

Utilization of host proteins by human and feline coronaviruses

by

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B.S., The University of Findlay, 2018  
D.V.M., Kansas State University, 2022

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology  
College of Veterinary Medicine

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

2026

## **Abstract**

Coronaviruses are important pathogens capable of infecting a wide range of species. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was the etiologic agent responsible for the COVID-19 pandemic, and cross-species transmission of SARS-CoV-2 was observed in a variety of species, mounting concern for animal reservoirs. Using lentivirus-based pseudoviruses carrying the spike protein (S) of SARS-CoV-2 parental and Omicron variants, we transduced CRFK cells stably expressing ACE2 of various species including dog, cat, mink, and white-tailed deer to assess entry potential. We found that pseudotyped viruses carrying S of the various SARS-CoV-2 Omicron strains efficiently entered cells expressing human or animal ACE2 comparably or better than those with parental strain. Host proteases also play an important role in coronavirus cell entry through proteolytic processing of S. Using the pseudoviruses carrying S from SARS-CoV-2 strains as well as SARS-CoV and Middle Eastern respiratory syndrome coronavirus (MERS-CoV), we assessed host proteases on virus entry and S cleavages using western blotting, entry/inhibition assays, and fusion assays. The presence of TMPRSS2 significantly increased the entry of all tested pseudoviruses and correlated with S cleavages and fusion events. The results suggest the importance of continual evaluation of SARS-CoV-2 variants in their susceptibility to animal species through ACE2, and comparable analysis of entry efficacies of SARS-CoV-2, SARS-CoV and MERS-CoV provide details of entry processes with host proteases among different coronaviruses.

Feline infectious peritonitis (FIP) is a fatal systemic disease of cats, which is caused by FIP virus (FIPV). Viral mutation and host factors including T cell immunity play critical roles in the development of FIP. Because the PD-1/PD-L1 axis is a major target for viral evasion mechanism against T cell immunity, we assessed the expression of PD-L1 in cells infected with various strains

of FIPV along with the subclinical biotype feline enteric coronavirus (FECV). While both viruses demonstrated robust viral replication at comparable levels, FIPV-1146, but not FECV-1683, significantly upregulated PD-L1 expression in two cell lines. We also assessed the PD-L1 expression in tissues of cats naturally infected with FIPV. PD-L1 mRNA levels in relation to viral load were highly variable among tissues and cats. On confocal microscopy, we found that PD-L1 and FCoV expression were clustered and largely co-localized in the tissues. These findings support further investigation into the role of PD-L1 and T cell responses in the development of FIP.

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Kyeong-Ok Chang

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## **Abstract**

Coronaviruses are important pathogens capable of infecting a wide range of species. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was the etiologic agent responsible for the COVID-19 pandemic, and cross-species transmission of SARS-CoV-2 was observed in a variety of species, mounting concern for animal reservoirs. Using lentivirus-based pseudoviruses carrying the spike protein (S) of SARS-CoV-2 parental and Omicron variants, we transduced CRFK cells stably expressing ACE2 of various species including dog, cat, mink, and white-tailed deer to assess entry potential. We found that pseudotyped viruses carrying S of the various SARS-CoV-2 Omicron strains efficiently entered cells expressing human or animal ACE2 comparably or better than those with parental strain. Host proteases also play an important role in coronavirus cell entry through proteolytic processing of S. Using the pseudoviruses carrying S from SARS-CoV-2 strains as well as SARS-CoV and Middle Eastern respiratory syndrome coronavirus (MERS-CoV), we assessed host proteases on virus entry and S cleavages using western blotting, entry/inhibition assays, and fusion assays. The presence of TMPRSS2 significantly increased the entry of all tested pseudoviruses and correlated with S cleavages and fusion events. The results suggest the importance of continual evaluation of SARS-CoV-2 variants in their susceptibility to animal species through ACE2, and comparable analysis of entry efficacies of SARS-CoV-2, SARS-CoV and MERS-CoV provide details of entry processes with host proteases among different coronaviruses.

Feline infectious peritonitis (FIP) is a fatal systemic disease of cats, which is caused by FIP virus (FIPV). Viral mutation and host factors including T cell immunity play critical roles in the development of FIP. Because the PD-1/PD-L1 axis is a major target for viral evasion mechanism against T cell immunity, we assessed the expression of PD-L1 in cells infected with various strains

of FIPV along with the subclinical biotype feline enteric coronavirus (FECV). While both viruses demonstrated robust viral replication at comparable levels, FIPV-1146, but not FECV-1683, significantly upregulated PD-L1 expression in two cell lines. We also assessed the PD-L1 expression in tissues of cats naturally infected with FIPV. PD-L1 mRNA levels in relation to viral load were highly variable among tissues and cats. On confocal microscopy, we found that PD-L1 and FCoV expression were clustered and largely co-localized in the tissues. These findings support further investigation into the role of PD-L1 and T cell responses in the development of FIP.

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## **Acknowledgements**

I extend my heartfelt gratitude to my major advisor, Dr. Kyeong-Ok Chang and my co-major advisor Dr. Yunjeong Kim for their guidance and support throughout the years. I am forever grateful to have been given the opportunity to learn from them, and for their willingness to accept a first year vet student excited about their FIP research.

I am also grateful for my committee members, Dr. Waithaka Mwangi, Dr. Pankaj Baral, and Dr. Kate KuKanich for their support, guidance, and challenging discussion, which helped me grow as a scientist.

I am grateful for David George who has mentored me throughout the years, and has taught me many useful lab techniques.

I would like to thank Krishani Perera for her mentorship, friendship, and patience throughout VRSP and my graduate studies. I am thankful for my lab mates, Luija Nathali and Uttama Acharjee for their friendship and teamwork throughout my graduate studies.

I am incredibly grateful for my family, and all of the support they have given me throughout the years. I am thankful to my parents for their unending love, support, and belief in me. They are the reason I have been able to make it this far in my career. I am grateful for my partner, Cynthia Quinn Noland, whose love and support has gotten me through my most challenging times. I am grateful for my son Wayland, whose love for me and love of animals and science brings joy to my life. Last but not least, I am thankful for my pets who have surrounded me with unconditional love.

## **Dedication**

I dedicate this dissertation to my parents for their unending love, support, and belief in me.

# Chapter 1 - Review of literature on coronaviruses

Portions of this chapter were derived from the following published articles:

Zabiegala, A., Kim, Y., & Chang, K. O. (2023). Roles of host proteases in the entry of SARS-CoV-2. *Anim Dis*, 3(1), 12. <https://doi.org/10.1186/s44149-023-00075-x>

Links:           PMCID: [PMC10125864](https://pubmed.ncbi.nlm.nih.gov/PMC10125864/)       DOI: [10.1186/s44149-023-00075-x](https://doi.org/10.1186/s44149-023-00075-x)

and

Zabiegala, A., Kim, Y., & Chang, K. O. (2025). Host susceptibilities and entry processes of SARS-CoV-2 Omicron variants using pseudotyped viruses carrying spike protein. *BMC Vet Res*, 21(1), 377. <https://doi.org/10.1186/s12917-025-04822-9>

Links:           PMCID: [PMC12108000](https://pubmed.ncbi.nlm.nih.gov/PMC12108000/)       DOI: [10.1186/s12917-025-04822-9](https://doi.org/10.1186/s12917-025-04822-9)

## 1.1 Classification of coronaviruses

Coronaviruses are positive sense single-stranded RNA viruses with a large genome of approximately 30kb and belong to the family *Coronaviridae* in the order *Nidovirales* (1, 2). Named after the Latin derivative for crown, coronaviruses were first described in 1968 after their appearance under electron microscope was found to resemble a “solar corona” (3). The subfamily *Orthocoronavirinae* of the family *Coronaviridae* is divided into four genera: alpha, beta, gamma, and delta coronaviruses. Coronaviruses are capable of infecting a wide range of species, with the alpha and beta coronavirus genera primarily responsible for mammalian infection. Alpha coronaviruses include human common cold strains HCoV-229E and HCoV-NL63, various swine coronaviruses such as transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV) and swine hemagglutinating encephalomyelitis, and feline coronaviruses. Beta coronaviruses include human common cold strains HCoV-OC43 and HCoV-HKU-1, as well as the more pathogenic severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern respiratory syndrome coronavirus (MERS), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Common beta coronaviruses in other species include mouse hepatitis virus (MHV), bovine enteritis and respiratory disease (BCoV), canine respiratory disease (CRCoV), as well as many strains that circulate within bat populations. Alternatively, delta and gamma coronaviruses primarily infect avian species, with the exception of the porcine deltacoronavirus HKU15.

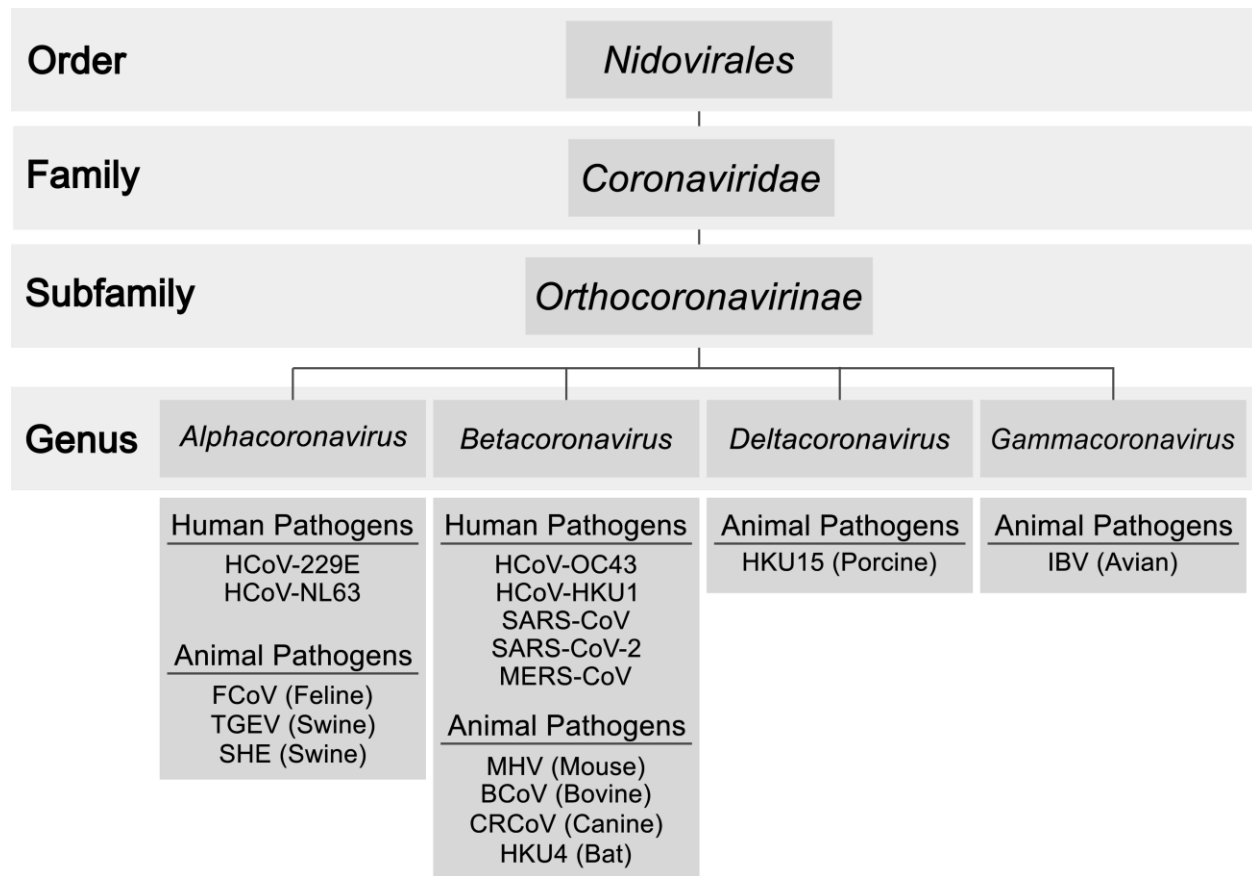


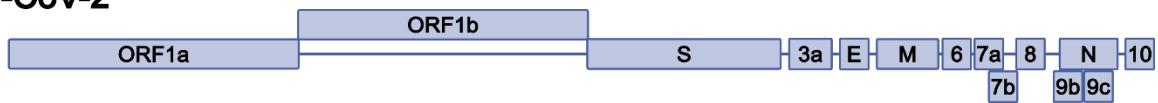
**Figure 1.1 Coronavirus classification**

Coronavirus classification by the International Committee on Taxonomy of Viruses (2).

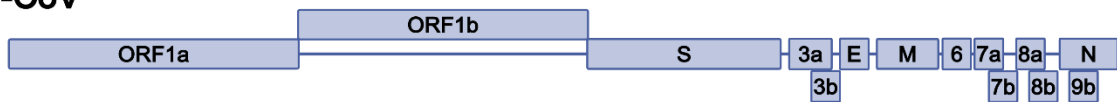
## 1.2 Genomic organization of coronaviruses

Coronaviruses are large RNA viruses with genomes of roughly 30kb arranged into approximately 10 ORFs (Fig. 1.2). The first ORF is divided into two portions ORF1a and ORF1b, which encode large, overlapping polypeptides encoding a majority of the nonstructural proteins of coronaviruses. The polypeptide pp1a is encoded by ORF1a while the pp1ab is the result of frameshifting between ORF1a and ORF1b. These large polypeptides are then cleaved by viral proteases such as the 3C-like protease (3CLpro) into numerous nonstructural proteins. The remaining genome consists of the structural proteins spike (S), envelope (E), nucleocapsid (N), and membrane (M), as well as various accessory proteins.

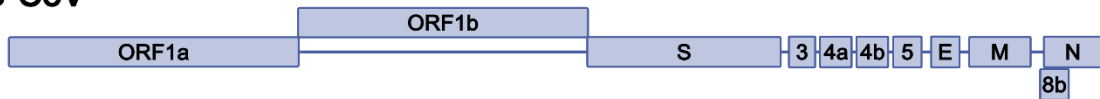
### SARS-CoV-2



### SARS-CoV



### MERS-CoV



### FIPV



**Figure 1.2 Coronavirus genomes**

Genomic organization of SARS-CoV-2, SARS-CoV, MERS-CoV, and FIPV.

## **1.3 Entry of Coronaviruses**

### **1.3.1 Spike protein**

The coronavirus spike protein (S) is a type I viral fusion protein that is responsible for viral attachment to the receptor and subsequent entry into its host cells (4). It is a functional trimer with each monomer having two parts, the S1 and S2 region (Fig. 1.3). The S1 contains the receptor-binding domain (RBD) which mediates viral attachment to the host cell functional receptor, while the S2 contains the hydrophobic fusion domain that facilitates viral membrane fusion and entry into the host cells (4). Following the release of the virus from cells, the RBD alternates between open ('up' and receptor accessible) and closed ('down' and receptor inaccessible) (Fig. 1.4), and open RBD binds to the functional receptor such as the angiotensin converting enzyme-2 (ACE2) for SARS-CoV and SARS-CoV-2 (5-8). Receptor binding causes a conformational change of S, resulting in exposure of the S2' site and the proteolytic cleavage leads to exposure of the fusion peptide to cell membrane (4, 9). The S2' site and the fusion peptide are well conserved among coronaviruses (4). Mutations in S are associated with changes in susceptible animal host, tissue tropism, virulence or pathogenicity (4).

