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Kristin Edwards April 10, 2024

The Impact of SARS-CoV-2 nsp5 Resistance Mutations on Antagonism of the Innate Immune Response

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Abstract

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Coronaviruses pose a significant threat to public health, with outbreaks such as SARS-CoV-1 and MERS-CoV underscoring the potential for severe respiratory illnesses. Since 2019, the emergence of SARS-CoV-2, causing COVID-19, has escalated into a global pandemic with devastating consequences. Understanding the molecular mechanisms of SARS-CoV-2 infection and immune evasion is crucial for developing effective therapeutic interventions. This paper presents an investigation into the impact of mutations in the SARS-CoV-2 non-structural protein 5 (nsp5) on the host innate immune response.

The study explored how the E166V mutation in nsp5 affects the ability of the virus to evade host immune surveillance. Using a SARS-CoV-2 replicon system, the project evaluated the interaction between nsp5 mutants and the retinoic acid-inducible gene I (RIG-I) pathway, a key component of the innate immune response. Luciferase assays demonstrated that while the E166V mutant has been linked to decreased replication fitness, there is no significant difference in interferon-beta (IFN-β) promoter activity between the two strains within a 24-hour timeframe. Additionally, the BA.1 E166V replicon demonstrated higher replication activity than the BA.1 WT mutant within the 24hr time course with equivalent activity at the 24hr time point. This suggests that decreased nsp5 activity may not significantly impact the antagonism of the RIG-I pathway within the first 24hr post-induction.

Future directions for research include extending the time course of both luciferase assays to capture prolonged immune responses, validating RIG-I expression and cleavage by nsp5 mutants through Western Blot analysis, and exploring the impact of mutations on the expression of interferon-stimulated genes (ISGs). Furthermore, the study proposes utilizing inhibitors such as nirmatrelvir to assess IFN-β stimulation in the context of antiviral resistance and exploring additional mutant strains to delineate key determinants of viral pathogenicity and immune evasion.

Overall, this investigation contributes to our understanding of how mutations in SARS-CoV-2 may impact host-virus interactions and informs the development of novel therapeutic strategies to combat COVID-19 and future coronavirus outbreaks. By elucidating the complex dynamics between viral genetics and host immune responses, this research aims to advance our ability to effectively manage and mitigate the impact of emerging infectious diseases.

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Introduction

Coronaviruses are a large family of viruses that can cause illness in animals and humans. Some coronaviruses are endemic, such as HCoV-229E and HCoV-OC43, which cause mild respiratory illnesses, typically resembling the common cold (1). These have circulated globally since the 1960s and are responsible for a significant proportion of common cold cases. These viruses can potentially cause severe illness in vulnerable populations such as the elderly and patients with underlying health conditions (1).

Coronaviruses are transmitted from person to person through respiratory droplets and close contact (2). They can also transfer from animals to humans. Bats are known to harbor various coronaviruses and often serve as intermediate hosts in the transmission of coronaviruses across animal populations and into the human population (2). When such crossover events occur, deadly consequences can ensue. In 2002-2003, Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-CoV-1) was responsible for an outbreak primarily in China which peaked at a mortality rate of 11% (3). Later in 2012, MERS-CoV (Middle East Respiratory Syndrome Coronavirus) emerged with a relatively high mortality rate of 34.4% (4). Coronaviruses have long been a point of concern for public health, but research efforts have significantly increased since 2020 in response to the ongoing global pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).

SARS-CoV-2 has caused over 770 million confirmed cases of Coronavirus Disease of 2019 (COVID-19) and over 7 million deaths worldwide in 4 years (5). The virus can be transmitted by individuals who are asymptomatic or presymptomatic (5). COVID-19 represents one of the greatest public health challenges of the 21st century, highlighting the importance of

global cooperation, scientific research, and public health measures in combating emerging infectious diseases.

Current options to prevent and treat COVID-19 include mRNA vaccines and monoclonal antibody therapies, both of which target the spike protein (6). Unfortunately, the spike protein has changed significantly as the virus has evolved into new strains since 2019 (6). Over the past four years, SARS-CoV-2 has evolved into several variants of concern (VOCs) the most recent of which include strains from the Omicron lineage (7). VOCs spread faster than earlier strains, evade neutralizing antibodies, and cause breakthrough infections against the original COVID-19 vaccines, making them difficult to control (6). Fortunately, these strains remain susceptible to small-molecule antivirals (8). In addition to understanding the molecular mechanisms underlying the replication and pathogenesis of SARS-CoV-2, it is crucial to understand how the virus interacts with infected host cells to develop new therapeutics to combat COVID-19.

Infection

SARS-CoV-2 and other coronaviruses belong to the family Coronaviridae, which comprises enveloped viruses with a single-stranded positive-sense RNA genome (1, 9, 10). The genome contains several open reading frames (ORFs) that encode various types of proteins (Figure 1). SARS-CoV-2 enters host cells through the interaction of its spike protein with the host cell receptor angiotensin-converting enzyme 2 (ACE2) (Figure 2). Once the spike protein binds to ACE2, the virus is internalized through endocytosis or direct fusion with the host cell membrane (Figure 2). After entry, the viral envelope fuses with the host cell membrane, releasing the viral RNA into the cytoplasm, where it serves as a template for the translation of viral proteins (Figure 2). (9, 10)

Translation of viral RNA yields three types of proteins: structural proteins (spike, envelope, membrane, and nucleocapsid), non-structural proteins (nsp1-16), and accessory proteins. The structural proteins are essential for the assembly of new viral particles. Non-structural proteins are involved in all aspects of viral replication, including RNA replication and transcription, as well as the modification of host cell processes to facilitate viral replication and evasion of the host immune response (Figure 2). The non-structural proteins are initially synthesized as large polyproteins, which are subsequently cleaved by viral proteases to produce functional proteins (Figure 1). Translation of ORF1a yields pp1a, and in the case of a ribosomal frameshift through ORF1b, pp1ab in produced (Figure 1). The polyproteins are processed into 16 individual non-structural proteins (nsp1-16) by two viral proteases: the main protease (Mpro, nsp5) and the papain-like protease (PLpro, part of nsp3). (9, 10)

