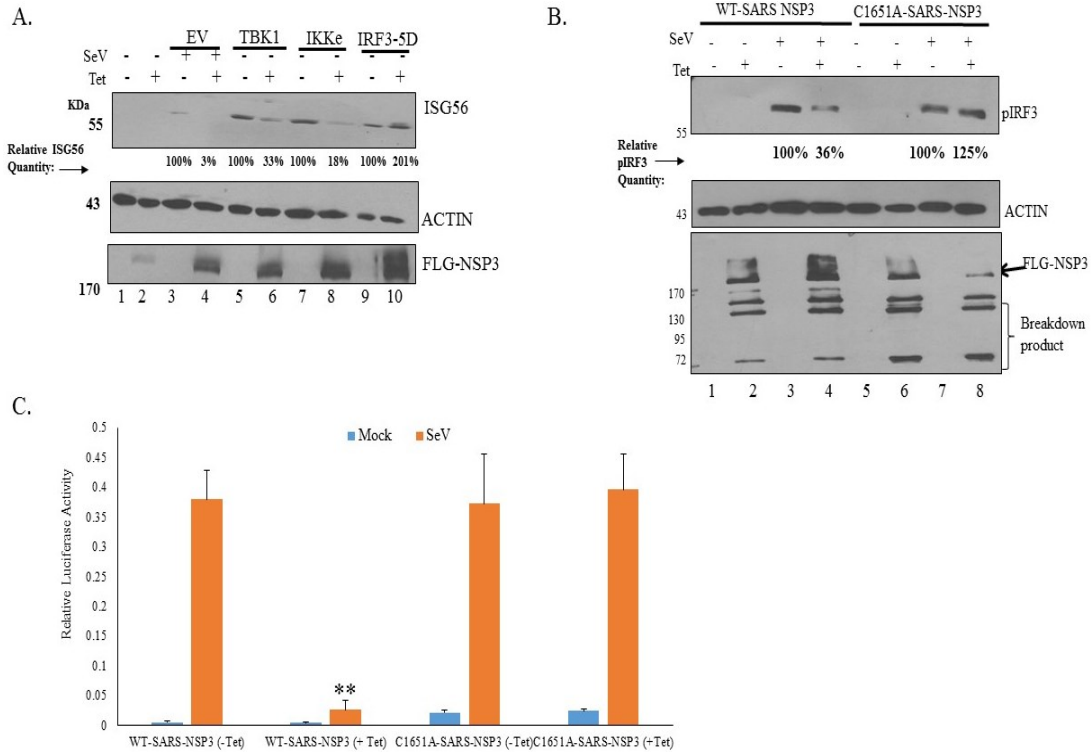
Figure 4.6.   SARS-nsp3 level of blockade dependent on its protease activity.

A) Immunoblot analysis of HA-SARS-NSP3, and ISG56 expression in HeLa-Fit-ScoV-nsp3 cells grown in the absence (-Tet odd-numbered lanes) or presence (Tet+ even-numbered lanes) of 2ug/ml f of Tet treatment for FLG-SARS-nsp3 expression cells that were transiently transfected with various signaling molecules within the TLR3 and RIG-1/MDA5 pathways above and below the level of IRF-3; SeV-infected cells (lane 3 and 4) serve as a positive control of the SARS-nsp3 blockade of ISG expression. (B) Immunoblot analysis of HA-SARS-nsp3, and pIRF3 expression in HeLa-Fit-ScoV-nsp3 and HeLa-Fit-ScoV-nsp3-C1651A cells grown in the absence (-Tet odd-numbered lanes) or presence (Tet+ even-numbered lanes) of 2ug/ml f of Tet treatment for HA-SARS-nsp3 and HA-SARS-nsp3-C1651A expression cells that were mock infected (lanes 1:2 and 5:6) or infected with SeV (100HAU; lanes 3:4 and 7:8) for 16 hrs. (C) Activation of IFNβ promotor, HeLa-Fit-ScoV-nsp3 and HeLa-Fit-ScoV-nsp3-C1651A cells were grown for 48 h in the absence (-Tet) (blue bars) or presence (+Tet) (red bars) of 2ug/ml Tet treatment to repress or de-repress FLG-SARS-nsp3 or FLG-SARS-nsp3-C1651A expression Subsequently, cells were co-transfected with pIFNβ-luc (80ng/well) and pRL-TK (20ng/well). Cells were then mock infected or infected with 100 HAU of SeV for 16 hrs prior to cell lysis and assayed for relative luciferase activity as a readout of IFN-β activity. The error bars represent standard deviation.