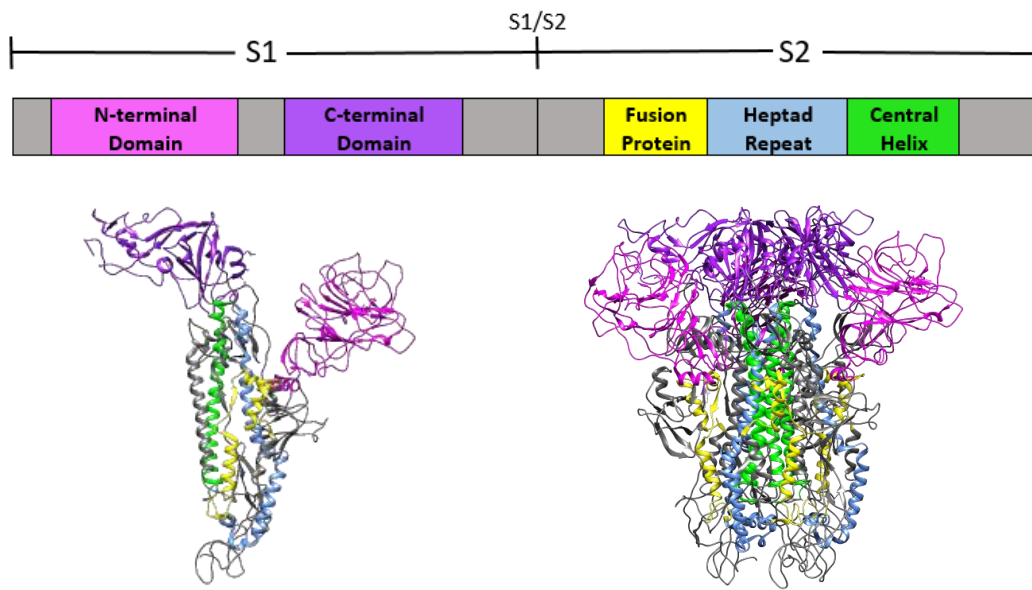


**Figure 1.3 Spike protein arrangement**

Arrangement of the coronavirus spike protein. Protein domains and structural arrangement of the monomer (left) and functional trimer (right) S protein of MHV (PDB: 6VSJ)

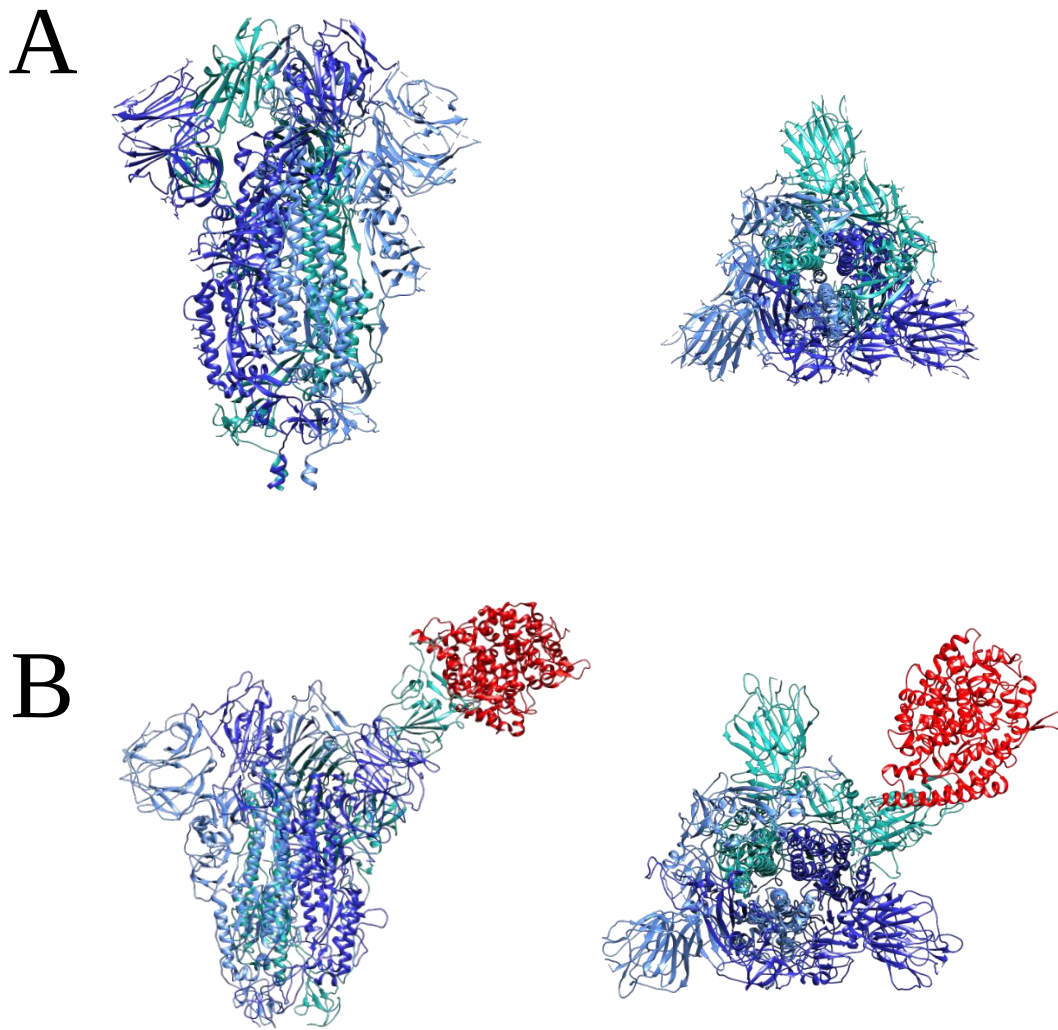


**Figure 1.4 Three dimensional structure of S protein bound to receptor**

(A) SARS-CoV-2 spike protein in unbound, “closed” position (PDB: 6VXX). (B) SARS-CoV spike protein (blue) in “open” position (PDB: 6ACK) bound to functional receptor ACE-2 (red) at the CTD of S1. This view is representative of the conformation of most coronavirus S proteins when bound to their functional receptors.

### **1.3.2 Receptors**

The S1 region of the coronavirus spike protein is responsible for receptor binding. For many coronaviruses of importance, the functional receptors have been identified. Certain receptors are shared among coronaviruses, such as angiotensin converting enzyme 2 (ACE2) which is the common functional receptor among the human coronaviruses SARS-CoV, SARS-CoV-2, and HCoV-NL63 (10-12). The functional receptor for MERS-CoV is DPP4, which is shared with bat coronaviruses related to MERS-CoV such as HKU4 (13-15). Aminopeptidase N (APN) is the identified functional receptor of various alphacoronaviruses such as the common cold HCoV-229E, TGEV in swine, and in serotype II feline coronaviruses (16-19). Human leukocyte antigen 1 (HLA-1) is also a shared receptor among various betacoronaviruses such as bovine coronavirus, common cold strain HCoV-HKU1, and canine respiratory coronavirus (20). However, receptors for various coronaviruses, including serotype I FCoVs, have not been identified.

**Table 1 Coronaviruses and their known receptors**

| <b>Name</b>                                                                             | <b>Year Identified</b> | <b>Species</b> | <b>Tissue Tropism</b>            | <b>NTD Receptor</b> | <b>CTD Receptor</b>                     |
|-----------------------------------------------------------------------------------------|------------------------|----------------|----------------------------------|---------------------|-----------------------------------------|
| <b>Alphacoronavirus</b>                                                                 |                        |                |                                  |                     |                                         |
| Common Cold (HCoV-229E) <b>(17, 21)</b>                                                 | 1965                   | Human          | Upper Respiratory                |                     | hAPN                                    |
| Common Cold (HCoV-NL63) <b>(12, 21)</b>                                                 | 2002                   | Swine          | Upper Respiratory                |                     | ACE-2                                   |
| Transmissible Gastroenteritis Virus (TGEV) <b>(16, 21)</b>                              | 1946                   | Swine          | Enteric                          |                     | pAPN                                    |
| Swine Hemagglutinating Encephalomyelitis (Vomiting and Wasting Disease) <b>(22, 23)</b> | 1959                   | Swine          | Enteric CNS                      |                     | NCAM                                    |
| Feline Coronaviruses (FCoV) <b>(18, 19)</b>                                             | 1963                   | Feline         | Enteric (FECV)<br>Systemic (FIP) |                     | Serotype 1: unknown<br>Serotype 2: fAPN |
| <b>Betacoronavirus</b>                                                                  |                        |                |                                  |                     |                                         |
| Mouse Hepatitis Virus (MHV) <b>(24, 25)</b>                                             | 1949                   | Mouse          | Liver<br>Enteric<br>CNS          | CEACAM-1            |                                         |
| Bovine Enteritis and Respiratory Disease (BCoV) <b>(20)</b>                             |                        | Bovine         | Enteric<br>Lower Respiratory     | Sialic Acids        | HLA-1                                   |
| Common Cold (HCoV-OC43) <b>(20, 25)</b>                                                 | 1967                   | Human          | Upper Respiratory<br>CNS         | Sialic Acids        |                                         |
| Common Cold (HCoV-HKU1) <b>(20, 25)</b>                                                 | 2005                   | Human          | Lower Respiratory                | Sialic Acids        | HLA-1                                   |
| Severe Acute Respiratory Disease (SARS/SARS-CoV-1) <b>(11, 25)</b>                      | 2002                   | Human          | Lower respiratory                |                     | ACE-2                                   |
| Covid-19 (SARS-CoV-2) <b>(10)</b>                                                       | 2019                   | Human          | Lower respiratory                |                     | ACE-2                                   |
| Middle Eastern Respiratory Syndrome (MERS) <b>(13)</b>                                  | 2012                   | Human          | Lower respiratory                |                     | DDP4                                    |
| Canine Respiratory Disease (CRCoV) <b>(20)</b>                                          | 1971                   | Canine         | Upper and Lower Respiratory      | Sialic Acids        | HLA-1                                   |
| SARS-like Coronavirus (SARSr-CoV) <b>(26)</b>                                           |                        | Bat            | Enteric                          |                     | ACE-2                                   |
| HKU4 <b>(14, 15)</b>                                                                    | 2006                   | Bat            | Enteric                          |                     | DPP4                                    |
| <b>Gammacoronavirus</b>                                                                 |                        |                |                                  |                     |                                         |
| Infectious Bronchitis Virus (IBV) <b>(27, 28)</b>                                       | 1930                   | Poultry        | Upper Respiratory                | Sialic Acids        |                                         |

### **1.3.3 Roles of host proteases**

Proteases are one of the most abundant proteins with human and animal genomes encoding approximately 600 proteases ( $\sim 2\%$  of the genomes) (29). Proteases are involved in many physiological processes such as digestion, protein activation/turnover, blood coagulation, wound healing, fertilization, cell differentiation and growth, cell signaling, immune response, and apoptosis (29). Moreover, proteases are involved in the cleavage and activation of proteins that are produced as proproteins, a process that requires tight regulation to protect cells from active (enzymatically or structurally) proteins in the wrong places. Therefore, protease activity is controlled by naturally occurring protease inhibitors to prevent uncontrolled, poorly regulated, or undesired protease activity (30). Several proteases have been implicated in the activation (cleavage) of SARS-CoV-2 S at various cleavage sites. Figure 1.5 summarizes overall structures of SARS-CoV-2, SARS-CoV and MERS-CoV S and the cleavage sites of various proteases.

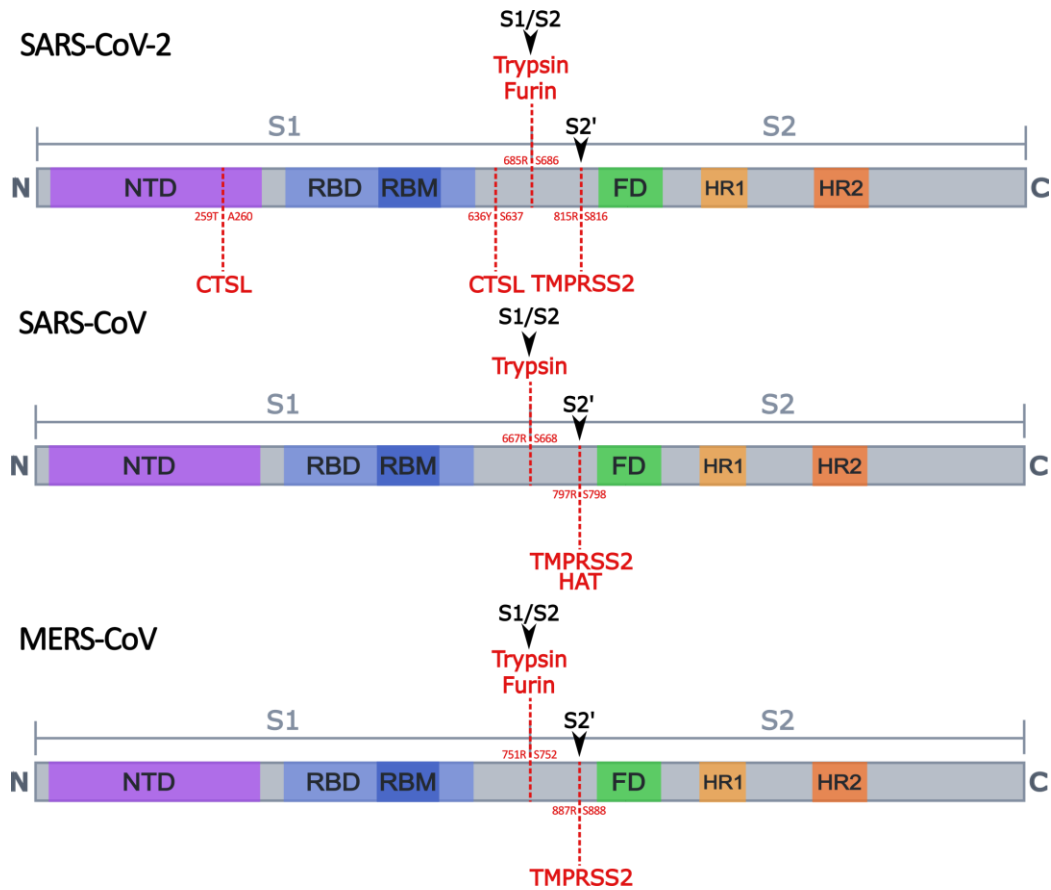


**Figure 1.5 Experimentally observed host protease cleavage sites in S of SARS-CoV-2, SARS-CoV and MERS-CoV.**

The S is divided into two portions: S1 and S2. S1 contains the receptor-binding domain (RBD) where the receptor-binding motif (RBM) interacts with the functional receptor (ACE2 or DPP4). The S2 domain contains the fusion domain, which is responsible for viral entry into the cell. In betacoronavirus entry studies, two cleavage sites have been observed as S1/S2 which is cleaved to cause a conformational change to facilitate binding to the functional receptor and S2' which is cleaved to expose the fusion domain to allow for viral entry. The S2' site is highly conserved among all coronaviruses and is cleaved by TMPRSS2. In SARS-CoV-2, the S1/S2 cleavage site can be cleaved by host furin and trypsin, and two cathepsin L cleavage sites have been observed in S1 region as pictured. SARS-CoV is unable to be cleaved by host furin but has been observed to be cleaved at the S1/S2 site experimentally by trypsin (31). In addition to TMPRSS2 cleavage at S2' site, human airway trypsin-like protease (HAT) has also been experimentally observed to have cleavage activity at the site. MERS-CoV S is able to be cleaved by the same proteases as SARS-CoV-2 in the S1/S2 and S2' sites.

### **Protease cleavage sites in S of SARS-CoV-2 and other coronaviruses**

The S1/S2 site divides S1 and S2, and Figure 1.6 shows the conservation of arginine/serine at the site among SARS-CoV, MERS-CoV and SARS-CoV-2. Most betacoronaviruses except SARS-CoV contain additional arginine residue(s) at the S1/S2 site as shown in Figure 1.6, which can be cleaved by furin (32-37). SARS-CoV-2 contains more arginine residues than other known betacoronaviruses with the polybasic cleavage motif (R-R-X-R-R motif) at S1/S2 site (Fig. 1.6). The polybasic motif matches the minimal basic motif of RXXR recognized by furin (33), a subtilisin-like protease present in the Golgi apparatus and is involved in the processing of a wide variety of proproteins into their active state (38, 39). As furin is ubiquitously present in most organs, it has been proposed that the presence of the polybasic motif mediates SARS-CoV-2 entry into different cell types in multiple organ systems. The polybasic site was reported to be associated with virulence and tropism of various viruses, most notably in the hemagglutinin protein of avian influenza (40-43). The polybasic cleavage site has been directly linked to severe and systemic pathogenesis (44, 45) in bird populations, whereas influenza strains lacking the polybasic cleavage site are typically limited to the respiratory and gastrointestinal tracts (46, 47). The S1/S2 cleavage facilitates more accessible RBD to the receptor ACE2 (48), and receptor binding causes a conformational change of the S, resulting in exposure of the S2' site, and the proteolytic cleavage leads to expose the fusion peptide to cell membrane (4, 9, 49-51). The cleavage of the S2' site allows for shedding of the S1 subunit and HR1 in S2 further undergoes a dramatic refolding transition, which triggers insertion of the fusion peptide into the target cell membrane (52-54). In the presence of type II transmembrane serine proteases (TTSPs) such as TMPRSS2 (50, 55-60), SARS-CoV-2 can utilize a direct entry route by fusion of the viral envelope to the cellular membrane (61, 62). In the absence of this protease, the virus has been shown to utilize an

endosomal entry route through processing of the S by endosomal cathepsins (61, 62). In SARS-CoV, though exogenous trypsin may have activity at the S1/S2 site *in vitro* (and gastrointestinal track *in vivo*), there is limited to no activation by host furin in entry studies of SARS-CoV (34, 59, 63, 64).