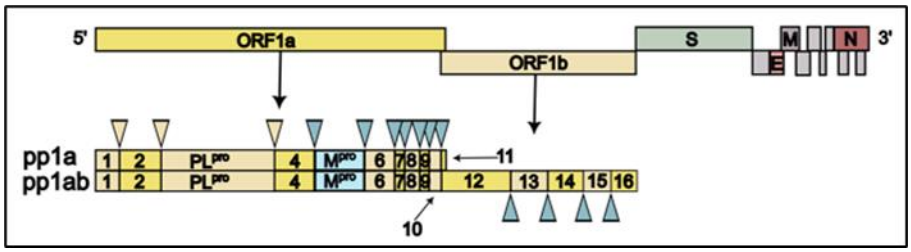

Figure 1. The viral RNA genome of SARS-CoV-2 is translated into structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N), in addition to polyproteins pp1a and pp1b. Polyproteins are processed into nsp1-16 by PLpro at 3 cleavage sites (tan) and nsp5 (Mpro) at 11 cleavage sites (blue).

Newly synthesized viral genomic RNA, along with the structural proteins, is assembled into new viral particles in the host cell endoplasmic reticulum-Golgi intermediate compartment (ERGIC). The viral particles bud into vesicles and are transported to the cell surface. Mature viral particles are released from the infected cell through exocytosis and can infect neighboring cells to initiate new rounds of infection (Figure 2). This process leads to the spread of the virus within the host organism. (9, 10)

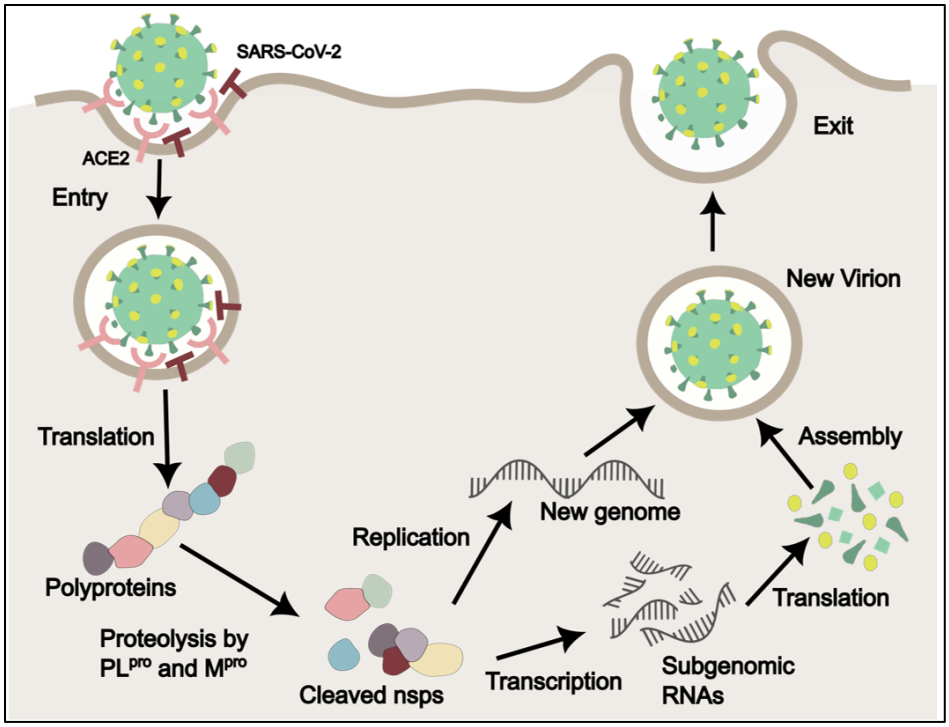

Figure 2. Overview of the SARS-CoV-2 infection cycle in a host cell.

Immune Response

SARS-CoV-2 interacts with the immune system through a complex interplay of viral proteins and host immune responses (9, 10). When the virus enters the body, it triggers various innate and adaptive immune responses aimed at neutralizing and eliminating the virus (9, 10). Cells recognize viral infections through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). These receptors recognize specific pathogen-associated molecular patterns (PAMPs) present on the virus. Upon recognition of viral RNA by PRRs, innate immune cells produce Type I interferons (IFNs) and proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (11). Type I IFNs play a crucial role in limiting viral replication and spread, while proinflammatory cytokines recruit additional immune cells, such as natural killer (NK) cells, to the site of infection and activate adaptive immune responses (9).

Once activated, NK cells can directly kill virus-infected cells through the release of cytotoxic granules containing perforin and granzymes. Dendritic cells process and present viral antigens to T cells, initiating adaptive immune responses. B cells produce virus-specific antibodies, including neutralizing antibodies, which can bind to viral particles and prevent them from infecting host cells. Antibody-mediated neutralization of the virus can limit viral replication and spread. (12)

This project focusses on the innate immune response stimulated by RLRs in the presence of SARS-CoV-2. Retinoic acid-inducible gene I (RIG -I) is a cytoplasmic receptor which recognizes viral RNA in the cytosol as a sign of viral replication (Figure 3). RIG-I has two caspase activation and recruitment domains (2CARDs) in the N-terminus, a central helicase domain (HD), and C-terminal domain (CTD). Under normal conditions, the 2CARDs interact with the HD in an inactive conformation (13) (Figure 4). When viral RNA is present, the CTD and HD bind to the viral RNA and release the 2CARDs which extend into the active conformation (Figure 4). Once in the active form, RIG-I oligomerizes, and the 2CARDs interact with the CARD from the mitochondrial activator of virus signaling (MAVS) protein (Figure 4) (13). The RIG-I and MAVS interaction induces the activation of interferon regulatory factor 3 (IRF3), IRF7, and nuclear factor kB (NF-kB) (13) (Figure 3). These active products function as transcription factors for the production of IFN-β (a Type I IFN) and pro-inflammatory cytokines, which act as chemoattractants to alert the immune cells in the body to a possible viral infection (Figure 3). IFN-β also serves as a signal to the infected cell and adjacent cells to amplify immune defense by inducing the expression of interferon-stimulated genes (ISGs) through the JAK/STAT pathway (Figure 3). ISGs are diverse in form and function, but they are collectively the first line of the innate immune response (14).