protease activity of the PLpro (135). Next, I determined whether the inhibition of IRF-3 activation by SARS-nsp3 is dependent on its protease activity. I conducted western blot analysis, determining the ability of SARS-nsp3 and a protease-deficient SARS nsp3 mutant (C1651A) to inhibit virus-induced IRF-3 phosphorylation. I found that when SARS-nsp3 was expressed, virus-induced phosphorylated IRF3 was reduced; however, in the presence of the catalytic mutant SARS-nsp3-C1651A, there was no observable reduction in phosphorylated IRF-3 expression **Figure 4.6B**. Quantification of the band intensity of pIRF3 further supported the results observed. In the presence of SARS-nsp3, pIRF3 band intensity decreased significantly by 64% **Figure 4.6B** (lane 4), yet in the presence of the SARS-nsp3 mutant lacking catalytical activity, pIRF3 band intensity was increased by 22%. Furthermore, SARS-nsp3 catalytic mutant is unable to inhibit the activation of the IFN-β promoter **Figure 4.6C**, suggesting that SARS-nsp3 may proteolytically cleave cellular proteins in this pathway to interfere with IRF-3 activation, thus inhibiting the expression of phosphorylated IRF-3. Alternatively, mutation of the C1651 residue in SARS-nsp3 PLpro domain may alter the overall structure of the nsp3 protein or its de-ubiquitinating activity, in ways that disrupt the ability to inhibit IRF-3 activation.

SARS-Nsp3 Mechanism of Action

It is well established that the TLR3 and RIG-I signaling cascades that elicit IFN-β gene induction converge at the level of TBK1 and IKK□, the kinases responsible for the phosphorylation and activation of IRF-3 (158). We have previously shown that SARS PLpro domain inhibits TLR3- and RIG-I-mediated IRF-3-dependent IFN-β induction by inhibiting IRF-3 phosphorylation, dimerization, and nuclear translocation (186). Additionally, we previously showed a physical interaction of the PLpro with IRF-3, in coimmunoprecipitation assays (186). Later studies have also associated the ability of CoV PLPs to inhibit type I IFN induction with their ability to antagonize STING, an important scaffolding molecule required for the activation of IRF-3, and this ability is mediated by catalytic-dependent and catalytic-independent activities (217-218). Despite the advances in understanding the mechanism by which SARS-CoV PLpro inhibits IRF-3-dependent signaling, evidence suggests that SARS PLpro interferes with the host innate immune responses by acting at a level that is downstream of TBK1 and IKK□ and in proximity to IRF-3. This evidence encouraged us to characterize potential mechanisms of SARS-nsp3 inhibition on IRF-3-dependent type I IFN induction. RIOK3 has been described as an essential novel adaptor protein required for the cytosolic nucleic-acid-induced type I IFN response (244). Furthermore, RIOK3 functions downstream of TBK1 and upstream of IRF-3 activation, making it a potential target of nsp3.