### Figure 1.6 Multibasic arginine residues

The multibasic arginine (R) residues observed in S of SARS-CoV-2 and MERS-CoV in the S1/S2 region allow for the cleavage of host furin, which shows a specificity for the motif RXXR. S2' is highly conserved among coronaviruses, as it is responsible for exposing the fusion domain to allow for cell entry

### TMPRSS2

TMPRSS2 is a transmembrane serine protease primarily expressed in lung and prostate tissue, but is also expressed in a lower amount in heart, liver and GI tract (65). The exact physiological function of TMPRSS2 is unknown at this time, as knockout mice showed no phenotypic changes when the protease was not expressed (66), though it has been linked to various processes through its role in cancer pathogenesis (67). Many studies have shown that the presence of TMPRSS2 is a major determinant of SARS-CoV-2 entry route into respiratory tracts (68, 69). The entry of SARS-CoV-2 in various tissues occurs through different pathways depending on the expression levels of TMPRSS2 and other receptors (54, 68). While it was shown that TMPRSS2 can cleave the S2' site (50) (Fig. 1.5), it is uncertain whether other sites such as S1/S2 are processed

by TMPRSS2. Some studies indicate that TMPRSS2 acts only on the S2' cleavage site (50), while others reported that it may have activity at both sites (58, 59). Though the cleavage potential of TMPRSS2 is still debated, it has been demonstrated in SARS-CoV (55), MERS-CoV (49), and SARS-CoV-2 (68) that the presence of TMPRSS2 is the determining factor for the entry route utilized, and that cells expressing this protease allow an increased viral entry and replication.

### **Cathepsins**

In the absence of TMPRSS2, SARS-CoV-2, as well as MERS-CoV, can utilize endosomal entry routes (55, 68, 70-72). Some reports showed that the SARS-CoV-2 Omicron variants may rely more on the endosomal entry than TMPRSS2 mediated cell entry (69), suggesting there are variations on the virus entry among SARS-CoV-2 variants. Cathepsins are mainly localized in the endolysosomes and perform a variety of functions such as processing proproteins, hormones and antigens (73, 74). The key cathepsin protease utilized by coronaviruses for cell entry is cathepsin L, a cysteine protease that preferentially cleaves peptide bonds with nonpolar, aromatic residues (75). It is present in most tissues, including respiratory epithelium, proximal tubules of kidney, cardiomyocytes, and glandular cells of duodenum and colon (65). Cathepsin L is also known to activate the entry of various viruses, including Hendra virus and Ebola virus (76, 77). SARS-CoV (62), MERS-CoV (78), and SARS-CoV-2 have the predicted cleavage sites of multiple cathepsins including cathepsin L and B in the S protein. While the precise cleavage sites of cathepsin are to be determined, cleavage of S protein upstream of the fusion peptide would allow priming of S2 for fusion of virus and the endosome membrane.

One group utilized a Protease-ase cleavages from MEROPS ANalyzed Specificities (PACMANS) system to predict and rank potential cleavage sites of SARS-CoV-2 S protein,

finding high probability of cleavage at sites near S1/S2 and near S2' (79). Experimentally, there were two sites observed by one group in S1 for cathepsin L cleavage on SARS-CoV-2 (80). Figure 1.7 summarizes the entry of SARS-CoV-2 in the presence or absence of TMPRSS2 on the membrane.

Other cathepsins were also reported to be involved in the activation of SARS-CoV-2 S protein. Those cathepsins include cathepsin B (CTSB), cathepsin K (CTSK), cathepsin S (CTSS), and cathepsin V (CTSV) (79), all of which are cysteine proteases that showed a similar preference for cleavage sites as cathepsin L. Cathepsin B is widely distributed throughout the body (65) and is responsible for activation of various proenzymes, prohormones and trypsin (73). It has fairly nonspecific cleavage sites (75), and had little to no effect on SARS-CoV-2 entry when cathepsin B selective inhibitors were used (81, 82). Cathepsin K is another cysteine protease with a preference of branched hydrophobic residues at the cleavage sites (83) and primarily expressed in osteoclasts for bone remodeling but also involved in Toll-like receptor signaling and processing of B-endorphin in the brain (73). Cathepsin S is present in a wide variety of tissues including ciliated cells of the nasopharynx, lung macrophages, and glandular cells in the GI tract and gall bladder (65) and involved in antigen processing and presentation and light chain processing (73). It has fairly nonspecific cleavage activation (75) but appears to favor branched hydrophobic residues like cathepsin K (83). Cathepsin V is widely distributed throughout the body (65) and involved in the regulation of natural killer cells and CD8+ cytotoxic cells (73). Like cathepsin L, cathepsin V appears to prefer aromatic hydrophobic amino acids (83). The current data which suggest the involvement of these cathepsins on the processing of SARS-CoV-2 S protein are mainly based on *in silico* modelling (79). Thus, the roles of these various cathepsins in the activation of coronavirus S protein remains to be determined experimentally.

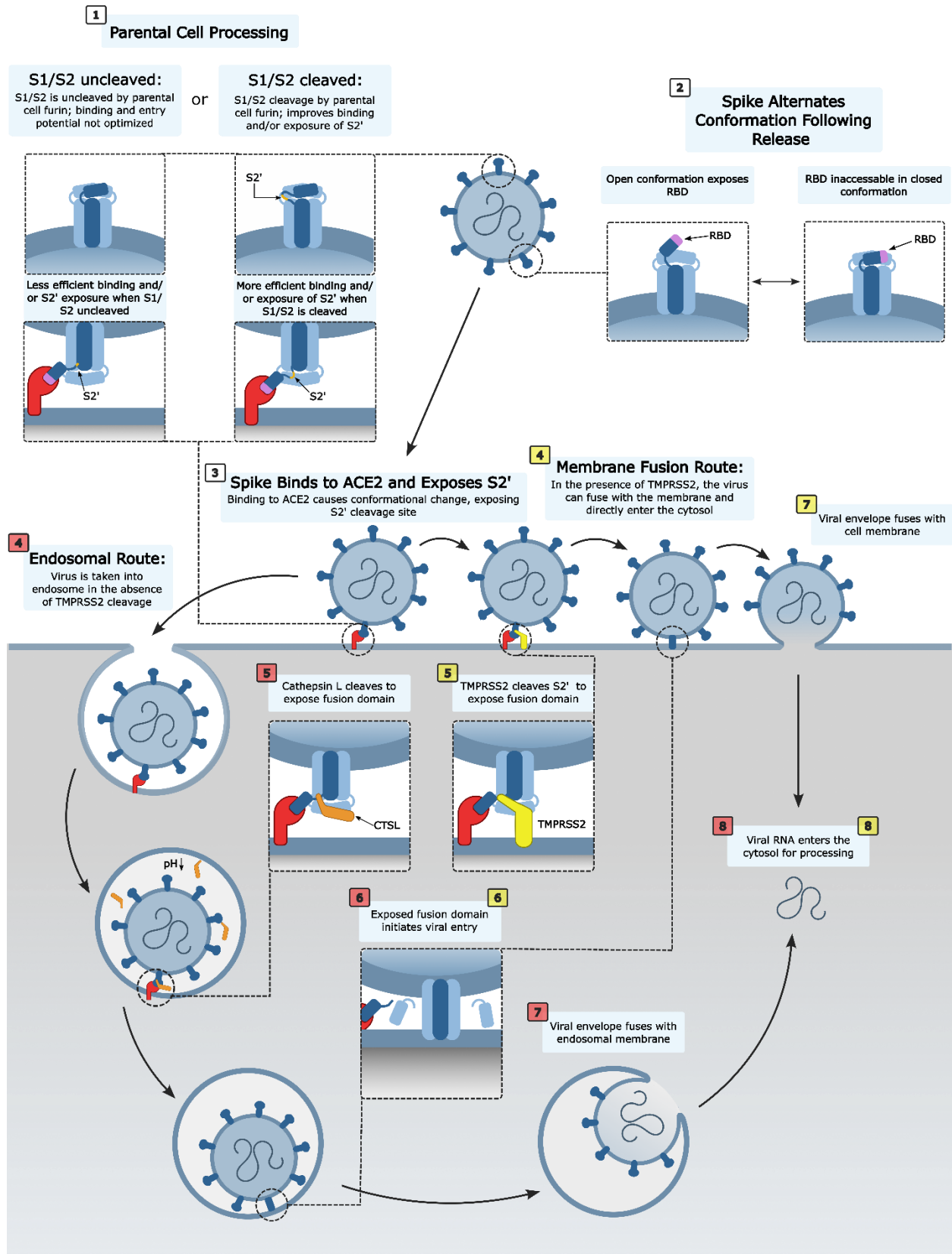


**Figure 1.7 Entry process of SARS-CoV-2**

Attachment and entry model of SARS-CoV-2. When SARS-CoV-2 is released by the parental cell, some S is cleaved by host furin. Cleavage by furin facilitates faster binding to the functional receptor ACE2. The binding of ACE2 to S induces a conformational change which exposes the S2' cleavage site. The presence or absence of TMPRSS2 dictates whether the virus enters through a fast membrane fusion or a slow endosomal route. In the absence of TMPRSS2, the virus is taken into an endosome where the pH will drop, activating cathepsin L. Cathepsin L cleaves S to initiate fusion to the endosomal membrane before release of viral RNA into the cytosol. In the presence of TMPRSS2, the S2' site is cleaved and the virus can fuse directly to the cell membrane, allowing for a more rapid entry of the viral RNA into the cell.

## **1.4 Human coronaviruses of significance**

Various coronaviruses circulate in human populations as common cold viruses and typically cause mild upper respiratory clinical signs. However, after the dawn of the 21<sup>st</sup> century, several coronaviruses emerged causing acute respiratory distress syndrome (ARDS). These novel coronaviruses emerged from zoonotic sources and cause severe respiratory infections with high case fatality rates. As SARS-related coronaviruses continue to circulate in bat populations, there is an ongoing risk for cross-species transmission of novel pathogenic coronaviruses in humans.

### **1.4.1 SARS-CoV**

In November of 2002, cases of a severe, atypical pneumonia of unknown etiology were observed in Foshan City, Guangdong Province, China. Initially, the outbreaks were contained to independent clusters within Guangdong Province among animal traders and healthcare workers. However, following a superspreader event in February 2003, cases began to arise in Hong Kong, Vietnam, Singapore, Thailand, Germany, and Canada (84-88). In March of 2003, the World Health Organization (WHO) issued a global alert for the severe pneumonia outbreaks as they continued

to spread to other countries, including the United States, where the CDC supported the study of this condition termed “Severe Acute Respiratory Syndrome.” Later that month the CDC identified a novel coronavirus as the likely causative pathogen of SARS (89). As a result of global cooperation, monitoring, intensive research efforts, and the implementation of travel alerts, the SARS-CoV outbreak was contained and human-to-human transmission was quelled by July 2003. WHO reports a total of 8,422 cases over 29 countries, with a total of 916 fatalities (90). Though the outbreak only lasted for approximately 6 months, SARS-CoV continued to be a focus of ongoing studies over the next decades.

It is now known that the reservoir species of SARS-CoV were bats, which are a common reservoir for many viruses including coronaviruses (91-93). The palm civet has been identified as the likely intermediate host, as strains found in masked palm civets at the time displayed approximately 99.8% homology with the strains isolated from humans (94-96). Workers with close contact with exotic food animals were over represented in the early SARS-CoV cases (97-99), further supporting the likelihood of live market palm civets as the intermediate host. Additionally, animal traders in Guangdong Province were found to have significantly higher IgG antibodies against SARS-CoV than vegetable traders (97, 98, 100). Other intermediate hosts such as raccoon dogs were assessed, but the strains found in other species displayed less homology to human isolates than the strains isolated from the palm civets (95, 96).

#### **1.4.2 MERS-CoV**

In 2012, the novel MERS-CoV was first identified in Saudi Arabia in a patient presenting with severe pneumonia and acute kidney failure (101). Symptoms of Middle Eastern respiratory syndrome (MERS) range from subclinical infection to pneumonia and severe systemic disease

(102). Unlike SARS-CoV and SARS-CoV-2, MERS-CoV is primarily transmitted by direct or indirect contact with the reservoir host: dromedary camels (103, 104). While reports of human-to-human transmission have been recorded, these instances primarily occur among close contacts in health care settings and households (103). Dromedary camels are currently the reservoir species responsible for human infection; however, it is likely MERS-CoV originated in bats due to the identification of coronaviruses in bats with similar homology (104). MERS-CoV continues to circulate today, primarily within the Arabian Peninsula where domesticated camels are raised for travel, trade, meat, and milk.

### **1.4.3 SARS-CoV-2**

#### **Emergence**

In late December 2019, an outbreak of pneumonia unknown origin were reported from Wuhan to the China National Health Commission (105). By January 2020, the virus had been sequenced and identified as a novel coronavirus. By the end of January, WHO declared the outbreak a global health emergency after the virus had spread to nearly 50 countries across the globe (106). Because of the knowledge gained during the first SARS-CoV outbreak, this novel coronavirus was quickly identified, and quarantine efforts were applied to prevent further transmission of the virus. However, due to the higher transmissibility of SARS-CoV-2 compared to its novel predecessors, the virus rapidly spread globally, causing the COVID-19 pandemic.

Bats are well known reservoirs of coronaviruses, and SARS-related coronaviruses have been identified in bat populations following the original SARS-CoV outbreak (107, 108). The closest isolate to SARS-CoV-2, termed RaTG13, was isolated from the stool of the horseshoe bat

*Rhinolophus affinis* in 2013 (109). Initially, the pangolin was thought to serve as an intermediate host, but further research was not supportive of this (110).

### **Variants and Epidemiology**

Throughout the COVID-19 pandemic, different strains of SARS-CoV-2 emerged with varying rates of transmissibility, antigenicity, and mortality. Five strains appearing as a threat to public health designated as variants of concern (VOC) have been identified throughout the pandemic: Alpha, Beta, Gamma, Delta, and Omicron (Pango lineages B.1.1.7, B.1.351, P.1, B.1.617.2, and B.1.1.529, respectively). However, as of mid-2022, only Omicron subvariants remain in circulation.

The Alpha variant carrying eight mutations in S was first documented in September 2020 in the United Kingdom and became the predominant strain in the UK by early 2021 (111). Compared to the parental Wuhan strain, the Alpha variant appeared to have increased transmissibility and mortality, but limited resistance to neutralization by monoclonal antibodies in vaccinated individuals (112-114). By the end of 2021, the strain was no longer observed in circulation.

The Beta variant was initially identified in South Africa in November 2020, and eight mutations were present in S. The Beta variant was observed to have higher transmissibility and improved capability of evading neutralizing antibodies compared to previous strains, though disease severity of the Beta variant was similar to the Alpha variant (115-117). By March 2022, the WHO designated the Beta variant as “previously circulating” as cases were no longer detected.

The Gamma variant was first detected in Brazil in late 2021 and spread to more than 36 countries (118, 119). With up to twelve mutations in the spike protein, the Gamma variant was

characterized as having increased transmissibility and viral load compared to previous strains. By March of 2022, WHO had designated the Gamma variant as “previously circulating.”

In India in the fall of 2020, the Delta lineage emerged with nine to thirteen mutations in S, depending on the sublineage. The Delta variant showed increased transmissibility and mortality in unvaccinated individuals than previous variants, and was the predominant circulating strain from late 2020 to early 2021 where it was reported in up to 119 countries (116, 120). By mid-2022, the Delta variant was classified as previously circulating.

The Omicron variant emerged in Botswana, late 2021 and rapidly became the dominant strain in circulation, and as of 2025, remains the only SARS-CoV-2 variant in circulation. Omicron subvariants are generally characterized by having over 30 mutations in S, increased transmissibility, and reduced virulence. However, each subvariant differs in these characteristics. In 2023, WHO updated its nomenclature as classifying independent Omicron sublineages as either variants of interest (VOI) or variants under monitoring (VUM) (121).

### **Zoonosis and reverse zoonosis**

Since the beginning of the COVID-19 pandemic, concerns were raised regarding cross-species transmission and the creation of an animal reservoir. Some of the most prominent animals naturally infected by SARS-CoV-2 were cats, minks, ferrets, dogs, white-tailed deer, and hamsters (Table 2). Cases of upper respiratory infections caused by SARS-CoV-2 in dogs and cats were observed in the early pandemic, with some documentation of animal-to-animal transmission, but limited evidence of animal-to-human transmission (122). SARS-CoV-2 infections in Syrian hamsters was also well reported, with natural infections observed in pet store populations (123-126). White-tailed deer were also a susceptible species of major focus due to the high prevalence

of SARS-CoV-2 in wild populations and demonstrated transmission from deer to deer without apparent symptoms (127). However, mink proved to be the species with the highest potential to become reservoir species. Mink were found to be highly susceptible to SARS-CoV-2, and often displayed severe systemic illness, severe respiratory disease, or sudden death (128). Throughout the pandemic, several outbreaks occurred in mink farms, some of which demonstrated mink-to-human transmission (129). However, these outbreaks were largely contained following mass culling of infected and exposed populations. As the pandemic progressed and Omicron became the dominant strain, fewer cases in animals were observed.