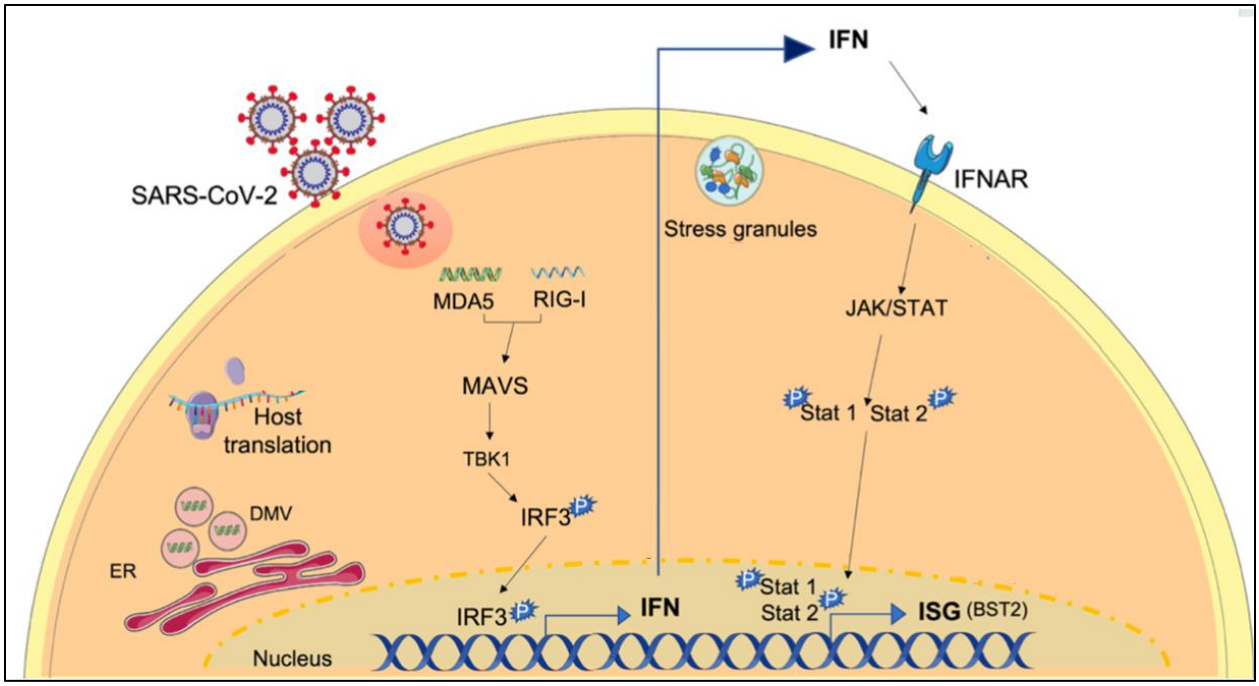

Figure 3. Overview of IFN stimulation response. Retinoic acid-inducible gene I (RIG-I) detects viral RNA in the cytosol, triggering a signaling cascade that produces Type I IFN and results in the translation of ISGs. Adapted from Chiale et al. (9).

Evasion of Immune Response

SARS-CoV-2 has evolved various strategies to evade host immune responses, including inhibition of antigen presentation, interference with cytokine responses, and modulation of IFN signaling pathways (15). The viral proteins ORF3a and ORF7a have been implicated in downregulating MHC class I expression on infected cells, which is crucial for presenting viral antigens to cytotoxic T cells (15, 16). The N protein has been implicated in inducing the expression of proinflammatory cytokines such as IL-6 and TNF-α, leading to an exaggerated and harmful inflammatory response (15, 17). Nsps antagonize the production and signaling of Type I IFNs, thereby impairing the host's innate immune response and facilitating viral replication within host cells (13).

The SARS-CoV-2 nsp5, also known as the main protease, is a dimeric cysteine protease required to process the viral polyproteins into the individual nsps required for viral replication

such as the replication complex (18) (Figure 1, 4). Viral proteases have been implicated in the cleavage of cytoplasmic host proteins that are involved in the production of Type 1 IFN. For example, the hepatitis C virus protease NS3/4A has been reported to cleave MAVS (19). Nsp5 has also been implicated in immune suppression mechanisms of SARS-CoV-2 by targeting the RIG-I pathway to evade the cell's innate immune response (13) (Figure 5). Nsp5 has been shown to cleave at the location of the conserved glutamine using its catalytic cysteine residue (Cys145) (13). The protease suppresses the RIG-I pathway by cleaving off the 10 most-N-terminal amino acids from RIG-I at the site of a glutamine residue (Gln10) located in the 2CARDs, which is essential for MAVS activation (13). The cleavage of these amino acids renders the 2CARDs unable to interact with the CARD on MAVS (Figure 4). Through these processes, nsp5 inhibits IFN-β induction through activation of RIG-I. However, it remains unknown whether mutations in nsp5 will affect the RIG-I cleavage mechanism.