To determine if SARS-nsp3 interacts with RIOK3 to interfere with IRF-3 activation, HeLa-Fit-HA-ScoV-nsp3 cells repressed or induced for SARS-nsp3 expression were transfected with a Flag-tagged RIOK3 construct. After 48 hours of transfection, the protein complexes were extracted and subjected to immunoprecipitation analysis. Cell lysates were assayed for both HA-tagged SARS-nsp3 and Flag-tagged RIOK3, and both proteins were found to be expressed robustly **Figure 4.7B**. Co-IP
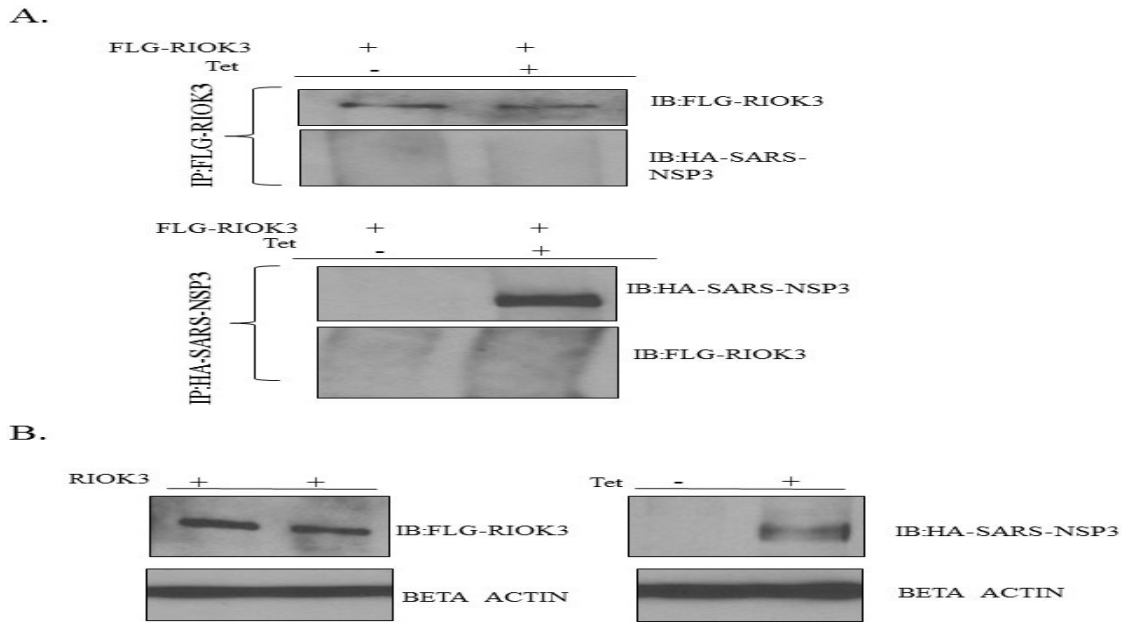
Figure 4.7.   SARS-nsp3 does not associate with RIOK3.

(A) Cell lysates were subjected to immunoprecipitation (IP) with a mouse anti-HA and anti-FLG antibody, followed by immunoblot analysis of HA-SARS-nsp3, and RIOK3-FLG. Data shown are representative of two independently conducted experiments. (B) HeLa-Fit-ScoV-nsp3 cells grown in the absence (-Tet odd-numbered lanes) or presence (Tet+ even-numbered lanes) of 2ug/ml f of Tet treatment for HA-SARS-NSP3 expression were transiently transfected with RIOK3-FLG plasmid for 48 hrs. Expression of HA-SARS-nsp3 and RIOK3 proteins in cell lysates was determined by immunoblot analysis.

experiments using anti-Flag or anti-HA antibodies revealed that SARS-nsp3 does not form a complex with RIOK3 **Figure 4.7A**. It has been reported that the DEAD-box helicase DDX3 is also a crucial component in the TBK1/IKKε-mediated activation of IRFs and ultimately the induction of type I IFNs (245-247). Most importantly, DDX3 is an important target for viruses to inhibit the induction of the antiviral innate response. For example, Hepatitis B virus polymerase functions to inhibit PRR-induced type I IFN induction by disrupting DDX3 and TBK1/IKKe complex (248). Likewise, DDX3 was identified as a host target of VACV protein, K7, which can inhibit SeV-induced IFN-β gene induction by inhibiting TBK1/IKKε-mediated IRF-3 activation (246). As with RIOK3, although cell lysates revealed robust expression of endogenous DDX3, following Co-IP experiments, no interaction was observed between HA-SARS-nsp3 and DDX3 (data not shown).