**Table 2 Documentation of SARS-CoV-2 in various animal species**

| Species                  | Evidence of natural infection | Documented Transmission                                                                                       | Clinical Signs                                                                                                                      | Prevalence of Seroconversion in populations                                                                  | Prevalence of positive viral loads in populations                                                                                 |
|--------------------------|-------------------------------|---------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|
| <b>Domestic cats</b>     | Yes                           | Cat to cat<br><br>Limited cat to human (105)                                                                  | Typically asymptomatic (106)<br><br>Minor upper respiratory signs<br><br>Possible myocarditis (107)                                 | Experimentally<br>Inoculated: consistently developed antibodies (108)<br><br>Naturally: 0-52% (105, 109-114) | Naturally: 0-40% (110, 111, 113, 115-118)                                                                                         |
| <b>Dogs</b>              | Yes                           | Limited dog to dog<br><br>Limited dog to human                                                                | Typically asymptomatic (106)<br><br>Minor upper respiratory signs (119)<br><br>Possible myocarditis (107)                           | Naturally: <1%-41% (109-114)                                                                                 | Naturally: 1%-28% (110, 111, 113, 115-118, 120)                                                                                   |
| <b>Ferrets</b>           | Yes                           | Prevalent ferret to ferret transmission experimentally (75% infection rate through direct contact) (121, 122) | Asymptomatic<br><br>Systemic Signs: fever and loss of appetite,<br><br>(more common with direct contact and inoculation) (108, 122) | Experimentally inoculated:<br><br>100% (108)<br><br>Naturally:<br><br>1.57% (123)                            | Experimentally inoculated: 100% (108, 124, 125)                                                                                   |
| <b>Mink</b>              | Yes                           | Mink to mink transmission<br><br>Mink to human transmission                                                   | Systemic<br><br>Respiratory (126)<br><br>Sudden death<br><br>Mortality rate: 35-55% (127)                                           | Naturally: 3-95% (128, 129)                                                                                  | Experimentally inoculated: 100% (126)<br><br>Naturally:<br><br>Documented in wild mink- 15% (130)<br><br>Farmed mink- 20.4% (128) |
| <b>Bovine</b>            | No                            | No transmissibility noted bovine to bovine                                                                    | Asymptomatic<br><br>Fever                                                                                                           | Few seropositive after inoculation (131)                                                                     | Zero to few experimentally infected (131-134)                                                                                     |
| <b>Pig</b>               | No                            | Unable to transmit pig to pig (135, 136)                                                                      | Asymptomatic                                                                                                                        | None in experimentally infected (108, 136)                                                                   | None to few experimentally infected, none infected through direct contact (135, 136)                                              |
| <b>Horse</b>             | Yes                           | Not documented                                                                                                | Asymptomatic (137)                                                                                                                  | Natural: 3.5-5.9% (137-139)                                                                                  | None experimentally (132) or naturally (133, 137, 138)                                                                            |
| <b>Rabbit</b>            | No                            | NA                                                                                                            | NA                                                                                                                                  | None-naturally infected (114)                                                                                | None experimentally (132, 133)                                                                                                    |
| <b>Syrian Hamster</b>    | Yes                           | Hamster to hamster (aerosol)                                                                                  | Upper and lower respiratory infection (140-144)<br><br>Vasculopathy (145)                                                           | 44% naturally (146)                                                                                          | 100% Experimentally Infected through inoculation, direct, and aerosol transmission (140, 141, 147)<br><br>Naturally 10% (146)     |
| <b>White-tailed deer</b> | Yes                           | Deer to deer direct contact (148)                                                                             | Asymptomatic                                                                                                                        | Naturally: 14.1-94.7% (149-152)                                                                              | Naturally: 11.2-82.5% (152-155)                                                                                                   |
| <b>Sheep and Goats</b>   | No                            | NA                                                                                                            | NA                                                                                                                                  | None                                                                                                         | None (132, 133)                                                                                                                   |

## **1.5 Feline coronavirus**

### **1.5.1 FCoV classification**

Feline coronaviruses are alphacoronaviruses capable of infecting domestic and wild felids. Unique to FCoVs is classification of the virus into two distinct serotypes and two distinct biotypes. FCoV serotypes are divided into serotype I and serotype II, based on the makeup of their spike protein. Whereas serotype I FCoVs have a spike protein entirely of feline origin, serotype II FCoVs have a spike protein that is recombinant of canine coronavirus (156). Serotype I FCoVs represent a majority of naturally occurring infections, but poses challenges in *in vitro* study due to poor growth in cell culture (157). Serotype II FCoVs, however, are better adapted to cell culture, but represent a minority of natural infections. The receptor for serotype II FCoVs has been identified as fAPN, though the receptor for serotype I FCoVs remains unknown.

Whereas FCoV serotypes are based off the makeup of S, the biotypes of FCoV are based off the cellular tropism and clinical signs. Feline enteric coronavirus is a ubiquitous infection among cat populations, causing inapparent to mild enteritis. FECVs infect the intestinal epithelium, are shed in the feces, and transmitted through the fecal-oral route. Shedding can be transient, reoccurring, or chronic over the periods of months or years, and reinfection is a common occurrence (158). Up 90% of kittens and one third of adult cats have been found to be shedding FECV upon shelter entry (159). While the FECV biotype causes a relatively benign infection, the feline infectious peritonitis virus (FIPV) biotype is responsible for causing a fatal systemic infection in which the viral tropism shifts from enterocytes to macrophages.

### **1.5.2 Feline infectious peritonitis**

Feline infectious peritonitis is a fatal condition caused by the virulent FCoV biotype FIPV. FIP can occur in up to 5% of cats infected with FCoV, and is most prominent in areas where there is a high population density such as shelters, catteries, or multi-cat households (160). FIP is most commonly seen in cats under two years of age or cats that are immunocompromised. Additionally, some breeds of cats are genetically predisposed to FIP, such as Bengals, Himalayans, and Ragdolls (161).

#### **Clinical Signs**

Cats that develop FIP are typically classified into either an effusive/wet form or noneffusive/dry form based on their clinical signs. Regardless of the form of disease, cats with FIP often display failure to thrive, inappetence, weight loss, and fever (158). The effusive form of FIP is characterized by inflammation of the visceral serosa and omentum with the accumulation of proteinaceous exudate and fibrin deposits within the body cavities. Abdominal distention is the most common initial clinical finding in cats with effusive FIP. Alternatively, the noneffusive form of FIP is characterized by granulomatous lesions on parenchymal organs such as kidneys, liver, mesenteric lymph nodes, central nervous system, and the eyes. In noneffusive FIP, neurologic signs, uveitis and chorioretinitis are some of the most prevalent clinical signs.

It is believed the manifestation of disease is dependent on the level of host immune response, and cats can often consecutively experience both forms of the disease (162). It is believed that the noneffusive form of FIP results from a partial immune response as the body attempts to sequester the virus in granulomatous lesions whereas the effusive form results from a lack of a proper immune response (162).

## **Pathogenesis**

The primary differentiating factor between FECV and FIPV is the shift in viral tropism from enterocytes to macrophages. While FECV can readily transmit between cats, FIPV is rarely transmitted between cats. It is generally accepted that FIPV arises from viral mutations within an individual cat, and several mutations have been implemented in this shift (158, 163, 164). Mutations in the ORF3c accessory protein leading to a truncated protein product have been shown to be detrimental to viral infection of enterocytes, but not monocytes and macrophages (18, 163, 165-168). Additionally, as the spike protein is a major factor of viral attachment and entry, it has been a focus of study in biotype shift. Two specific amino acid changes (M1058L and S1060A) in the fusion domain of the spike protein have been linked to the dissemination of FCoV to extraintestinal tissues (168, 169). While these mutations are not observed in FCoV isolated from the gastrointestinal tract, a majority of FCoV in other tissues contains at least one of these mutations. Mutations in the ORF 7b accessory protein were initially thought to be involved in biotype shift, however, further evidence indicated it is likely not the case (18, 170, 171).

### **1.5.3 FCoV and the immune system**

In addition to viral mutations, the host immune response has been shown to be a strong contributing factor to the resistance to, or development of, FIP. In particular, cell-mediated immunity appears to be vital in preventing FIP development in cats infected with FCoV. Studies have found that healthy cats in endemic environments of FIP, or healthy cats with systemic FCoV infection have higher levels of circulating FCoV-specific T cell populations, as well as follicular hyperplasia in lymph nodes (172-175). Alternatively, one of the most prominent changes noted in

cats that develop FIP is a marked T cell depletion, and lymphatic tissue changes such as reduced T cell zones (172, 176).

Humoral immunity has also been shown to play a significant role in the development of FIP. The presence of FCoV specific antibodies has been shown to increase the susceptibility of developing FIP due to antibody dependent enhancement (177, 178). Additionally, FIP granulomatous lesions that have not yet undergone necrosis have been shown to have an ample population of B cells and plasma cells producing coronavirus-specific antibodies, whereas T cell populations in these lesions remained scarce (179). Recurrent or persistent infection with FCoV has been correlated with the induction of high FCoV-specific antibody titers in cats, which could contribute to the development of FIP (172, 174).

IFN- $\gamma$  is a prominent cytokine in inflammatory processes and cell-mediated immunity. Studies have shown that clinically normal cats infected with FCoV typically have increased IFN- $\gamma$  mRNA expression in peripheral blood leukocytes (180, 181). In contrast, cats with FIP have shown low IFN- $\gamma$  expression in peripheral blood leukocytes, but strong expression within FIP lesions (182). It has been observed that cats living in catteries with a high prevalence of FIP cases had higher serum IFN- $\gamma$  levels compared to cats living in catteries with a low prevalence of FIP, further supporting that cats resistant to FIP have stronger IFN- $\gamma$  driven cell-mediated immunity than those that succumb to disease (183). Additionally, the emergence of a single FIP case within a previously unaffected cat population has been associated with increasing serum IFN- $\gamma$  levels in other cats in the cattery, potentially due to high levels CD8<sup>+</sup> T cells observed in asymptomatic FCoV-infected cats (184).

## **1.6 PD-1/PD-L1 axis**

### **1.6.1 Programmed death protein and its ligands**

Programmed death protein (PD-1) and its ligands (PD-L1 and PD-L2) are potent mediators of the cytotoxic T cell response. PD-1 is a type I membrane protein in the CD28 family and is expressed on both T cells and B cells, although its expression is more consistently observed on activated T cells (185-188). It is also found on other immune cells like NK cells, monocytes, and dendritic cells (185-188). PD-L1, which is expressed on most cell types, and PD-L2, which is primarily expressed on hematopoietic cells, are both members of the B7 family. Binding of PD-1 to one of its ligands inhibits the T cell receptor phosphorylation cascade, ultimately inhibiting T cell activity (185-188).

### **1.6.2 Factors in PD-L1 expression**

PD-L1 expression is largely controlled by inflammatory signaling (189). The most prominent inducer of PD-L1 expression is IFN- $\gamma$ , which is a crucial cytokine in inflammatory responses and tissue homeostasis (189). Upon binding to its receptor on the target cells, IFN- $\gamma$  signals through the JAK-STAT pathway, primarily via STAT1 to recruit various transcription factors (189). In PD-L1 expression, the interferon regulatory factor-1 (IRF-I) transcription factor appears to play a crucial role in IFN- $\gamma$  mediated PD-L1 expression (190). In addition to IFN- $\gamma$ , type I interferons IFN- $\alpha$  and IFN- $\beta$ , are capable of inducing PD-L1 in various cell types (191-193). While PD-L1 induction has been found to occur in the presence of various inflammatory cytokines, it is typically either variable between cell types or the mechanism of induction is unclear (194).

### **1.6.3 PD-1/PD-L1 in disease**

Alterations in PD-1/PD-L1 signaling have been linked to various disease processes including cancer, autoimmune conditions, and viral infections. In cancer and viral infections, upregulation of PD-L1 can provide a means of immune evasion of cancer cells and virus infected cells. Many cancerous cells have been found to upregulate PD-L1 to attenuate T cells in the tumor microenvironment and blockades of this signaling axis have been shown to restore T cell activity against tumor cells. Similarly, in viral infections, the upregulation of PD-1/PD-L1 provides a mechanism of evasion of effector T cells and can allow for persistent infections. Viruses including lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), influenza virus, herpes simplex virus (HSV), respiratory syncytial virus (RSV), hepatitis B virus (HBV), human cytomegalovirus (CMV), Rabies virus, Hanta virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have all been shown to employ the PD-1/PD-L1 axis as a mechanism of immune evasion (195-208). The use of checkpoint inhibitors in some of these viral infections, including LCMV, HSV, and SIV has been found to revive exhausted T cells and lower viral titers (209-212).

## **Chapter 2 - Host Susceptibilities and entry processes of SARS-CoV-2 Omicron variants using pseudotyped viruses carrying spike protein**

This chapter is derived from the following published article:

Zabiegala, A., Kim, Y., & Chang, K. O. (2025). Host susceptibilities and entry processes of SARS-CoV-2 Omicron variants using pseudotyped viruses carrying spike protein. *BMC Vet Res*, 21(1), 377. <https://doi.org/10.1186/s12917-025-04822-9>

Links:        PMCID: [PMC12108000](https://pubmed.ncbi.nlm.nih.gov/PMC12108000/)        DOI: [10.1186/s12917-025-04822-9](https://doi.org/10.1186/s12917-025-04822-9)

## **2.1 Abstract**

The zoonotic potential has been well studied for SARS-CoV-2 and its earlier variants, but the information for Omicron variants and SARS-CoV is lacking. In this study, we generated lentivirus-based pseudoviruses carrying spike protein (S) of SARS-CoV-2, parental and Omicron variants including BA.1.1, BA.4/5, XBB.1 and JN.1 to assess the entry into cells expressing human or animal ACE2 including dogs, cats, and white-tailed deer. Using these pseudoviruses, along with pseudoviruses carrying S of MERS-CoV and SARS-CoV, we assessed the protease processing of these various S through western blotting, entry/inhibition assays, and fusion assays. The results showed that overall, pseudotyped viruses carrying each S of SARS-CoV-2 Omicron strains efficiently entered cells expressing human or animal ACE2 comparably (BA.1.1 and JN.1) or better (BA.4/5 and XBB.1) than those with parental strain. In addition, the entries of pseudotyped viruses carrying S of SARS-CoV were also efficient in the cells expressing human or animal ACE2. The presence of TMPRSS2 significantly increased the entry of all tested pseudoviruses including those with S of MERS-CoV, SARS-CoV, and SARS-CoV-2, with BA.1.1, JN1, and XBB.1 Omicron having the largest fold increase. When cathepsin inhibitors were examined to assess their inhibitory effects on entry of parental and Omicron variants, they were significantly less effective in the entry of Omicron variants compared to parent strain, suggesting Omicron strains do not depend on the endosomal route compared to parental strain.

## **2.2 Introduction**

Coronaviruses are enveloped viruses that have a single stranded, positive-sense RNA genome in the Coronaviridae family. Many coronaviruses cause diverse diseases in a wide range of animals, including humans. Novel coronaviruses have periodically surfaced in human

populations from animal to human spillover events, which include severe acute respiratory syndrome coronavirus (SARS-CoV), Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV) and most recently SARS-related coronavirus 2 (SARS-CoV-2). SARS-CoV outbreaks that occurred in 2003 caused severe respiratory illness and spread from China to more than a dozen countries (213) before it was no longer reported since 2004 (89). MERS-CoV was first reported in Saudi Arabia in 2012 and has been mainly found in countries in and near the Arabian Peninsula with occasional outbreaks outside the Middle East (214). MERS-CoV transmission is mainly by direct or indirect contact with dromedary camels with limited person-to-person transmission (214). SARS-CoV and MERS-CoV have mortality rates of approximately 13% and 35%, respectively (215, 216). In late 2019, another novel coronavirus, SARS-CoV-2 unexpectedly emerged (217). Coronavirus disease (COVID-19), caused by SARS-CoV-2, is a mild to severe respiratory illness with an increased mortality in populations with underlying health conditions or of advanced ages (218). Unlike its two novel coronavirus predecessors, SARS-CoV-2 has rapidly spread worldwide resulting in the World Health Organization's declaration of the COVID-19 pandemic in March 2020. Since the first report of SARS-CoV-2, multiple antigenically distinct variants have emerged over the years, each time eventually replacing earlier variants. Compared to the parental reference Wuhan-Hu-1 strain, the Omicron variants have more than 30 mutations on the spike protein (S) and at least 15 mutations in the receptor-binding domain (RBD), which is substantially more than other variants of concern (82). The Omicron variant has continuously evolved since the emergence of the first dominant variant BA.1 (B.1.1.529.1) in late 2021 (219) and is composed of several major sub-lineages BA.1.1 (B.1.1.529.1.1), BA.2 (B.1.1.529.2), BA.3 (B.1.1.529.3), BA.4/5 (B.1.1.529.4/ B.1.1.529.5) and their recombinant variants such as XBB (220). JN.1, a more recent variant which was first

detected in August of 2023 (221), was the most prominent strain in the beginning of 2024 (222, 223). As of January 2025, the main SARS-CoV-2 circulating strains are descendants of the JN.1 subvariant (224).