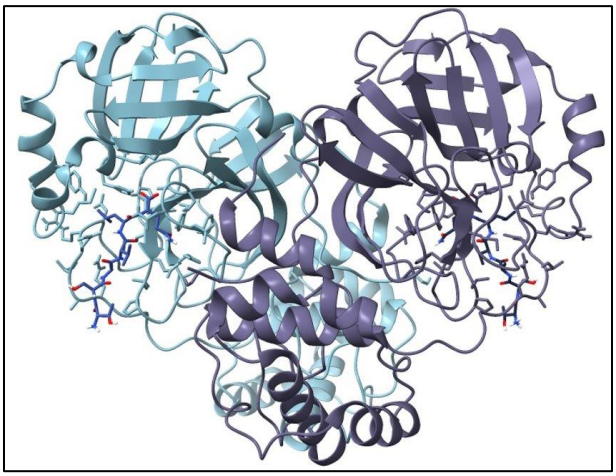

Figure 4. The structure of the main protease (nsp5), a dimeric cysteine protease which is mainly responsible for cleaving pp1a and pp1ab into functional nsps to be used in the viral replication process. (PBD: 7MB4)

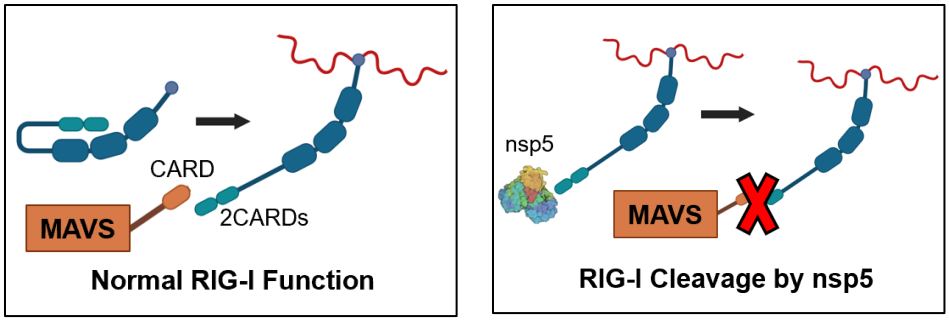

Figure 5. RIG-I activation and signaling through interacting with MAVS to stimulate the production of IFN-β. nsp5 inhibits the production of IFN-β by preventing MAVS activation through cleavage of the 2CARDs on RIG-I.

Current Treatments and Mutations

Although nsp5 is well conserved with only one mutation in Omicron compared to the Washington strain (P132H), nsp5 mutations have begun to appear in patients with the increased use of nsp5-targeting inhibitors to treat COVID-19 (20, 21). One drug of interest is Paxlovid™ by Pfizer, Inc. (USA): an antiviral approved for clinical use that targets SARS-CoV-2 nsp5 (22). Paxlovid™ is a combination of a nsp5 inhibitor, nirmatrelvir (NIR), and ritonavir, which improves the NIR pharmacokinetic profile (22). NIR mimics the peptide substrate of nsp5 and inhibits the active site by forming a covalent bond between its nitrile group and the catalytic cysteine residue of nsp5 (Cys145) (Figure 6) (22). This drug has proven to be effective in preventing severe disease, hospitalizations, and death from COVID-19 (23). However, reports began circulating in 2022 of Paxlovid-treated patients who experienced a rebound of COVID-19 infection and symptoms 2 to 8 days after recovery from initial infection (24). Additionally, clinical reports and passage studies have identified nsp5 mutations associated with resistance to NIR (20, 25, 26, 27). Although mutations in the nsp5 active site can decrease enzymatic activity, preliminary data also suggest that the Omicron strains have higher tolerance for mutations in

nsp5 than the original Washington (WA1) strain (28). It is unknown how these mutations affect cleavage of host targets.

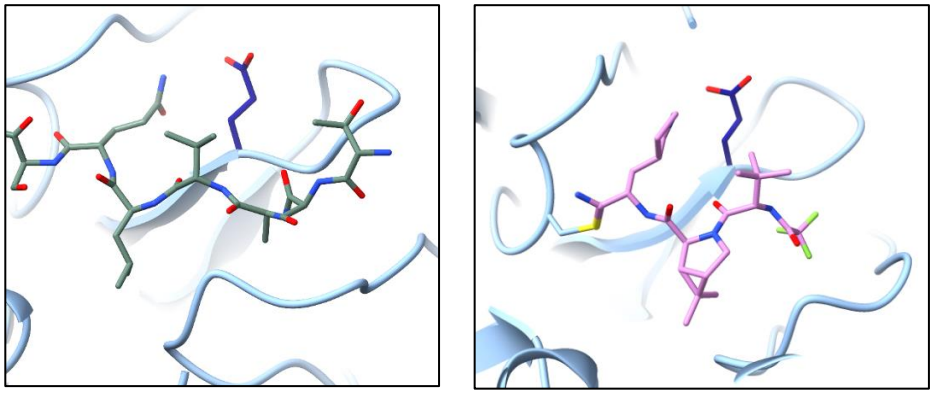

Figure 6. BA.1 WT nsp5 in complex with the nsp4/5 cleavage site substrate (green) (left, PDB: 7DVP) and in complex with NIR (pink) (right, PDB: 7TLL).

Experimental Aims

Mutations in nsp5 can affect both the activity and susceptibility of nsp5 to current antivirals. One such NIR resistant mutation seen in patients and passage studies is E166V, which confers up to 100-fold resistance (20, 21, 28, 29). Position 166 of nsp5 is located in the active site; a mutation from the negatively charged polar glutamic acid to the nonpolar valine residue confers NIR resistance by reducing the binding efficacy between protease and substrate (Figure 7) (28). This mutant has also been linked to COVID-19 rebound infections in patients treated with multiple rounds of Paxlovid (20, 21). However, there is an apparent decrease in replication fitness associated with E166V, as commonly occurs with resistance mutations (28, 29, 30). While previous studies have characterized the resistance profile of this nsp5 mutation, none have investigated any possible changes in interactions between protease and host proteins caused by substitutions that impact nsp5 activity.