Discussion

The type I IFN (IFN-α/β) response is a crucial early antiviral defense mechanism utilized by the host to combat invading viral pathogens. Type I IFNs function to establish an antiviral state within infected and neighboring cells. IFN-β and -α1 gene expression is mediated by the transcription factors IRF-3, NF-κB, and AP-1. Once translated and secreted, IFN-β and -α1 bind to their cognate cell surface receptors in an autocrine and paracrine fashion. This induces the JAK/STAT signaling pathway, which induces an extensive range of interferon-stimulated genes (159,242-243). IRF-3 is also an essential regulator of a subset of pro-inflammatory genes such as RANTES and CXCL10, and can directly mediate the transcription of certain ISGs with ISRE sites including ISG15 and ISG56 (156). Viruses must battle these immune responses to propagate successfully; as a result, many viruses encode proteins that interfere with the initial induction of type I IFNs, blocking JAK/STAT signaling or inhibiting the antiviral state at a later step.

The initial observation that SARS-CoV induces a poor IFN response led to the assumption that the genome encodes antagonists of the IFN system. SARS-CoV has created a number of strategies to inhibit type I IFN production. We have shown previously that the PLpro domain contained within nsp3 is a potent IFN antagonist (186). The PLpro domain was shown to inhibit IFN-β by blocking IRF-3 phosphorylation, dimerization and nuclear translocation (186). Subsequent studies have also shown that the PLpro domain blocks IRF-3-mediated IFN induction by diverse mechanisms (194). However, the PLpro domain is contained within nsp3, a 213 kDa membrane-associated replicase product. To understand fully the pathogenesis of SARS-CoV infection, studies are needed to elucidate the mechanisms by which SARS-nsp3 interacts with its host and subverts the host antiviral response. A detailed understanding of this interaction in the context of the full-length nsp3 protein may yield important information that informs development of effective antiviral therapeutics that can be used to attenuate highly pathogenic coronaviruses' replication and pathogenesis.

In the present study, I demonstrate that expression of SARS CoV full-length nsp3 protein can inhibit IRF-3-mediated antiviral defenses. In particular, inducible expression

of nsp3 was shown to block the activation of virus-induced IFN-β promoter **Figure 4.1** and ISG56 expression **Figure 4.2A**. SARS-CoV replication requires the proteolytic processing of SARS-CoV replicase polyprotein into individual nsp proteins by two virally encoded cysteine proteases, 3CLpro and the PLpro. Using an autoproteolytic mechanism, the PLpro is responsible for the proteolytic cleavage of the amino terminal end of the viral polyprotein, resulting in the release of nsp1, nsp2, and nsp3 (120, 134). SARS-CoV proteolytic process requires an active site containing a classical catalytic triad composed of amino acids Cys1651, His1812, and D1826 (120, 134). Interestingly, I observed that the SARS-nsp3 C1651A protease mutant completely lost the ability to block expression of pIRF3, suggesting that this residue is indispensable for SARS-nsp3-mediated inhibition of IRF-3 activation **Figure 4.6B**. It is possible that this specific mutation of the catalytic site may alter the conformation of the protein, thus affecting the substrate binding event that drives the cysteine protease catalytic mechanism.