Viral entry into host cells plays a key role in host susceptibility and tissue tropism. With SARS-CoV and SARS-CoV-2, the virus-cell interaction occurs between the spike protein (S) and the cell receptor angiotensin converting enzyme-2 (ACE2). Coronavirus S forms a trimer, and each monomeric S is composed of two parts, the S1 and S2 subunits. The S1 subunit contains the receptor binding domain (RBD) that directly interacts with ACE2, and the S2 subunit has the hydrophobic fusion peptide at the S2' site, which is implicated in viral fusion and entry. SARS-CoV-2 S has polybasic amino acids at the S1/S2 junction, which is subjected to cleavage by furin by the parental cell prior to release (reviewed in (225)). The polybasic cleavage site at the S1/S2 junction found in SARS-CoV-2, but not in SARS-CoV, has been implicated in increasing cleavage and virus transmissibility (reviewed in (225)), likely due to a more accessible RBD to ACE2 (48). During viral circulation, the S protein can adopt an “open” or “closed” position where the RBD is only accessible in the “open” position for binding of ACE2 or antibodies targeting the RBD (226, 227). Conformational change in the S following receptor binding allows S2' site cleavage by cellular proteases, such as transmembrane serine protease 2 (TMPRSS2), which exposes the fusion peptide to the cell membrane for direct fusion (4, 9, 49-51). Previous research demonstrated that SARS-CoV-2 enters cells via two distinct routes: when TMPRSS2 is present on the cell membrane, SARS-CoV-2 preferentially utilizes a direct entry route by fusion of the viral envelope to the cellular membrane, but in the absence of this protease, SARS-CoV-2 enter cells via an endosomal entry route where proteolytic processing of S is mediated by cathepsins

(68). These two distinct routes have also been described for SARS-CoV (228) and MERS-CoV (229).

As it became evident that SARS-CoV-2 was a global health threat, identification of animal species that are susceptible to this virus infection has become important to find potential animal reservoirs, which led to extensive research on the zoonotic risk of domestic and wild animals. Previous research has shown that many species are susceptible to SARS-CoV-2 infection in experimental settings and/or naturally, with or without clinical signs or virus shedding. Additionally, some animal species including white-tailed deer, mink, and hamsters have demonstrated animal-to-animal virus transmission (220). We and others have previously reported that SARS-CoV-2 interacts with ACE2 from various animal species using lentivirus-based pseudoviruses carrying the spike protein (S) of various SARS-2 strains and cells stably expressing animal ACE2 (225, 230). The animal ACE2s used in this study include human, horse, dog, cat, Syrian golden hamster, mink, white-tailed deer, bovine, and dromedary camel. As these studies have mainly focused on parental or earlier variants, in this study, we examined whether the mutations in the S of SARS-CoV-2 BA.1.1, BA.4/5, JN.1, or XBB.1 Omicron subvariants change viral entry efficiency in cells expressing animal ACE2 compared to their infectivity of cells expressing human ACE2. We hypothesize that high rates of mutations present in S gene of Omicron subvariants significantly affect their binding capabilities and entries to the cells expressing various animal ACE2. We also examined the entry of pseudovirus carrying SARS-CoV S, which also utilizes ACE2 as the receptor, in these cell lines. To investigate the differences in protease activity on S of SARS-CoV, MERS-CoV, and SARS-CoV-2 parental and Omicron subvariants, we performed western blots of concentrated pseudoviruses, entry assays of the pseudoviruses into cells expressing the appropriate functional receptor (ACE2 for SARS-CoV

and SARS-CoV-2 S and DPP4 for MERS-CoV S) with or without TMPRSS2, and fusion studies of each parental S and its respective functional receptor with or without TMPRSS2.

## **2.3 Materials and methods**

### **2.3.1 Cells and Plasmids**

Human embryonic kidney 293T (HEK293T) and Crandell-Rees feline kidney (CRFK) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) or Eagle's Minimal Essential Medium (MEM) supplemented with 10% or 5% fetal bovine serum respectively, 100 U/ml penicillin and 100 µg/ml streptomycin. Generation of the CRFK cells stably expressing ACE2 from human, horse, dog, cat, Syrian golden hamster, mink, white-tailed deer, bovine and dromedary camel was previously reported by our lab (230). Generation of the plasmid expressing the S of SARS-CoV-2 parental strain, designated as pAbVec-SARS2-S, was also reported by us (230). The plasmids expressing the S of SARS-CoV, MERS-CoV, Omicron BA.1.1, Omicron XBB.1, and Omicron JN.1 subvariants were purchased from Sino Biologicals (Wayne, PA) and the plasmid expressing Omicron BA.4/5 was obtained from InvivoGen (San Diego, CA). The mutations found in the RBD of SARS-CoV-2 Omicron variants compared to parental SARS-CoV-2 RBD (231-233) are shown in Table 3. The plasmids carrying S of MERS-CoV or HCoV-EMC/2012 were cloned into the pSI plasmid after the full-length, codon-optimized S gene of each virus was synthesized from Integrated DNA technology (Coralville, IA) for this study.

**Table 3 Mutations found in the RBD of SARS-CoV-2 Omicron variants compared to parental SARS-CoV-2 RBD**

Mutations found in all four omicron strains are indicated by bolded text, and residues that form hydrogen bonds between parental SARS-CoV-2 parental S and human ACE2 are underlined (231-233).

| Omicron BA.1.1      | Omicron BA.4/5      | Omicron JN.1        | Omicron XBB         |
|---------------------|---------------------|---------------------|---------------------|
| <b>G339D</b>        | <b>G339D</b>        | I332V               | <b>G339H</b>        |
| R346K               | S371F               | <b>G339H</b>        | R346T               |
| S371L               | <b>S373P</b>        | K356T               | L368I               |
| <b>S373P</b>        | <b>S375F</b>        | S371F               | S371F               |
| <b>S375F</b>        | T376A               | <b>S373P</b>        | <b>S373P</b>        |
| <u><b>K417N</b></u> | D405N               | <b>S375F</b>        | <b>S375F</b>        |
| <b>N440K</b>        | R408S               | T376A               | T376A               |
| <u>G446S</u>        | <u><b>K417N</b></u> | R403K               | D405N               |
| <b>S477N</b>        | <b>N440K</b>        | D405N               | R408S               |
| <b>T478K</b>        | L452R               | R408S               | <u><b>K417N</b></u> |
| E484A               | <b>S477N</b>        | <u><b>K417N</b></u> | <b>N440K</b>        |
| <u>Q493R</u>        | <b>T478K</b>        | <b>N440K</b>        | V445P               |
| <u>G496S</u>        | E484A               | V445H               | G446S               |
| <u><b>Q498R</b></u> | F486V               | <u>G446S</u>        | N460K               |
| <u><b>N501Y</b></u> | <u><b>Q498R</b></u> | N450D               | <b>S477N</b>        |
| <u><b>Y505H</b></u> | <u><b>N501Y</b></u> | L452W               | <b>T478K</b>        |
|                     | <u><b>Y505H</b></u> | L455S               | E484A               |
|                     |                     | N460K               | F486S               |
|                     |                     | <b>S477N</b>        | F490S               |
|                     |                     | <b>T478K</b>        | <u><b>Q498R</b></u> |
|                     |                     | N481K               | <u><b>N501Y</b></u> |
|                     |                     | Δ483                | <u><b>Y505H</b></u> |
|                     |                     | E484K               |                     |
|                     |                     | F486P               |                     |
|                     |                     | <u><b>Q498R</b></u> |                     |
|                     |                     | <u><b>N501Y</b></u> |                     |
|                     |                     | <u><b>Y505H</b></u> |                     |

### 2.3.2 Generation of pseudoviruses

Pseudotyped viruses containing the S genes of interest were generated using a second-generation lentiviral packaging system with a packaging plasmid psPAX2 (Addgene, Watertown, MA), a reporter plasmid pLenti-luc (Addgene, Watertown, MA), and an envelope plasmid carrying the S gene of SARS-CoV, MERS-CoV, or SARS-CoV-2 (parental, Omicron BA.1.1, BA.4/5, JN.1, or XBB.1 variant) as previously described by us (230). Briefly, one-day old

HEK293T cells in a 6-well plate were transfected with 1 ug of PsPAX2, 1 ug pLenti-luc and 100 ng to 1 ug S using Lipofectamine 2000 (Thermo Fisher, Waltham, MA). Based on transfection efficiency with S expression, 100 ng per well was used for Omicron BA.1.1, Omicron JN.1, and Omicron XBB.1 S while 1 ug was used for SARS-CoV-2 parental and Omicron BA.4/5 S, SARS-CoV S, and MERS-CoV S. The cells were then incubated at 37 °C for 48 hrs and the supernatant containing pseudoviruses was then collected. Cell debris was removed by centrifugation at 400 x g for 10 min before storage at -80°C. Pseudovirus titers were determined using a Lenti-X p24 Rapid Titer Kit (Takara, San Jose, CA) (230).

### **2.3.3 Western blot analysis of pseudoviruses**

Western Blot analysis was performed for pseudoviruses containing the S gene of parental strain of SARS-CoV-2, SARS-CoV, and MERS-CoV, and the Omicron BA.1.1, BA.4/5, JN.1, or XBB.1 strain of SARS-CoV-2. Pseudoviruses were concentrated by ultracentrifugation with 30% sucrose cushion at 100,000 X g for 2 hrs. Virus pellets were resuspended at 100-fold concentrated volume, and proteins were resolved in a 10% Novex Bis-Glycin gel (Thermo Fisher, Waltham, MA) and transferred to a nitrocellulose membrane. The membrane was incubated with convalescent human serum against SARS-CoV-2 (230) or anti-FLAG antibodies for SARS-CoV and MERS-CoV (Abcam Fremont, CA). Chemiluminescent signals were visualized by FOTO/Analyst Luminary/FX Systems (Fotodyne Inc, Harland, WI).

### **2.3.4 Pseudovirus entry assay**

Entry efficiencies of SARS-CoV-2, parental or Omicron variants, or SARS-CoV into cells expressing animal ACE2 were evaluated and compared to the entry into cells expressing human

ACE2 using the pseudovirus entry assay in CRFK cells expressing ACE2 from human, horse, dog, cat, Syrian golden hamster, mink, white-tailed deer, bovine or dromedary camel. Briefly, one-day old CRFK cells expressing each ACE2 in a 24 well plate were transduced with the pseudoviruses carrying S of SARS-CoV, SARS-CoV-2 parental or Omicron BA.1.1, BA.4/5, JN.1, or XBB.1 subvariants and further incubated for 30 hrs. Cell lysates were prepared and firefly luciferase activity was determined using the luciferase reporter assay system (Promega, Madison, WI) on a luminometer (GloMax® 20/20 Luminometer, Promega, Madison, WI) following the manufacturer's direction.

The effects of TMPRSS2 on the entry of the pseudovirus containing the aforementioned S genes were determined by transducing each in HEK293T cells expressing ACE2 or DPP4 with or without TMPRSS2. Briefly, one-day old HEK293T cells were transfected with pIRES-Neo-hACE2-FLAG (230) or pCMV6-hDPP4 (Origene, Rockville, MD) with or without pCMV6-hTMPRSS2 (Origene). The plasmid expressing Renilla luciferase, pRL-CMV (Promega), was included as an expression control. The firefly luciferase activity was normalized to the Renilla luciferase activity.

### **2.3.5 Fusion Assays**

To assess the roles of TMPRSS2 in the S-mediated entry of SARS-CoV, parental SARS-CoV-2, or MERS-CoV, a fusion assay was performed. HEK293T cells at 70% confluency were transfected with plasmids expressing S of SARS-CoV, parental SARS-CoV-2, or MERS-CoV, and the respective functional receptor with or without TMPRSS2. For SARS-CoV and SARS-CoV-2 fusion studies, pIRES-Neo-hACE2-FLAG plasmid (230) and an aforementioned plasmid expressing S of SARS-CoV (pCVM3-SARS-CoV-S) or SARS-CoV-2 (pAbVec-SARS2-S) with

or without the TMPRSS2 plasmid (pCMV6-hTMPRSS2) were transfected as described above. For MERS-CoV, a plasmid expressing DPP4 (pCMV6-hDPP4) and MERS-CoV S with or without the TMPRSS2 plasmid (pCMV6-hTMPRSS2) were transfected. Twenty-four hrs post-transfection, the cells were fixed in 4% paraformaldehyde, stained with Hematoxylin and Eosin (#ab2458807, Abcam), and mounted in glycerol. Images were acquired at 40x magnification using an SDL camera through a Zeiss Axiovert 200M microscope. The number of nuclei involved in syncytia formation at 20x magnification were manually counted over 5 fields of view and the average over the 5 fields was calculated per sample.

### **2.3.6 Cathepsin inhibitor studies**

Well-known cathepsin L inhibitors including MDL28170 and Z-FL-CHO (both from Sigma-Aldrich, St. Louis, MO) were tested against Omicron strains compared to parental strain using the pseudovirus entry assays in CRFK cells expressing hACE2. The enzyme assay of cathepsin L inhibition was done with cathepsin L inhibitor kit from Abcam (Waltham, MA) per manufacturer's instruction. The cell entry inhibition assay with pseudotyped virus carrying S of SARS-CoV-2 (parental, BA.4/5, XBB.1, JN.1, and BA.1.1) was performed in CRFK cells expressing hACE as previously described (234, 235). In this assay, the cells were incubated with DMSO (0.1%) or serial dilutions of MDL28170 or Z-FL-CHO immediately after cells were transduced with the pseudotyped virus carrying each S. After the transduction, cells were incubated for 48 hrs, and luciferase activities were measured. The dose-dependent inhibition curve for each compound was prepared for both enzyme and cell-based assays, and the 50% effective concentration (IC<sub>50</sub> for enzyme assay and EC<sub>50</sub> for cell-based assay) values were determined by GraphPad Prism software using a variable slope (GraphPad, La Jolla, CA).

### **2.3.7 Statistical analysis**

Data consists of at least three independent experiments. Statistical analysis was performed using GraphPad Prism software version 9.5 (San Diego, CA). A two-tailed Wilcoxon matched-pairs signed rank test was performed to assess the change in entry of SARS-CoV, SARS-CoV-2 parental, and SARS-CoV-2 Omicron BA.1.1, BA.4/5, JN.1, and XBB.1 into the different ACE2 expressing cell lines. A one-tailed Mann-Whitney test was used to assess the effect of TMPRSS2 expression on the parental pseudoviruses when compared to the functional receptor alone and to assess the effect on TMPRSS2 expression of number of nuclei present in syncytia formation in the fusion assays.

## **2.4 Results**

### **2.4.1 Entry efficiencies of pseudoviruses carrying S of SARS-CoV-2 Omicron strains or SARS-CoV compared to SARS-CoV-2 parental strain in cells expressing human or animal ACE2**

When the entry efficiencies of SARS-CoV-2 parental strain in cells expressing animal ACE2 were compared to human ACE2, there was a significant increase in the pseudovirus entry in cells expressing horse (3.6-fold), dog (3.39-fold), cat (2.47-fold), hamster (8.93-fold), rabbit (11.39-fold), white-tailed deer (4.09-fold), and cow (3.82-fold) ACE2 compared to human ACE2. In addition, there was a significant decrease in entry into mink ACE2 (0.35-fold) and no significant difference in camel ACE2 compared to human ACE2 (Fig. 2.1A). For Omicron strains, cells expressing cow or camel ACE2 were highly susceptible for the entries of all four Omicron strains compared to human ACE2. On the other hand, entries of all four pseudotyped viruses

carrying S were decreased in cells expressing mink ACE2 compared to human ACE2 (Fig. 2.1B-E). Overall, BA.4/5 and XBB.1 have similar trends as parental strain, showing there were increased entries to horse, dog, cat, WTD, cow and camel ACE2, decreased entries to mink ACE2 compared to human ACE2 (Fig. 2.1C and E). For BA.1.1 and JN.1, while there were increased entries to cow and camel ACE2, there was decreased entry into cells expressing horse, dog, and mink ACE2 compared to human ACE2 (Fig. 2.1B and D).