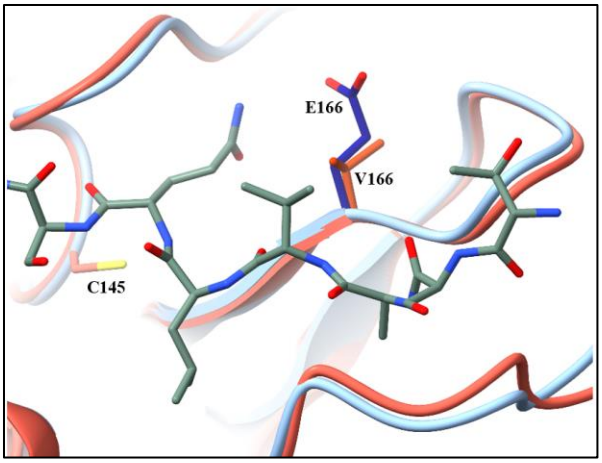

Figure 7. BA.1 WT nsp5 in blue in complex with the nsp4/5 substrate (green) (PDB: 7DVP) aligned with E166V nsp5 in red (PDB: 8SMB).

This project seeks to understand the impact of resistance mutations on cleavage of host targets of nsp5. I will investigate the hypothesis that the decrease in nsp5 cleavage activity caused by E166V will result in increased IFN-β production compared to WT. To answer this question, I will use a SARS-CoV-2 replicon system to determine how the E166V mutation may alter the way that nsp5 cleaves RIG-I and the resultant change in levels of IFN-β production while also taking into consideration the associated decrease in replication fitness.

My results indicate that the design of the assay is an effective protocol to evaluate changes in IFN-β promoter activity over time using a noninfectious replicon system. Using this design, I determined that within the first 24 hours post-induction of IFN-β stimulation, there is no significant difference in in IFN-β promoter activity between BA.1 WT and BA.1 E166V SARS-CoV-2 replicons, nor is there an apparent difference in replicon fitness between the strains.

Methods

Cell Line

HEK293T cells, a commonly used human embryonic kidney cell line, can serve as a valuable tool for studying innate immune responses to viral infections (31). The cell line was selected for this project due to its high transfection efficacy (31). The RIG-I protein expressed in HEK293T cells is considered catalytically inactive due to its inability to hydrolyze ATP, which is a crucial catalytic activity required for its normal function in detecting viral RNA and initiating downstream signaling pathways (31, 34). However, RIG-I in HEK293T cells may still retain its ability to recognize viral RNA and initiate downstream signaling pathways, albeit with reduced efficiency compared to the fully active form of the protein. Catalytically active RIG-I plasmid was exogenously expressed in the cell line for each experiment to compensate for this effect.

Replicon System

SARS-CoV-2 virus is a biosafety level 3 (BSL-3) agent that requires specialized facilities. However, our lab has developed a SARS-CoV-2 replicon system which recapitulates all steps of viral replication but cannot produce new virions, making it safe to use at a BSL-2 level (30). The SARS-CoV-2 replicons contain the majority of the viral genome but lack the spike, envelope, and membrane structural proteins required to form virions (30, 32). Reporters and/or selectable markers take the place of these structural proteins (30). The replicons used incorporate a nano-luciferase (NLuc) reporter. The NLuc reporter system is a bioluminescence assay that measures viral replication to assess viral fitness.

Plasmids

To measure IFN-β stimulation, a reporter was expressed from IFNβ-pGL3 plasmid containing Firefly luciferase gene under the control of the IFN-β promoter, IRF3 (gift from Dr. Bunsuk Hahm). Higher levels of Firefly luciferase luminescence indicate increased activity of the IFN-β promoter and, by extension, higher levels of IFN-β production. The reporter was co-transfected with either the catalytically active pUNO1-hRIGI plasmid (Invivogen) or the control mCherry plasmid. This experiment included two replicons derived from the omicron BA.1 variant of SARS-CoV-2 (WT and E166V mutant) (30). Analysis of the relative amounts of NLuc and Firefly luciferase (measured 6, 12, and 24h post transfection) were used to compare both replication fitness and IFN-β promoter activity.

All assays were performed using three biological replicates with triplicate wells per condition. Controls for this experiment involved including/excluding the SARS-CoV-2 replicon and/or RIG-I. Polyinosinic-polycytidylic acid [poly(I:C)] (Invivogen), a synthetic analog of viral dsRNA, was used as a positive control in place of replicon to induce the RIG-I pathway (33). mCherry served as a negative control plasmid transfected in place of RIG-I.

IFN-β Stimulation Assay

50,000 HEK293T cells were plated in a 96-well plate. The next day, cells were transfected with 100ng of total DNA comprising 60ng of IFNβ-pGL3 and 40ng of the RIG-I plasmid (Invivogen) or mCherry plasmid using jetPRIME transfection reagent (Polyplus) according to manufacturer's instructions. Two hours post-transfection, IFN-β response was induced by transfection with either 10ng of [poly(I:C)] (Invivogen) or 84ng of total DNA comprising 70ng of replicon (WT or E166V) and 14ng of a plasmid containing SARS-CoV-2 nucleocapsid (N) using jetPRIME transfection reagent. The N plasmid is included to increase

replicon activity. At 0, 6, 12, and 24 hours post-induction, cells were washed with PBS and lysed with Reporter Lysis Buffer (Promega). 20µl of lysates were freeze-thawed and centrifuged at 10,000g for 2 minutes. Supernatants were stored at -20˚C. Luminescence was measured on a GloMax Navigator microplate luminometer (Promega) according to manufacturer instructions for Luciferase Assay Reagent (Promega) after adding 100µl of reagent to lysates. (Figure 8)