Although the evidence presented above suggests a protease-dependent mechanism for type I IFN antagonism, it is important to consider that the DUB/delSGylation activity of SARS nsp3 PLpro domain which may contribute to regulation of innate immunity is dependent on the same active site. Therefore, I cannot exclude the possibility that SARS-nsp3 may use the DUB/delSGylation activity to inhibit IRF-3 activation. Furthermore, it is also possible that the C1651A mutation may alter the overall folding of the nsp3 protein, disrupting the interaction of nsp3 with potential signaling proteins in the IRF-3 activation pathway it targets, including iRF-3 itself. Thus, to better understand the role, if any, of the catalytic activity in facilitating SARS-nsp3 PLpro inhibition of IRF-3 activation, it is essential to examine additional mutations in the PLpro domain, such as the other two catalytic sites, in the context of the full-length SARS-nsp3 protein. Nonetheless, we demonstrated that the ability of SARS-CoV nsp3 to antagonize IRF-3-mediated gene expression is dependent on the PLpro domain **Figure 4.2**.

Activation of the type I IFN system results in the stimulation of a signal transduction cascade that induces hundreds of ISGs, producing an antiviral state in host cells and stimulates the adaptive immune responses (249). VSV is highly sensitive to IFNs, and the presence of type I IFNs blocks VSV replication, as seen when stimulated with TLR3 and RIG-I ligands to induce an antiviral state; however, this effect was partially reversed by expression of SARS-nsp3 **Figure 4.4A** and **Figure 4.4B**. In contrast, SARS-nsp3-delPLpro mutant lost the ability to compromise the establishment of an antiviral state **Figure 4.4C**, demonstrating that SARS-nsp3 functions as a negative regulator of TLR3- and RIG-I-mediated induction of the antiviral state, which is dependent on the PLpro domain, in a biological assay system. Interestingly, our previous results and data published by others have shown that the PLpro domain has the capacity to inhibit IRF-3-dependent gene expression by blocking IRF-3 activation (186,194, 217,218). Here, I show that SARS-nsp3 inhibitory effect was bypassed by the expression of a phospho-mimetic IRF-3 5D, a constitutively active form of IRF-3, indicating that nsp3 interferes with IRF-3-mediated signaling in the vicinity of IRF-3, prior to its activation **Figure 4.6A**. Most recently, Chen et al indicated that SARS-CoV PLpro negatively regulates IRF-3- dependent antiviral innate immune responses by targeting the IRF-3 scaffolding protein STING (217). In contrast to our observations and those of

others, Matthews et al observed that SARS-CoV PLpro can inhibit IRF-3 signaling at a step after IRF-3 activation, suggesting another antagonistic strategy of PLpro (250). It is unclear why this effect was not observed by us and other research groups.

The PLpro domain may employ multiple mechanisms to inhibit IRF-3 signaling; however, the detailed mechanism by which nsp3 inhibits IRF-3 activation has not been described. In search for SARS-nsp3 interacting molecules, I identified two potential cellular targets that function downstream of TBK1/IKKε and in the proximity of IRF-3. It is well established that DDX3 is a component of the antiviral innate immune signaling pathway leading to type I IFN induction. DDX3 contributes to the upregulation of type I IFNs induction through the formation of a complex with IKKε or TBK1 (245-247). IKKε phosphorylation of DDX3 is required for the recruitment of IRF-3 into the complex (245). Interestingly, similar to STING, DDX3 functions as a bridging adaptor linking IKK-related kinases to IRF-3. A recent study identified RIOK3 as a novel adaptor protein that is crucial for IRF-3-mediated antiviral defenses (244). This study demonstrated that RIOK3 functions downstream of TBK1 and upstream of IRF-3 activation. Furthermore, RIOK3 physically formed a complex with both IRF-3 and TBK1 and is required for the interaction between TBK1 and IRF-3 (244). However, upon our investigation, I found that SARS-nsp3 does not interact with RIOK3 **Figure 4.7A** or DDX3 (data not shown). It is possible that the experimental conditions used above do not promote SARS-nsp3 interaction with DDX3 or RIOK3, or that such interactions may be transient and weak. Thus, it is important to further determine the potential interaction of SARS-nsp3 with DDX3 and/or RIOK3, and search for other potential targets of SARS-nsp3. While the mechanism of action of nsp3 was undefined in this study, future experiments are underway to elucidate the precise mechanism by which SARS-nsp3 inhibits IRF-3-dependent antiviral innate immune responses.