The pseudoviruses carrying S of SARS-CoV efficiently entered cells expressing human or animal ACE2 at comparable levels to S of SARS-CoV-2 (Fig. 2.2A). When the entry efficiencies of SARS-CoV in cells expressing animal ACE2 were compared to human ACE2 (Fig. 2.2B), there was a significant increase of entry in dog (2.4-fold), cat (6.29-fold), hamster (6.58-fold), white-tailed deer (4.22-fold), cow (4.64-fold), and camel (16.58-fold) ACE2 expressing cells. While there was a significant decrease in mink ACE2 expressing cells, no significant change was observed in horse ACE2 compared to human ACE2 (Fig. 2.2B). None of the tested pseudoviruses showed entry capabilities into CRFK cells lacking exogenous ACE2.

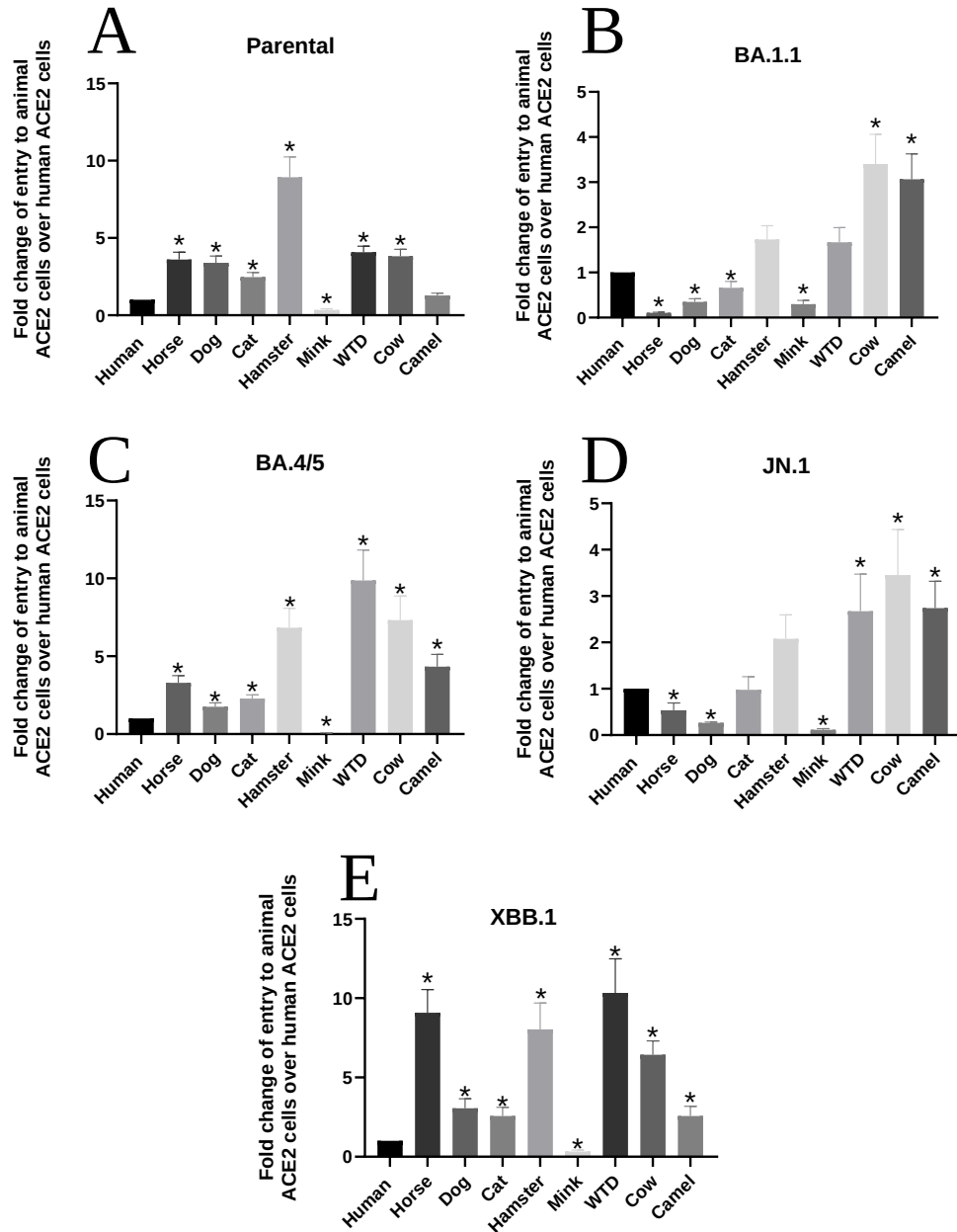


**Figure 2.1 Entry of pseudoviruses carrying S into cells expressing human or animal ACE2**  
 Entry of pseudovirus carrying S of SARS-CoV-2 parental (A) or Omicron subvariants BA.1.1 (B), BA.4/5 (C), JN.1 (D), or XBB.1 (E) into cells expressing human or animal ACE2. The entry of each pseudovirus into human, horse, dog, cat, hamster, mink, white-tailed deer (WTD), cow, or camel ACE2 expressing cells was measured using a Luciferase reporter system. Fold changes of entry into cells expressing each animal ACE2 were compared to cells expressing human ACE2. Statistical differences are indicated with an asterisk (\*,  $P < 0.05$ ).

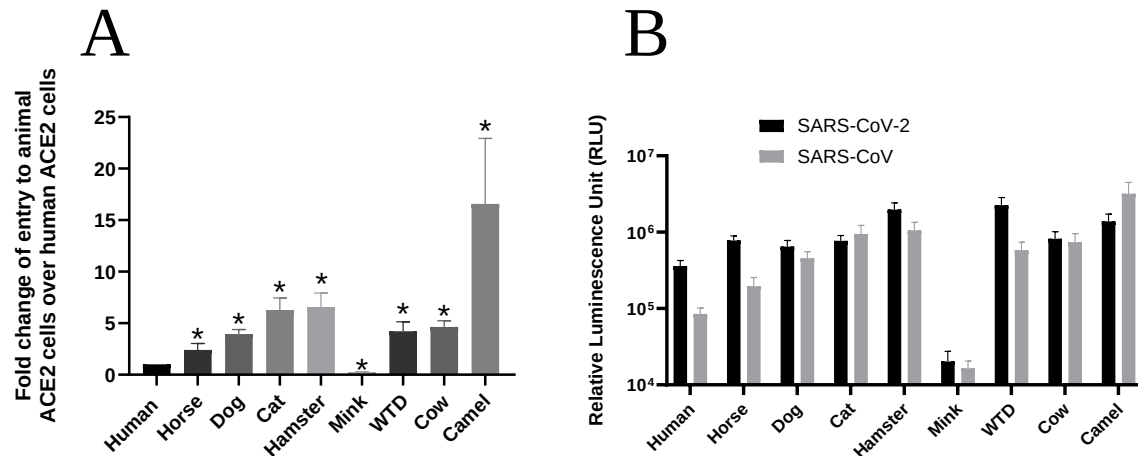


**Figure 2.2 Entry of pseudoviruses carrying S of SARS-CoV into cells expressing human and animal ACE2**

The entry of each pseudovirus into human, horse, dog, cat, hamster, mink, white-tailed deer (WTD), cow, or camel ACE2 expressing cells was measured using a Luciferase reporter system. (A) Entry of SARS-CoV as compared to SARS-CoV-2 in each cell type. (B) Fold changes of entry into each cell types were compared to cells expressing human ACE2. Statistical differences are indicated with an asterisk (\*,  $P < 0.05$ ).

#### 2.4.2 Protease activity on the S1/S2 and S2' cleavage sites of S of SARS-CoV, MERS-CoV, and SARS-CoV-2 parental strain and Omicron subvariants

The S1/S2 and S2' cleavage sites of SARS-CoV, MERS-CoV, and SARS-CoV-2 parental and select Omicron variants are shown in Fig. 2.3A, with the basic residues near each cleavage site highlighted in yellow. Western Blot analysis (Fig. 2.3B) was performed to assess processing of the S1/S2 cleavage site of SARS-CoV, MERS-CoV, and SARS-CoV-2 parental strain and Omicron subvariants BA.1.1, BA.4/5, JN.1, and XBB.1 spike protein using concentrated pseudoviruses. Band sizes of approximately 180 or 100 kDa correspond to full-length, uncleaved S or cleaved S2 domain, respectively. While SARS-CoV S remained primarily unprocessed, a majority of MERS-CoV S were processed prior to release from the parental cell, consistent with

the presence or absence of the multibasic cleavage site at S1/S2 in MERS-CoV S and SARS-CoV S respectively. Interestingly, while SARS-CoV-2 contained a multibasic cleavage site promoting S1/S2 processing by host furin, only approximately half of parental SARS-CoV-2 S was processed, whereas a majority of the Omicron subvariants were almost entirely cleaved.

To understand the differences in TMPRSS2 processing of these various S, HEK293T cells expressing human ACE2 (or DPP4 for MERS-CoV pseudoviruses) with and without TMPRSS2 were transduced with pseudoviruses carrying S of SARS-CoV, MERS-CoV, or SARS-CoV-2 parental strain and Omicron subvariants BA.1.1, BA.4/5, JN.1, and XBB.1 (Fig. 2.3C). The entry of all pseudoviruses assessed significantly increased in the presence of TMPRSS2, though at varying levels. The presence of TMPRSS2 was shown to significantly increase SARS-CoV by approximately 10-fold, MERS-CoV by approximately 46-fold, SARS-CoV-2 parental strain by approximately 34-fold, and Omicron subvariants BA.1 by approximately 181-fold, BA.4/5 by approximately 35-fold, JN.1 by approximately 232-fold, and XBB.1 by approximately 108-fold. None of the tested pseudoviruses showed entry into HEK293T cells lacking exogenous ACE2 or DPP4.

In addition to entry studies, fusion assays were performed using S of parental SARS-CoV-2, SARS-CoV, and MERS-CoV (Fig. 2.4A). HEK293T cells co-transfected with S from parental SARS-CoV-2, SARS-CoV, or MERS-CoV and their functional receptor with or without TMPRSS2 were used to assess the fusion capabilities of each S. The numbers of nuclei involved in syncytia formation per 20x view was counted over five different fields of view and averaged for the comparative analysis. In cells co-transfected with parental S of SARS-CoV-2 or SARS-CoV with ACE2 alone, expression of both proteins resulted in efficient syncytia formation with counted nuclei at an average of 32.5 and 20.8 respectively. For cells co-transfected with S of

MERS-CoV and DPP4, little fusion was observed with an average of 2.8 nuclei in syncytia formation. When these cells also expressed TMPRSS2, the average numbers of nuclei in syncytia per field were significantly increased for SARS-CoV-2 (70.6 nuclei), SARS-CoV (65.4 nuclei), or MERS-CoV S (10.8 nuclei). Representative fusion images can be seen in Fig. 2.4B.

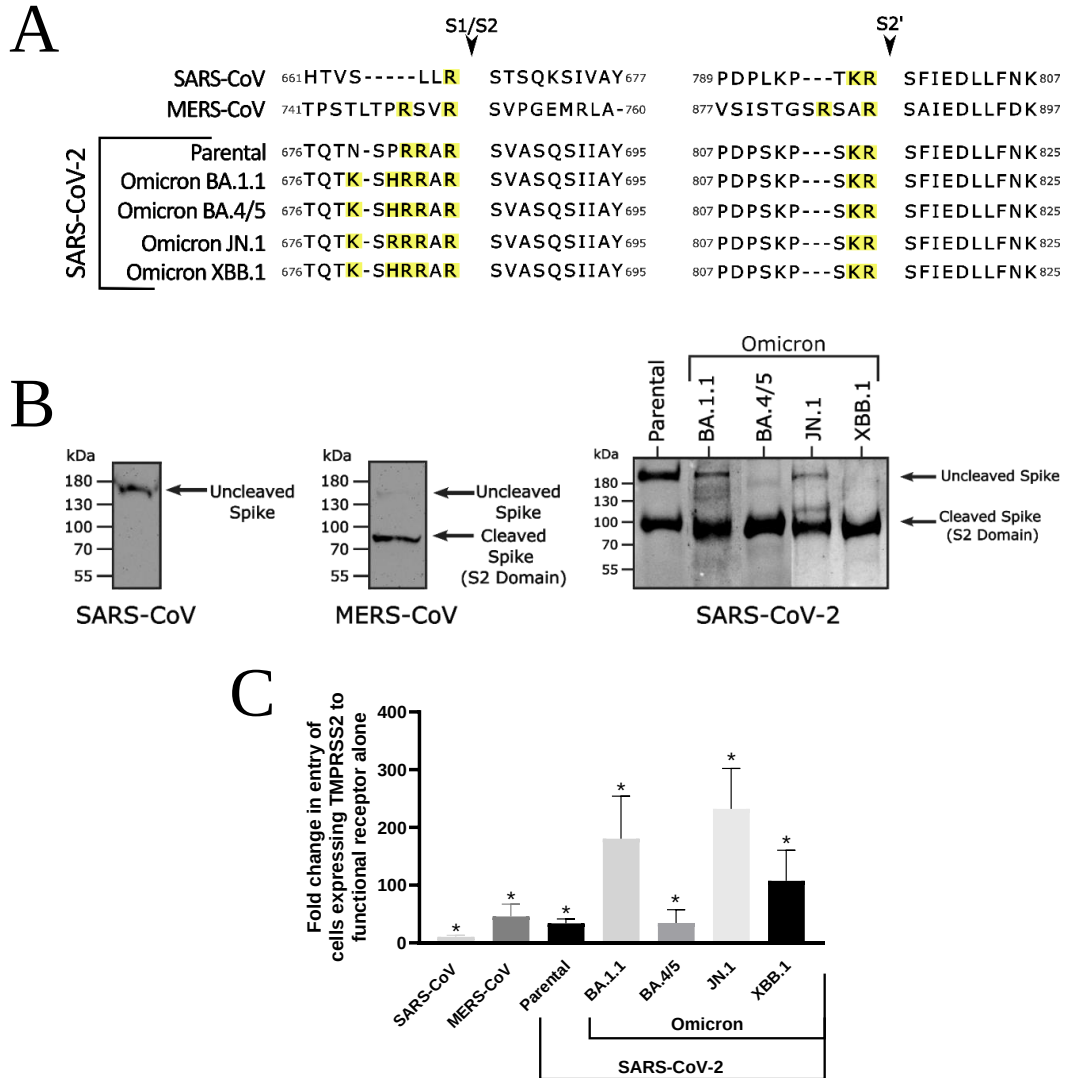
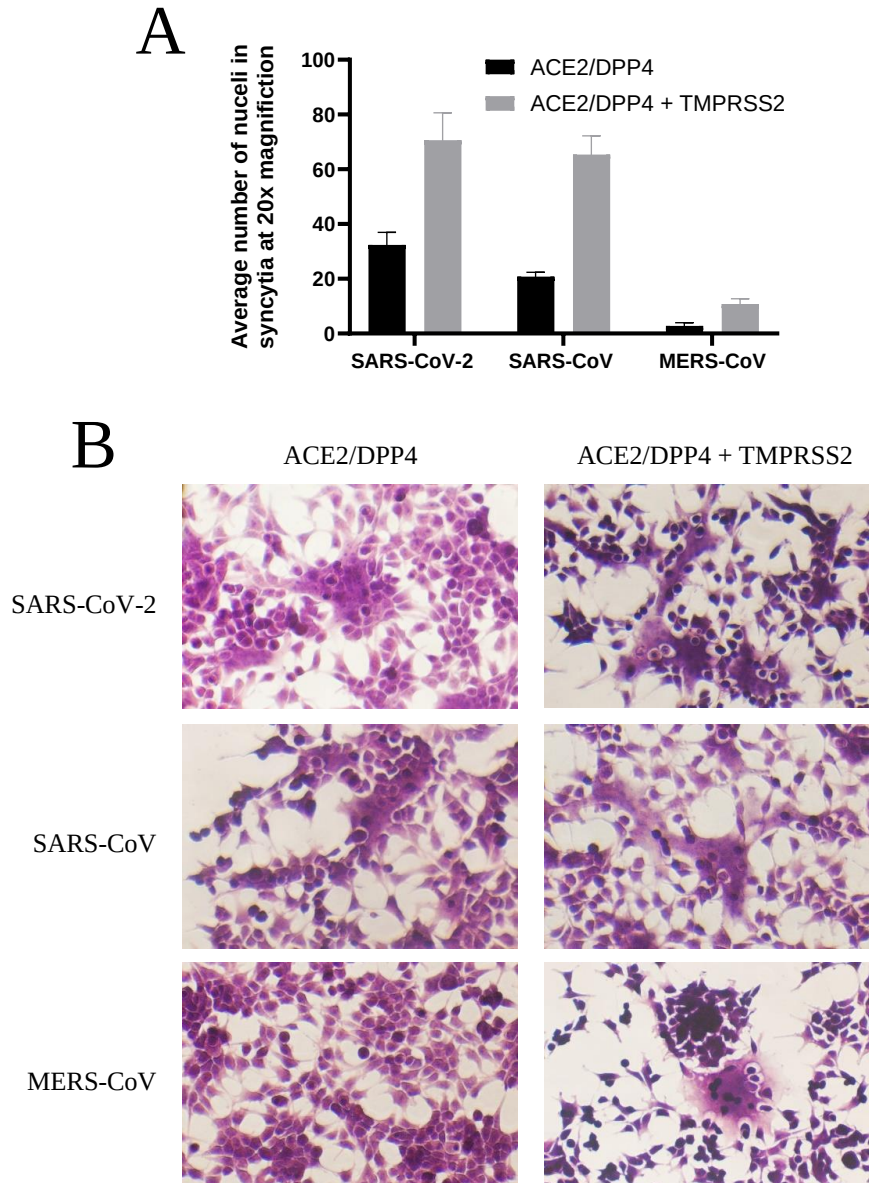


**Figure 2.3 Protease activity against S of SARS-CoV, MERS-CoV, and SARS-CoV-2**

(A) Sequences of the S1/S2 junction and S2' cleavage site of S from SARS-CoV, MERS-CoV, and SARS-CoV-2 parental and Omicron BA.1.1, BA.4/5, JN.1, and XBB.1. Basic residues near each cleavage sight are shown in yellow highlights. (B) Western blot analysis of S cleavage from concentrated pseudoviruses. Full-length blots can be found in the supplementary materials (Supplementary figure 1). (C) The entry of pseudoviruses expressing S of SARS-CoV and SARS-CoV-2 (parental and Omicron subvariants) into cells expressing the functional receptor ACE2 with or without TMPRSS2, and the entry of pseudoviruses expressing MERS-CoV S into cells expressing the functional receptor DPP4 with or without TMPRSS2. Asterisks show statistical differences ( $p < 0.05$ ) of the entry in the presence of TMPRSS2.