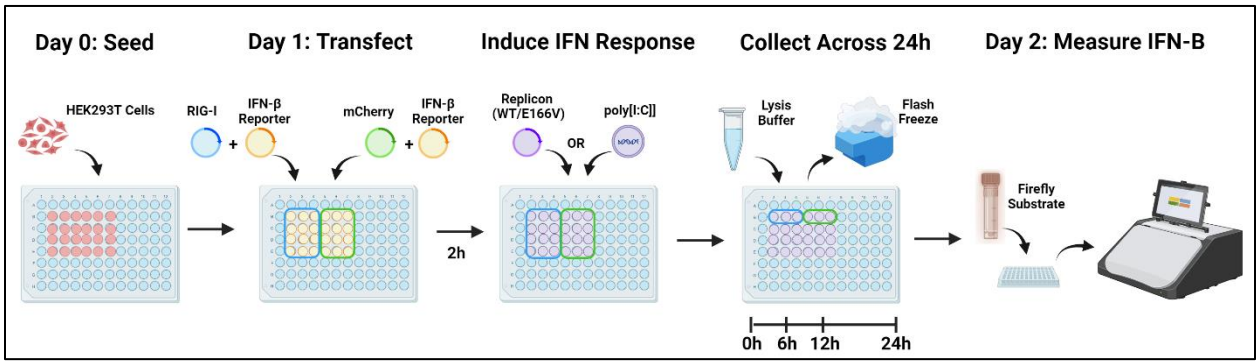

Figure 8. The IFN-β Stimulation Assay utilizes the Firefly luciferase reporter to measure IFN-β promoter activity, which correlates to IFN-β production, between BA.1 WT and BA.1 E166V SARS-CoV-2 replicons.

Replicon Fitness Assay

The replicon fitness assay follows the same initial protocol as the IFN-β stimulation assay described above. At the 6, 12, and 24hr collection times, 100uL of a Promega Nano-Glo™ substrate/lysis buffer (Promega) was added to cells in media and dispensed immediately into a 96 well-plate. After a 2-minute incubation period, NLuc luminescence was measured on a GloMax Navigator microplate luminometer (Promega) according to manufacturer instructions. (Figure 9)

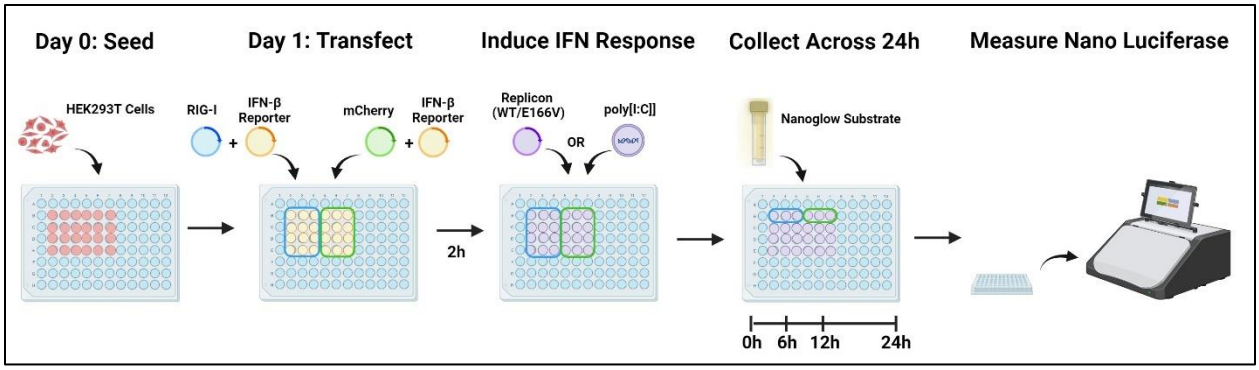

Figure 9. The Replicon Fitness Assay utilized the NLuc reporter incorporated into the SARS-CoV-2 replicon system to measure replication of BA.1 WT and BA.1 E166V replicons.

Results

Validation of IFN-β Assay Design using poly[I:C]

The design of the IFN-β stimulation assay relies on the premise that the catalytically active RIG-I transfected into cells provides the machinery to produce an IFN-β response to viral RNA which can be quantified using the Firefly luciferase reporter under the IRF3 promoter. This design was validated using the known RIG-I inducer, poly[I:C]. Results indicate a significant difference in IFN-β promoter activity between RIG-I and mCherry transfected cells in the presence of poly[I:C] (Figure 10). This confirms that the RIG-I and Firefly reporter plasmids work as expected, and the differences between samples can be attributed to the activity of the RIG-I pathway and resultant IRF3 activity, which is associated with IFN-β production.