In conclusion, our investigations have demonstrated that SARS-nsp3 protein is a bona-fide interferon antagonist, which acts through PLpro-mediated suppression of IRF-3 activation. SARS-CoV encodes many antagonists of the host innate immune response, including open reading frame 6 (ORF6) protein, nucleocapsid, ORF3b, NSP1, NSP3, and the PLpro domain (113, 114,186,191,192,194). Many of these IFN antagonists have evolved different strategies to counteract the IFN system by inhibiting IFN synthesis and/or signaling. By rapidly inhibiting type I IFN gene induction, SARS-CoV is able to suppress the immediate early antiviral innate immune response, resulting in the successful establishment of infection. The existence of multiple IFN antagonists in the SARS-CoV genome exemplifies the importance of understanding the biology of virus and host interactions.

The multifaceted strategies circumventing the initial host innate immune response evolved by SARS-CoV likely support the replication and spread of this virus in different cell types and different species. Results from this study demonstrate that SARS-nsp3 is a key player in this context, suggesting a critical role for this protein in pathogenesis and disease outcome. This work provides further insight into SARS-CoV antagonism of the host innate immune response in a biologically relevant setting. Characterization of virulence factors responsible for negatively regulating the IFN response is an essential

component in the development of vaccines and therapeutics aimed at disrupting this critical aspect of viral pathogenesis. Future studies should be directed towards identifying the precise mechanism of SARS-nsp3-PLpro-mediated suppression of IRF-3 activation.

CHAPTER 5.   DISCUSSION

An In-vitro System for Comparative Studies of Human Coronavirus (CoV) Nonstructural Protein 3 (Nsp3)

The replication of coronaviruses is a highly orchestrated and complex process. To date, coronaviruses contain the largest known RNA genome. Comparable to many other positive-sense, single-stranded RNA viruses, coronaviruses' genomic replication and transcription are mediated by a large replication complex that is attached to rearranged intracellular host membranes (118). Specifically, coronaviruses induce membrane rearrangements called double-membrane vesicles (DMVs), which function as a structure for viral genome replication and perhaps offer protection from the host defenses (121-122). The important role of coronavirus nsp3 in virus replication has been shown by several earlier reports (123,124). Nsp3 has been shown to interact with numerous of other viral nonstructural proteins involved in replication and transcription and, as such, may serve as a scaffolding protein for the recruitment of these viral non-structural proteins to the site of DMVs. Angelini et al demonstrated that the full-length and the C-terminal-truncated form of nsp3 have membrane disarranging and proliferation abilities. Furthermore, working in concert, nsp3, nsp4, and nsp6 have the ability to induce DMVs which are similar to those observed in SARS-CoV infected cells (123).

Most importantly, nsp3 houses the coronavirus-encoded papain-like protease (PLP), which plays a pivotal role in viral polyprotein processing and replication. All human coronaviruses, except SARS-CoV and MERS-CoV, encode two PLPs. When two PLPs are encoded, it is the PLP2 that functions similarly to SARS-CoV PLpro. The PLpro mediates cleavage of the replicase polyproteins 1a and 1ab at 3 specific sites, generating 3 mature nonstructural proteins (nsp) (from nsp1–3) (3,120, 259). It is well established that the PLpro domain plays a role in antagonism of the host innate immune response. To date, the study of coronaviruses' PLP domain has been largely based on utilizing truncated nsp3 constructs that represent specific domains, yet the way that the full-length nsp3 protein contributes to antagonizing the antiviral host response to enable successful virus infection is not clearly understood. I attempted to address this demand by generating stable cell lines that express, in a tetracycline-regulated fashion, the full-length nsp3 protein of human coronaviruses OC43, NL63, MERS-CoV and SARS-CoV, respectively. Studies in the full-length nsp3 protein context will lead to a greater understanding of the complex organization of SARS CoV viral proteins encoding the viral proteases, as well as provide insights to their functional relationships.