#### Figure 2.4 Fusion assay results

(A) The average numbers of nuclei in syncytia per 20x field are shown for parental S of SARS-CoV-2, SARS-CoV, and MERS-CoV. (B) Representative fusion images with the H&E stain after co-transfection with S from SARS-CoV-2, SARS-CoV, or MERS-CoV and ACE2/DPP4 with or without TMPRSS2 shown at 40x magnification.

### 2.4.3 Effects of cathepsin L inhibitors on the entry of Omicron strains compared to parental strain in the pseudotype virus assay

In the absence of TMPRSS2, SARS-CoV-2 enters the cell through an endosomal route and S is processed by host cathepsins, primarily Cathepsin L. Both MDL28170 and Z-FL-CHO were highly potent against cathepsin L with IC<sub>50</sub>, 0.01 or 0.015  $\mu$ M in the enzyme assay, respectively (Table 4). In the virus entry assay, while both inhibited the entry of pseudotyped viruses carrying S from parental or omicron SARS-CoV-2, there was greater inhibition against parental compared to omicron strains (> 10-fold,  $p < 0.01$ ) (Table 4). The EC<sub>50</sub> of MDL28170 against parental SARS-CoV-2 was approximately 0.017  $\mu$ M, while the EC<sub>50</sub> for BA.1.1, BA.4/5, JN.1, and XBB.1 was approximately 0.36, 0.34, 0.30, and 0.36  $\mu$ M, respectively. Similarly, the EC<sub>50</sub> of Z-FL-CHO against SARS-CoV-2 was approximately 0.023, while the EC<sub>50</sub> of BA1.1, BA.4/5, JN.1, and XBB was 0.34, 0.41, 0.37, and 0.42, respectively.

**Table 4 Effects of cathepsin L inhibitors on the entry Omicron strains in the pseudotype virus assay expressing S**

|                                                                      | EC <sub>50</sub> ( $\mu$ M)<br>Virus entry assay |                  |                  |                  |                  | IC <sub>50</sub> ( $\mu$ M)<br>Enzyme assay |
|----------------------------------------------------------------------|--------------------------------------------------|------------------|------------------|------------------|------------------|---------------------------------------------|
|                                                                      | Parental Wu                                      | BA45             | XBB              | JN-1             | B11              | Cath L                                      |
| MDL28170                                                             | 0.01 $\pm$ 0.16                                  | 0.34 $\pm$ 0.13* | 0.36 $\pm$ 0.05* | 0.30 $\pm$ 0.03* | 0.36 $\pm$ 0.01* | 0.01 $\pm$ 0.03                             |
| Z-FL-CHO                                                             | 0.02 $\pm$ 0.01                                  | 0.41 $\pm$ 0.04* | 0.42 $\pm$ 0.01* | 0.37 $\pm$ 0.02* | 0.34 $\pm$ 0.04* | 0.015 $\pm$ 0.02                            |
| Numbers are mean $\pm$ SD of three independent results. * $p < 0.05$ |                                                  |                  |                  |                  |                  |                                             |

## 2.5 Discussion

Previous research using pseudoviruses carrying S of SARS-CoV-2 and cells expressing human or animal ACE2 has shown that several mutations in the RBD in variant strains can alter viral entry (236-238). Omicron variants have accumulated more mutations throughout of the genome including S (Table 3) compared prior variant types. Many studies have been focused on the biological relevance of the mutations influencing the receptor bindings. It was shown that some mutations in S of Omicron variants including K417N (BA.1.1, BA.4/5 and JN.1), G446S (BA.1.1), E484A (BA.1.1 and BA.4/5), G496S (BA.1.1), and Y505H (BA.1.1, BA.4/5 and JN.) were associated with decreased binding affinity to human ACE2, while mutations such as S477N (BA.1.1, BA.4/5 and JN.1), and N501Y (BA.1.1, BA.4/5 and JN.1) increased the binding affinity in a compensatory manner (239). In addition, an increase in the amount of positively charged amino acids in the RBD of Omicron variants has been found to decrease the stability of S trimer in the closed position, causing them to adopt an open position more frequently than the parental strain (227, 240). It was also shown that although K417N mutation conserved in the Omicron variants decreases a binding efficiency to ACE2, it increases the stability of an open conformation which overall contributes to effective binding (237).

We have previously reported the entry of pseudoviruses carrying S of SARS-CoV-2 parental strain and variants of concern (VOC) including alpha, beta and delta variants into cells expressing human or animal (domestic and wild) ACE2 (230). In this study, we extended to the studies with S from Omicron variants including BA.1.1, BA.4/5, JN.1, and XBB.1. The results showed that the overall entry patterns to cells expressing each animal ACE2 of Omicron BA.4/5 and XBB.1 were similar to each other and to parental strain (Fig. 2.1A, C and E). In addition, overall entry patterns to cells expressing each animal ACE2 of Omicron BA1.1 and JN.1 were

similar to each other (Fig. 2.1B and D). Among the cells expressing animal ACE2, cow and camel ACE2 were efficiently mediated the entries of all tested Omicron strains compared to human ACE2 (Fig. 2.1B-E). On the other hand, the entries of all tested Omicron strains were decreased to mink ACE2 compared human ACE2 (Fig. 2.1B-E). This is consistent with our lab's previous work (230) as well as a report from Damas et al. (241) predicting weak binding of SARS-CoV-2 S to mink ACE2 using in silico analysis. It is not clear why there is the discrepancy between natural infections in minks and poor entry with mink ACE2. It is possible that additional host proteins are required for efficient entry for minks. There were variable entry efficiencies into cells expressing horse, dog, cat, hamster and WTD ACE2 among Omicron strains (Fig. 2.1B-E). Li et al. (237) reported that S of Omicron BA.1 and BA.2 enhanced binding and entry efficiency with some animal ACE2, including rodents and bats, suggesting a potentially extended host range. The S of BA.4/5 are closely related to BA.2 with two additional substitutions in the RBD (L452R and F486V) and a 493Q that is a mutation reverted to the parental strain. Additional studies (238, 242, 243) showed that that Omicron BA.1, BA.2 and BA.3 have higher binding capacity to many animal ACE2 or increased entry compared to the parental strain using the pseudoviruses carrying S. Regarding BA.4/5, a binding assay study (238) showed that BA.4/5 share similar enhanced binding affinity to many animal ACE2 with BA.1-3. Motozono et al. (244) reported that S of BA.4/5 variant had increased stability in the open conformation by the L452R mutation which is not present in BA.1.1 or JN.1., and this may be the reason for increase of entries to cells expressing most of tested animal ACE2 in this study (Fig. 2.1C). There were efficient entries of the most pseudoviruses carrying parental or Omicron strain into cells expressing cow and horse ACE2, the species showing limited evidence of virus infection *in vivo* (131-134, 137, 138, 245, 246) suggesting there might be additional factors involved in viral replication and pathogenicity.

Both SARS-CoV and SARS-CoV-2 use ACE2 as the receptor for successful viral entry and infection. Since the last known human SARS-CoV cases in 2004, its zoonotic potential was studied with wild animals found at the live market in China, including palm civets (*Paguma larvata*), raccoon dogs (*Nyctereutes procyonoides*), and ferret badgers (*Meilogale*) (96), and domestic species including cats and ferrets (247, 248). Using the entry assay with cells expressing human or animal ACE2, we found that pseudoviruses carrying S of SARS-CoV entered the cells at efficiency comparable to those of SARS-CoV-2 (parental) in most tested cells (Fig. 2.2A and B). SARS-CoV and SARS-CoV-2 are Sarbecoviruses that use ACE2 as a cellular receptor and have a 75% amino acid homology in their S (249), thus it is not surprising that they have similar ACE2 bindings and entry capacities. It would be interesting to see whether this host susceptibility trend could be extended to other members of Sarbecoviruses that utilize ACE2 as a receptor. Our results indicated an efficient entry ability in many animal ACE2 expressing cells such as hamster, cat, and white-tailed deer, all of which have been shown to harbor natural infections (Table 2).

ACE2 is the receptor molecule that is indispensable for SARS-CoV-2 and SARS-CoV infection, whereas DPP4 is the necessary receptor molecule for MERS-CoV. It is believed that sequential proteolytic processing at the S1/S2 junction in the parental cell and the S2' cleavage site in the target cell is required for virus entry. Both S of SARS-CoV-2 and MERS-CoV contain polybasic amino acids at the S1/S2 junction (RRXR and RXXR, respectively), but SARS-CoV has a single Arg (Fig. 2.3A). The presence of polybasic residues at the S1/S2 junction is shown to allow processing by a wider range of cellular proteases such as furin, which efficiently cleave R-X-(R/K/X)-R sequence (250), and it was reported that furin efficiently cleaves the S1/S2 junction of S of SARS-CoV-2 (50, 251) and MERS-CoV (78). In our Western blot analysis, about half of S of SARS-CoV-2 parental strain pseudoviruses from HEK293T cells were

proteolytically processed, yielding an uncleaved band and a cleaved band that corresponds to the S2 domain (Fig. 2.3B). While the parental SARS-CoV-2 S was only approximately 50% cleaved, the Omicron subvariants were almost entirely cleaved (Fig. 2.3B). All Omicron spike proteins tested contain the mutations N679K and either P681H (BA.1.1, BA.4/5, XBB.1) or P681R (JN.1). These mutations create additional basic residues at the multibasic furin cleavage site (Fig. 2.3A). The P681H found in the Alpha and Mu variants as well as the P681R mutation found in the Delta variant have been found to increase processing at the S1/S2 site, with more cleavages with the mutation of P681R than P681H (252-254). Additionally, the N679K mutation has also been attributed to more cleavages by furin in fluorogenic cleavage assays, lending to improved cleavage of S1/S2 compared to previous variants only containing a mutation at P681 (255). Similar to the Omicron subvariants, MERS-CoV S, which also contains a multibasic sequence at S1/S2, was majorly processed. Interestingly, though there are fewer basic residues at the S1/S2 cleavage site than the parental SARS-CoV-2 S, MERS-CoV-S appeared to be primarily cleaved (Fig. 2.3B). In contrast, the majority of S of SARS-CoV remained intact (Fig. 2.3B), as SARS-CoV S lacks the residues necessary for furin processing, which is consistent with previous reports (57, 59).

While the processing of S1/S2 occurs in the parental cell prior to release, it is believed that following the binding of SARS-CoV, MERS-CoV, and SARS-CoV-2 to their respective functional receptor, these coronaviruses can utilize TMPRSS2-mediated entry via fusion at the cell membrane or endosomal pathway in TMPRSS2-negative cells (57, 68). It is speculated that furin-mediated cleavage of the S1/S2 junction works in concert with TMPRSS2 to promote viral entry (251, 256). The aim of our study was to understand the extent to which TMPRSS2 acts on S of these coronaviruses in comparison to one another using HEK293T cells expressing ACE2

for SARS-CoV and SARS-CoV-2 and DPP4 for MERS-CoV with or without TMPRSS2. As expected, entry of these coronaviruses all significantly increased when TMPRSS2 was expressed, though at varying magnitudes. Consistent with the speculation that TMPRSS2 works in conjunction with furin cleavage, SARS-CoV, which is majorly uncleaved at S1/S1 (Fig. 2.3B), had the lowest fold increase in entry (10-fold) with TMPRSS2 expression (Fig. 2.3C). SARS-CoV-2 parental strain, in which only approximately 50% of S is cleaved (Fig. 2.3B), had a moderate increase in fold change when TMPRSS2 was expressed (34-fold) as opposed to when only ACE2 was expressed (Fig. 2.2C). The spike proteins of MERS-CoV and SARS-CoV-2 Omicron BA.1.1, BA.4/5, JN.1, and XBB.1 were all majorly cleaved at S1/S2 in the parental cell (Fig. 2.3B). Interestingly, while Omicron BA.1.1, JN.1, and XBB.1 pseudoviruses showed a marked increase in entry with the presence of TMPRSS2 (181-fold, 232-fold, and 108-fold respectively), the increase in entry for MERS-CoV and SARS-CoV-2 Omicron BA.4/5 was more moderate (46-fold and 35-fold respectively)(Fig. 2.3C).

Fusogenicity is implicated in virulence and immune evasion of coronaviruses, and the ability of SARS-CoV, SARS-CoV-2, and MERS-CoV S to mediate cell-to-cell fusion has been independently demonstrated both *in vitro* and *in vivo* (257). TMPRSS2 expression has been linked to viral-induced syncytia formation, which is thought to contribute to viral pathogenicity *in vivo*. Infected syncytia is a common pathological finding in lung tissues of patients infected with SARS-CoV-2 (258). When Iwata-Yoshikawa et al. (259) inoculated TMPRSS2 knockout mice with various strains of SARS-CoV-2, they showed significant reduction in viral load and pathological lung changes compared to their wild-type counterparts. Additionally, they found the use of TMPRSS2 inhibitor Nafamostat significantly reduced the viral infectivity *in vivo*. Hampering both the direct membrane fusion entry route and the cell-to-cell transmission that

would occur during fusion events makes TMPRSS2 inhibitors an attractive therapy for SARS-CoV-2 treatment. Our goal in this study was to comparatively assess the utilization of TMPRSS2 in fusogenicity of SARS-CoV, SARS-CoV-2, and MERS-CoV in relation to each other, and to our entry study. To study this, we co-transfected each S and respective receptor with or without TMPRSS2. In agreement with the entry studies, in the presence of TMPRSS2, more syncytia were formed by S of SARS-CoV-2, SARS-CoV, and MERS-CoV. The S of SARS-CoV-2 or SARS-CoV resulted in more robust syncytia formation than S of MERS-CoV with or without TMPRSS2 (Fig. 2.4A). Less syncytia formation by S of MERS-CoV may be due to the usage of different receptor from S of SARS-CoV/SARS-CoV-2.