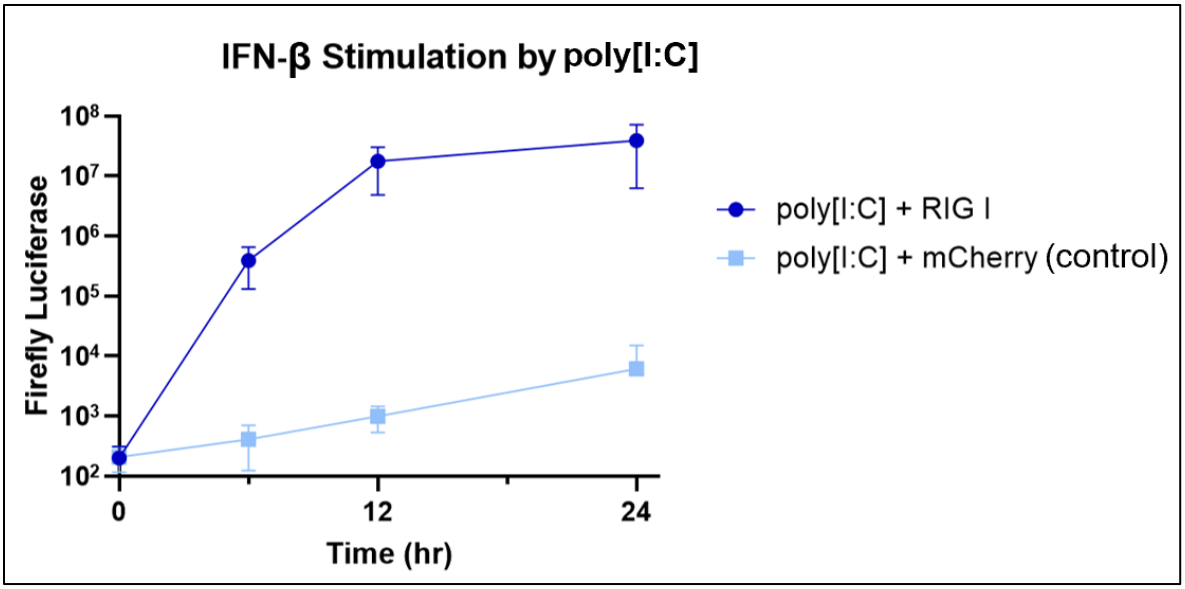

Figure 10. Firefly luciferase reporter signal significantly increases over the time course when induced by poly[I:C]. Cells with exogenously expressed RIG-I produce significantly higher levels of Firefly luciferase signal than cells transfected with the control plasmid. The Firefly luciferase reporter is under the control of the IRF3 promoter and serves as a measure of IFN-β stimulation.

IFN-β Stimulation by SARS-CoV-2 Replicons

BA.1 WT or BA.1 E166V replicons were transfected into cells to induce the IFN-β response. As expected, IFN-β stimulation, measured by Firefly luciferase signaling, significantly increased over the time course when induced by replicon (Figure 11). Cells with exogenously expressed RIG-I produced notably higher levels of Firefly luciferase signal than samples containing mCherry. Interestingly, although the E166V replicon produced a slightly higher average Firefly luciferase signal than WT, there was no significant difference in the IFN-β promoter activity between the WT and E166V replicons in the presence or absence of catalytically active RIG-I (Figure 11). This indicates that replicons containing E166V mutant nsp5 are capable of inhibiting the RIG-I pathway as efficiently as WT replicons.

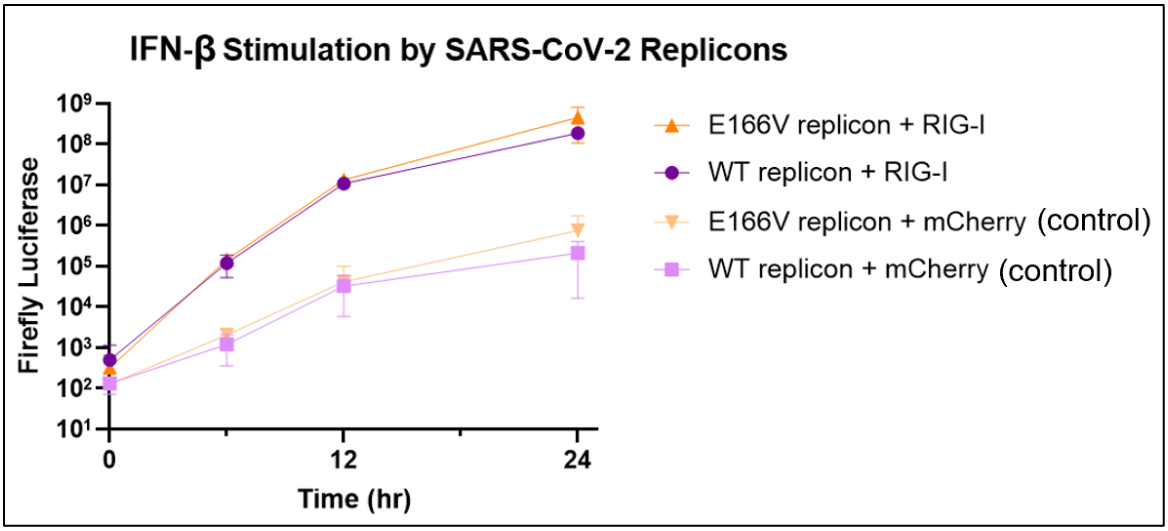

Figure 11. SARS-CoV-2 replicons induced an IFN-β response, measured by Firefly luciferase, which significantly increased over the 24hr time course for all conditions. Cells transfected with RIG-I produced higher Firefly luciferase levels than cells transfected with the control mCherry plasmid. Interestingly, there is no significant difference in Firefly luciferase signal between BA.1 WT and BA.1 E166V replicons in the presence or absence of RIG-I.

Replicon Fitness Comparison

To understand the level of replicon activity during this experiment, NLuc signal was measured at the indicated time points (Figure 12). Unexpectedly, the BA.1 E166V mutant demonstrates higher replication activity than the BA.1 WT mutant within the 24hr time course with equivalent levels at the 24hr time point (Figure 12). This result is contrary to the results reported by Lan et al. for this mutation; however, it should be noted that the time course of this experiment is restricted to 24hr while the timepoint used by Lan et al. occurred at 48 hours post-transfection (30).