In order to study the functional role of coronaviral nsp3 in regulation of the host innate immune response, I desired to produce stable cell lines that permits the integration of the full-length nsp3 expression construct into the host cell chromosome, cells that could be used over many experiments, and with expression that is consistent and comparable between experiments. Therefore, I utilized the Flp-In T-Rex-system to create stable cell lines expressing coronaviral full-length nsp3 proteins in a tetracycline-regulated manner **Figure 3.3A-E**. This system is a very useful tool for functional studies

because it allows for site-specific integration and stable expression of a gene of interest (GOI), providing single-copy isogenic cell lines. Thus, integration should occur into the same genomic locus in every clone. As a result, all clones should be identical (Invitrogen). Furthermore, Flp-In expression involves introduction of an Flp Recombination Target (FRT) site into the genome of the mammalian cell line of choice. Thus, integration is site-specific, occurring at the FRT site (229). Single-copy site-specific integration should eliminate the integration of multiple copies of integrants and random sites of integration into the genome of the host cell. Thus, this system provides comparable protein-expression levels within the same batch of cells. In addition, utilizing one of the strongest mammalian promoters and regulatory elements from the tetracycline-resistance operon for transcription of the GOI should permit highly inducible expression of the protein of interest.

Several lines of evidence suggest that this system would be useful for generating stable cell lines in which inducible expression levels of the GOI are homogenous, permitting the evaluation of the function of different coronaviral nsp3 proteins. However, a particular observation demonstrated from IFA staining and Western blot analysis is considerable variation in Tet-inducible expression levels among the full-length nsp3 protein of human coronaviruses OC43, NL63, MERS-CoV and SARS-CoV (data not shown). Interestingly, this phenomenon was also observed via transient, ectopic expression among the full-length nsp3 proteins **Figure 3.4A**, where the nsp3s would be constitutively expressed from the strong CMV promoter. In contrast, upon the addition of tetracycline into the culture medium, HeLa-Fit-ScoV-nsp3 cells demonstrated robust expression of SARS-nsp3 **Figure 3.5A**, and this expression is comparable to HeLa-Fit-ScoV-nsp3-delPLP cells expressing SARS-nsp3-delPLP **Figure 3.5A**. I postulate that the variation in nsp3 expression levels among HeLa-Fit-OC43-nsp3, HeLa-Fit-NL63-nsp3, and HeLa-Fit-MERS-nsp3 stable cells is not attributed to size of nsp3 because SARS-CoV encodes the largest nsp3, yet HeLa-Fit-ScoV-nsp3 stable cells expressed SARS-nsp3 most robustly.

It is important to note that, although belonging to the same family of viruses, there are differences in the amino acid sequence of each coronaviral nsp3 gene (130). Thus it is possible that HCoV-OC43, HCoV-NL63, and MERS-CoV codons are suboptimal for expression in mammalian cells, as compared to that of SARS-CoV. It is possible that the disproportionate levels of expression may be due to suboptimal efficiencies in the transcriptional and/or translational regulation of specific coronaviral nsp3 proteins. Further experiments are necessary to address the elements contributing to poor protein expression of HCoV-OC43, HCoV-NL-63 and MERS-CoV-nsp3s in mammalian cells. This is key to a comprehensive understanding of whether CoVs' nsp3s may differ in their effect on host innate immunity. Interestingly, the data provided in this body of work suggest that SARS-CoV nsp3 sequence is optimal for expression in mammalian cells; which may explain the rather large number of global SARS cases that spread rapidly in a short period of time, highlighting the propensity of SARS-CoV to adapt readily to the human host thereby allowing efficient human-to-human transmission. I have generated an isogenic Tet-inducible gene-expression model system in HeLa cells that stably express SARS-CoV-nsp3 and SARS-CoV-nsp3-delPLP **Figure 3.5A**. I have demonstrated that

HeLa-Fit-ScoV-nsp3 cells express SARS-CoV nsp3 robustly and that expression is comparable to HeLa-Fit-ScoV-nsp3-delPlpro cell line **Figure 3.5A**. Employing such inducible cell lines can be useful for SARS-CoV-nsp3 functional studies, providing insight into SARS pathogenesis.

SARS CoV Nsp3 Regulates Innate Immune Functions

Viruses are exceptionally diverse and have evolved to infect and cause disease in almost all life forms, including humans. Needless to say, viruses can have a devastating effect on public's health. Prime examples are, the Spanish influenza (H1N1) pandemic in 1918, the transmission of the avian influenza virus (H5N1) to humans, and the sudden emergence of two highly pathogenic coronaviruses that causes severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). Thus, continued studies of viral pathogens are necessary (42, 260, 261). Viral pathogenesis is the process by which a virus causes disease in its host (1). A fundamental property of viruses is their complete dependence on a living host for replication. Therefore, it must be noted that the pathogenesis of a given virus is a combination of many multifaceted elements unique to a specific virus and its individual host. This intimate relationship defines the nature of the disease. A crucial component in understanding viral pathogenesis is defining the fundamental mechanisms that determine the pathogenesis of infection between a host with mild disease and the host that undergoes severe consequences. Thus, understanding viral virulence genes and factors that contribute to disease will aid in the pursuit of identifying specific targets for antiviral therapeutic interventions intended to prevent the development of severe disease.

Host cells respond to invading viruses by initiating host innate immune responses characterized by production type I interferons (IFN-α and β) and establishment of an antiviral state (113,154-156, 241). The host innate immune response is a rapid first line of defense mechanism against viral pathogens. SARS-CoV evades the innate immune response through an intricate combination of virus-host interactions that disrupt intracellular signaling pathways and weaken the antiviral actions of IFN. Viral regulation of the host innate immune response disrupts the relationship between innate and adaptive immunity, providing an environment conducive to SARS-CoV replication and spread. One viral protein that is likely a significant virulence factor of SARS-CoV is the PLpro. The PLpro domain has been shown to have interferon-antagonistic properties, which offer SARS-CoV an advantage in its battle against the host innate immune defenses (186, 194, 217). This influence is mediated, at least in part by the ability of the PLpro to inhibit the activation of IRF-3, a transcription factor required for the induction of IFN-β (159, 186, 194, 217, 220, 242).

Increasing evidence has shown that the PLpro possesses various IFN-antagonistic properties (186, 194, 217, 258); however, little is known about the function of the full-length nsp3 in disrupting IRF-3-dependent innate immunity. SARS-CoV PLpro is contained within nsp3, a 213 kDa membrane multi-domain-associated replicase product (3). In order to have a comprehensive understanding of the pathogenesis of SARS-CoV,

studies clarifying the mechanisms by which SARS-nsp3 interacts with its host and disrupts the host antiviral response are necessary. A detailed understanding of this interaction in the context of the full-length nsp3 protein may result in biologically relevant knowledge that potentially aid in the development of effective antiviral therapeutics, which target highly pathogenic coronaviruses' replication and mitigate pathogenesis.

In this body of work, I tried to determine the molecular mechanism by which SARS-nsp3 inhibits the IRF-3-dependent antiviral response. While the detailed mechanism by which nsp3 inhibits IRF-3 activation still remains undefined, I have provided evidence that the full-length nsp3 protein of SARS-CoV functions as a bona-fide interferon antagonist, inhibiting IFN synthesis by acting at a level proximal to IRF3 **Figure 4.6A**. Furthermore, this ability is mediated by the PLpro domain and perhaps in part, the catalytic activity **Figure 4.6B**. Future experiments that address the mechanism of SARS-nsp3-mediated suppression of IRF-3 activation are essential to delineate the exact role that nsp3 plays in SARS pathogenesis.

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VITA

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