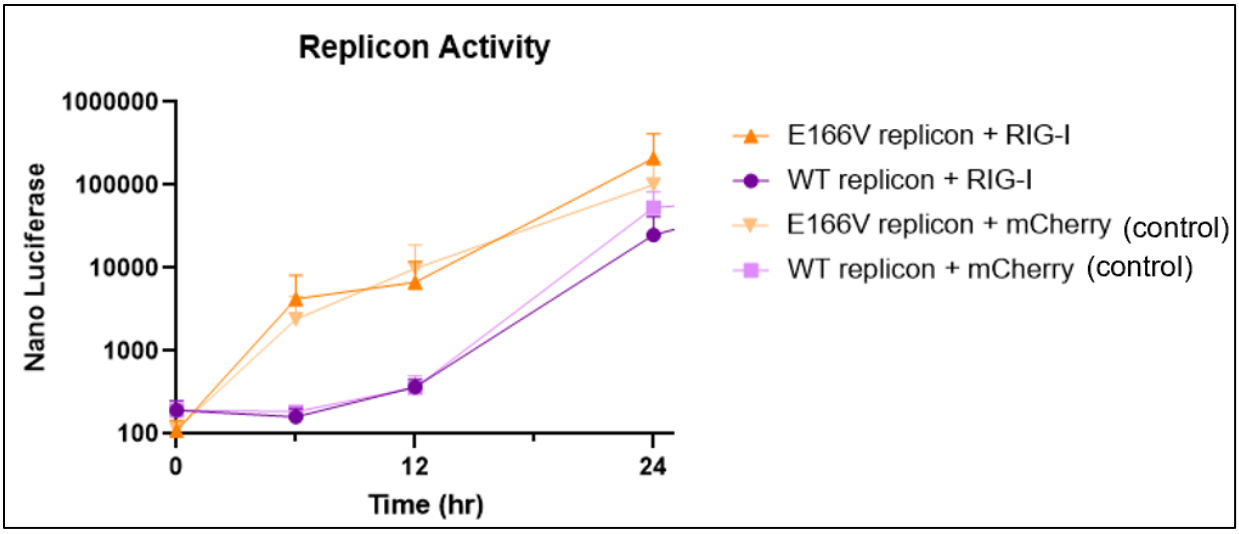

Figure 12. NLuc reporter production by BA.1 WT and BA.1 E166V replicons over a 24hr time course. During this time, the BA.1 E166V mutant appears to have higher replication activity than the BA.1 WT strain prior to 24hr and equivalent levels at 24hr.

Discussion & Future Directions

Understanding the dynamics of antiviral resistance and viral fitness is crucial for elucidating host-virus interactions and developing effective antiviral strategies. SARS-CoV-2 employs various strategies to evade the host immune response, showcasing its adaptability and complexity in immune evasion mechanisms (9, 12). In this project, I have designed and implemented two luciferase assays which can be used in tandem to gain insight into the complex interplay of SARS-CoV-2 antiviral resistance and resultant changes in the innate immune response.

Specifically, this assay is intended to determine how mutations in nsp5 may affect the resultant immune response by interfering with the cleavage mechanism of RIG-I, one of several immune evasion strategies by SARS-CoV-2. However, there does not appear to be a significant difference in IFN-β promoter activity, measured by Firefly luciferase under the control of the IRF3 promoter, between the BA.1 WT and BA.1 E166V strains within the 24-hour time course.

There are several possible explanations for this trend. Possibly, nsp5 activity may not be the main mechanism of IFN-β antagonism, so a decrease in fitness caused by the nsp5 mutation does not result in a physiologically relevant decrease in IFN-β production. Another possibility is that the E166V mutation does not substantially reduce replicon fitness, contrary to previous assumptions. We are aware of preliminary data which indicates a significant decrease in replicon fitness at 48 hours post-induction. However, over the course of this experiment, which was limited to the first 24 hours post-induction, the BA.1 E166V mutant replicon does not demonstrate this decreased fitness. Moving forward, several avenues of research can build upon the findings presented in this study.

First, extending the time course of the IFN-β stimulation assay beyond the 24-hour window up to 72 hours will capture the full scope of the IFN-β response (34). Further investigation of the temporal dynamics of IFN-β promoter activity will provide insight into prolonged immune activation and potential mechanisms of immune exhaustion or tolerance that may not be apparent within the first 24 hours of induction. The replicon fitness assay must also be extended to 72 hours to consider a potential correlation between replicon fitness and IFN-β stimulation.

Western Blot validation is needed to confirm the expression of RIG-I within cells. Western Blot can also be used to confirm RIG-I cleavage by the nsp5 mutant, E166V, to confirm the role of the cleavage mechanism in suppressing the host innate immune response. While this mechanism is confirmed in WT nsp5, it is important to confirm in the mutant as well (13). This experimental validation will strengthen our understanding of the molecular mechanisms employed by the virus to evade detection and clearance by the host immune system.

Utilizing RT-qPCR to measure the production of interferon-stimulated genes (ISGs) and IFN-β RNA in cells transfected with WT or E166V replicons will provide valuable data regarding the impact of viral mutations on innate immune signaling pathways. In the event that nsp5 activity is not the main mechanism of IFN-β antagonism, RT-qPCR analysis of various ISGs could identify different effects on parallel innate immune responses which converge with the RIG-I pathway. Comparative analysis between WT and mutant replicons will elucidate the effects of viral genetic variation on host antiviral responses.

Incorporating inhibitors such as nirmatrelvir or other compounds to assess IFN-β stimulation in the context of antiviral inhibition and resistance will offer insight into potential therapeutic interventions. I hypothesize that the NIR antiviral treatment or drug-resistant mutations affecting the SARS-CoV-2 nsp5 proteolytic efficiency will lead to an increase in IFN-β promoter activity, and by extension, IFN-β production. Evaluating the ability of potential antivirals to modulate IFN-β production in the presence of WT or E166V replicon will help to optimize therapeutic efficacy and mitigate the emergence of drug resistance.

Exploring other mutant strains of interest, such as the E166V/L50F double mutant, will delineate key determinants of viral pathogenicity, immune evasion, and therapeutic resistance. Investigating the synergistic or antagonistic effects of multiple mutations on viral pathogenesis and immune evasion mechanisms provides valuable insights into the dynamics between viral genetics and host immune responses.

COVID-19 presents an on-going global threat that requires the development of effective therapeutics. The investigation of mutations that confer antiviral resistance and the mechanisms by which they disrupt in the immune system provides insight into how current antiviral treatments can be improved. By continuing to explore the interaction of viral evasion strategies

and host immune responses, we can advance our understanding of viral pathogenesis and the interplay between antiviral resistance and innate immune suppression. This will inform innovative approaches for combating viral infections. Advancing this field of study will help develop new ways to combat future unique coronavirus outbreaks in the same manner that research on SARS-CoV and MERS-CoV informed and expedited efforts to treat SARS-CoV-2 and COVID-19 (35).

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