There are numerous evidences (260, 261) showing SARS-CoV-2 Omicron strains are more infectious with the improved binding to ACE2 (262), and less virulent (260, 261) compared to earlier strains. Some early studies showed that SARS-CoV-2 Omicron strains were less likely rely on TMPRSS2 than parental strain for viral entry (262, 263). Peacock et al. (264) showed that SARS-CoV-2 Omicron strains were found to replicate efficiently in nasal epithelium, which lacks TMPRSS2. However, other studies showed the important role of TMPRSS2 for Omicron strains *in vitro* (265) and *in vivo* (259, 266). In TMPRSS2 knockout animals, the infection of SARS-CoV-2 Omicron strain resulted in severe loss of infection and pathogenicity (259, 266). Regarding endosome-mediated entry of Omicron strains, Sakurai et al (267) reported SARS-CoV-2 Omicron variant progressively adapted to use the endosome route by comparing the inhibitory activity of E64d (cathepsin L inhibitor) between a Delta strain and several Omicron strains. In this study, we used two cathepsin L inhibitors to examine their effects on virus entry. There were significant differences (greater than ten-fold) in EC50 of MDL28170 and Z-FL-CHO against Omicron strains compared to parental strain (Table 4), suggesting Omicron strains do not depend on the

endosomal route compared to parental strain. It is possible that more efficient S1/S2 cleavage of Omicron strains than parental strain (Fig. 2.3B) resulted in increased EC50 values. The differences between ours from Sakurai's results may be due to using different pseudoviruses, SARS-CoV-2 strains, cell types, and cathepsin L inhibitors.

Though the pseudovirus system is a safe and fast method for virus analysis with wide applications, it is not without limitations. While this system is good for assessing the entry capability of a virus, it cannot assess the replication efficiency. Both SARS-CoV and SARS-CoV-2 have demonstrated the ability to enter, but not replicate in various human and animal cell lines (268). Therefore, this assay should be validated using live viruses. Additionally, we used cells exogenously expressing ACE2, DPP4 and TMPRSS2, which may lack endogenous proteins that aid or inhibit viral entry that may be present in cells naturally susceptible to the tested coronaviruses.

In summary, our results suggest the importance of continual evaluation of SARS-CoV-2 variants in their susceptibility to animal species, as well as monitoring entry routes and performing inhibitor screening of emerging strains. Comparable analysis of entry efficacies of SARS-CoV-2 variants as well as SARS-CoV and MERS-CoV provides detailed information regarding entry processes among different coronaviruses.

# **Chapter 3 - Differential induction of PD-L1 expression in cells infected with Feline Infectious Peritonitis Virus and Feline Enteric Coronavirus**

This chapter is derived from the following published article:

Zabiegala, A., Kim, Y., & Chang, K. O. (2025). Differential induction of PD-L1 expression in cells infected with feline infectious peritonitis virus and feline enteric coronavirus. *Virology*, 614, 110734. <https://doi.org/10.1016/j.virol.2025.110734>

Link: <https://www.sciencedirect.com/science/article/pii/S0042682225003484?via%3Dihub>

### 3.1 Abstract

Feline infectious peritonitis (FIP) is a fatal systemic disease of cats, which is caused by FIP virus (FIPV), a virulent biotype of feline coronavirus (FCoV). In a small number of cats infected with feline enteric coronavirus (FECV), virus mutation may emerge that enable infection of monocytes and macrophages, leading to the development of FIP. This cell tropism shift, along with impaired T cell response, plays a critical role in FIP development. Programmed cell death protein (PD-1) and its ligands (PD-L1 and PD-L2) play crucial roles in immune responses as an immune checkpoint system. The PD-1 axis downregulates T cell response and is reported to be involved in immune evading mechanisms of various viruses. In this study, we used two virus strains FIPV-1146 and FECV-1683 corresponding to FIPV and FECV biotypes, respectively, to study the role of PD-L1 expression as a potential mechanism for FIP development. While both viruses demonstrated robust viral replication at comparable levels, FIPV-1146, but not FECV-1683, significantly upregulated PD-L1 expression in both cell lines. Infection of Fcwf-4 cells with FIPV-Black strain also increased PD-L1 levels. Co-culture studies using Jurkat cells and Fcwf-4 cells infected with FIPV-1146 or FECV-1683 showed that PD-L1 induction by FIPV-1146 attenuated T cell activation. The cell signaling profiling assays showed that FIPV-1146, but not FECV-1683, induced IFN- $\beta$  signaling pathway activation, which is a well-known regulator for PD-L1 expression. The elevated PD-L1 levels by FIPV may lead to dampened T cell response, allowing persistent infection in macrophages and development of the FIP clinical disease.

### 3.2 Introduction

Coronaviruses are positive stranded RNA viruses belonging to the family Coronaviridae, which comprises genera of alpha-, beta-, gamma-, and deltacoronaviruses (1, 4). As RNA viruses,

they undergo frequent mutation and recombination during replication. These processes have contributed to the emergence of genetically diverse coronaviruses capable of causing a wide range of diseases across various mammalian and avian hosts (269, 270). Feline infectious peritonitis (FIP) is caused by FIP virus (FIPV), a feline coronavirus (FCoV) biotype associated with fatal systemic disease in domestic and wild cats, and was first described in the 1960s (19). The clinical signs of FIP include fluid accumulation in body cavities, lethargy, fever, weight loss, and neurological or ocular abnormalities depending on the form of the disease (271-273). Feline enteric coronavirus (FECV), another biotype of FCoV, typically causes unapparent or mild enteritis with minimal clinical significance. However, unlike FIP, which occurs in a small number of cats, FECV infection is common and widespread among cats (274). Although the exact mechanisms remain elusive, there is a consensus that FIPV arises through mutations of FECV within individual cats during intestinal infection, resulting in altered viral tropism and, ultimately, multi-organ disease (158, 163, 164). Genetic analysis studies on FIPV and FECV have indicated that three viral genes may be involved in the change in viral tropism: ORF 3c and ORF 7b accessory genes and spike (S) gene (32, 51, 163, 168, 275-277). A functional ORF 3c accessory protein appears necessary for successful viral replication in the intestinal epithelium (163, 168), but is not necessarily required for systemic infection or the development of FIP (18, 163, 165-168). The deletion in the ORF 7b accessory protein was initially proposed to be associated with FIP development. However, the findings of deletions in 7b genes in FECV field viruses and during cell culture adaptation suggest those mutations may arise independently of FCoV biotype (18, 170, 171). Coronavirus S protein plays a key role in viral attachment and entry. Cleavage at the S1/S2 site by host proteases triggers a conformational change in the spike (S) protein, which activates the fusion peptide located near the S2' site, facilitating viral entry into host cells (32, 51,

276). Since changes in viral tropism are likely contributed by the S protein, several studies have investigated the structural and functional characteristics of the S protein of FIPV and FECV (168, 169, 277). One or both amino acid mutations (M1058L and S1060A) within the highly conserved fusion domain of S protein have been identified in the majority of FIPVs or FECV from extra-intestinal tissues (168, 169), which suggest that while the S protein play a role in tropism changes, a multifactorial interplay involving viral mutations, host immune responses, and cellular tropism is likely required for the development of FIP.

Cell-mediated immunity, primarily driven by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), plays a crucial role in the elimination of virus-infected cells. Upon recognizing virus-infected cells or cancer cells, NK cells or CTLs release cytotoxic granules containing perforin and granzymes, which kill the target cells. In addition to viral factors described above, it has been well documented that T cells and cell-mediated immunity play a crucial role in FIP development as host factors (162, 177). Virus-specific antibodies have been shown to provide insufficient protection or may even exacerbate disease progression through antibody-dependent enhancement of viral entry into monocytes and macrophages (158, 162, 177). Despite the importance of cell-mediated immunity in the pathogenesis of FIP, the mechanisms through which virus–T cell interactions contribute to disease progression are poorly understood.

Programmed cell death protein (PD-1) and its ligands (PD-L1 and PD-L2) play crucial roles in cell-mediated immune responses as a checkpoint system. PD-1 is a type 1 transmembrane protein expressed on the surface of T cells that, when bound to its ligands, inhibits the T cell receptor phosphorylation cascade, ultimately suppressing cytotoxic T cell activity (185-188). While PD-L1 is expressed on a majority of cell types, PD-L2 is only expressed on hematopoietic cells such as macrophages, dendritic cells, B cells and T cells (278-284). Many viruses including

include lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), influenza virus, herpes simplex virus (HSV), respiratory syncytial virus (RSV), hepatitis B virus (HBV), human cytomegalovirus (CMV), Rabies virus, Hanta virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exploit the PD-1 axis as a strategy for immune evasion by inducing PD-L1 expression *in vitro* or *in vivo* (195-208). Inhibiting this axis using monoclonal antibodies (mAbs) has been shown to help clear certain viral infections *in vivo*, including LCMV, SIV, and HSV (209-212), which underscores its crucial role in antiviral immune responses.

Previous studies showed that FIPV-1146 infection in CRFK cells upregulated PD-L1 expression, and elevated levels of PD-L1 have also been observed in peripheral blood mononuclear cells (PBMCs) from cats with confirmed FIP (285). Although increased PD-L1 expression has been observed, the role of FIPV-mediated modulation of the PD-1 axis in the pathogenesis of FIP remains largely unknown. In this study, we used two distinct FCoV biotypes, FIPV-1146 (WSU-79-1146) and FECV-1683 (WSU-79-1683), to study the role of the PD-1 axis in Crandell-Rees Feline Kidney (CRFK) cells and *Felis catus* whole fetus (Fcwf)-4 cells (feline fetal macrophage cells) as a potential mechanism contributing to FIP development. An additional FIPV strain (FIPV-Black) was also included in the study.

Real-time quantitative reverse transcription (qRT)-PCR results and immunofluorescence assay (IFA) with confocal microscopy demonstrated that PD-L1 expression was induced in CRFK and Fcwf-4 cells following infection with FIPV-1146 and FIPV-Black strains. In contrast, FECV-1683 infection did not result in upregulation of PD-L1 expression. Furthermore, co-culture studies of Jurkat cells and FIPV-1146-infected cells revealed that virus-induced PD-L1 expressions attenuated T cell activation. We also demonstrated that FIPV-1146, but not FECV-1683, induced

IFN- $\beta$  signaling, a known upstream regulator of PD-L1 expression (286, 287). These findings suggest that elevated PD-L1 levels, driven by IFN- $\beta$  signaling induced by FIPV biotype, may dampen T cell responses, thereby contributing to the development of clinical FIP.

### **3.3 Materials and methods**

#### **3.3.1 Cells and viruses**

CRFK cells and Fcwf-4 cells were obtained from American Type Cell Culture Collection (ATCC; Manassas, VA). Cells were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS). Human embryonic kidney 293T (HEK-293T) cells were purchased from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Human monocyte THP-1 cell line was purchased from ATCC and maintained in RPMI supplemented with 10% FBS. All cells were supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin and were incubated at 37°C with 5% CO<sub>2</sub>. FIPV-1146 (FIPV-WSU-79-1146), FECV-1683 (FECV-WSU-79-1683) and Sendai virus (SEV) were obtained from ATCC (Manassas, VA). FIPV-Black was kindly provided by Cornell University (Drs. Whittaker and Diel).

#### **3.3.2 Plasmids, interferons, and antibodies**

The plasmid-based cell signaling profiling systems including pIFN $\beta$ -luc, pISRE-luc, pNF- $\kappa$ B-luc, pSTAT3-luc, and pMYC-luc were obtained from Takara Bio (San Jose, California). The plasmid (pNFAT-Luc, Plasmid #10959) expressing luciferase under nuclear factor of activated T-cells (NFAT) was a gift from Toren Finkel. The plasmid expressing feline PD-L1 (pCMV6-fPD-L1) was generated by synthesizing the full-length, codon-optimized gene with FLAG Tag

sequence at the 3'-end (Integrated DNA technology, Coralville, IA), which was subsequently cloned into pCMV6-hPD-L1 with FLAG-tag purchased from Origene (Rockville, MD). Recombinant human IFN- $\gamma$  (hIFN- $\gamma$ ) and feline IFN- $\gamma$  (fIFN- $\gamma$ ) were obtained from R&D systems (Minneapolis, MN), and type I interferon (IFN-I) proteins were purchased from Sigma-Aldrich (St Louis, MO). Polyclonal guinea pig (GP) antibody against FIPV was obtained from Antibodies-online.com (Limerick, PA). Monoclonal mouse anti-FIPV mAb was purchased from MyBioSource (San Diego, CA). Rabbit recombinant monoclonal anti-PD-L1 antibodies RM1012 and 28-8 were purchased from Abcam (Waltham, MA). Goat anti-mouse IgG conjugated with Alexa Fluor 647 and goat anti-rabbit IgG conjugated with Alexa Fluor 488 were purchased from ThermoFisher (Waltham, MA). Anti-mouse secondary mAb conjugated with FITC and anti-FLAG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD3/CD28 mAbs were obtained from Cell Signaling technology (Danvers, MA).

### **3.3.3 Real-time qRT-PCR for hPD-L1, fPD-L1, and FCoV**

Total RNA of cell lysates was extracted using Qiagen RNeasy Mini purification kit (Germantown, MD). RT-qPCR was performed using the SuperScript One-Step RT-PCR system with Taq DNA Polymerase (Thermo Fisher) for fPD-L1 and fPD-L2. RT-qPCR for FCoV was performed using the SYBR Green real-time PCR kit (Thermo Fisher). The first cycle for cDNA synthesis and pre-denaturation was performed at 50°C for 30 min followed by 95°C for 2 min. Forty cycles of denaturation, annealing and extension were performed at 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec, respectively. Appendix table 1 contains the sequences of primers and probes used for real-time RT-qPCR. Relative mRNA levels of PD-L1 and PD-L2 were

calculated using the  $\Delta\Delta C_t$  comparative method (288). Virus titers were calculated as TCID<sub>50</sub>/mL using qRT-PCR data, based on a standard curve generated from serial dilutions of the viral stock.

### **3.3.4 Viral replication kinetics and quantitative analysis of fPD-L1 and fPD-L2 expression in Fcwf-4 and CRFK cells infected with FIPV-1146 or FECV-1683**

Viral replication kinetics were assessed in Fcwf-4 and CRFK cells infected with FIPV-1146 or FECV-1683. Briefly, one-day-old Fcwf-4 and CRFK cells in a 12-well plate were infected with FIPV-1146 or FECV-1683 at an MOI of 1 and subsequently incubated at 37°C with 5% CO<sub>2</sub>. Cell lysates were collected at 1, 4, 8, 10, 12 and 24 hpi for total RNA extraction. Total RNA was extracted for Real-time RT-qPCR for FCoV with primers targeting FCoV N gene (Appendix table 1).

To assess the PD-L1 expression in response to FIPV-1146 or FECV-1683, one-day-old CRFK cells and Fcwf-4 cells were infected with FIPV-1146 or FECV-1683 at an MOI of 0.1 and 2. The cells were then incubated at 37°C with 5% CO<sub>2</sub> for 4 or 24 h. Cell lysates were collected at 4 and 24 hpi, and total RNA was extracted from the cell lysates for real-time RT-qPCR for fPD-L1, fPD-L2 and FCoV N gene. The fPD-L1 expression in Fcwf-4 cells following infection with FIPV-Black was also assessed. Briefly, 3-day-old confluent Fcwf-4 cells in 12-well plates were infected with various 0.05, 0.2 or 0.5 MOI of FIPV-Black strain. The cells were then incubated at 37°C with 5% CO<sub>2</sub> for 50 h before the cell lysate was collected for total RNA extraction and RT-qPCR for fPD-L1 and FCoV N gene.

### **3.3.5 Immunofluorescence confocal microscopy**

For confocal microscopy, Fcwf-4 cells or CRFK cells were seeded onto a Lab-Tek II CC2 chamber slide (Fisher Scientific, Pittsburgh, PA) and grown to 90% confluency. The cells were infected with a control (media), FIPV-1146, or FECV-1683 at a MOI of 1, and subsequently incubated for 24 h at 37°C with 5% CO<sub>2</sub>. The cells were then fixed with increasing concentrations of paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 5-min intervals. Initially, cells were treated with a 1:1 ratio of media to 4% paraformaldehyde [prepared in PBS (pH 7.4)] and incubated at 37°C for 5 min, followed by a 1:2 ratio of media to 4% paraformaldehyde under the same condition. Finally, cells were incubated with 4% paraformaldehyde alone for at least 15 min at RT. Cells were then treated with 50mM Glycine (Fisher Scientific, Pittsburgh, PA) for 5 min at RT to quench free aldehydes, washed three times with PBS, and permeabilized with 0.1% Triton-x100 (Fisher Scientific, Pittsburgh, PA) in PBS 10 min at RT. After three washes with PBS, the cells were incubated with a blocking buffer of 2% nonfat dried milk in PBS with 0.05% Tween20 for 1 hr at RT. The cells were then incubated with two rabbit anti-PD-L1 antibodies (28-8 and RM1012) and mouse anti-FCoV antibodies diluted in blocking buffer at 4°C overnight. Anti-FCoV antibodies were diluted in a 1:1,000 ratio, anti-PD-L1 clone 28-8 was diluted at a 1:100 ratio, and anti-PD-L1 RM1012 clone was diluted at a 1:200 ratio. Cells were washed three times with PBS for 5 min before incubation with an anti-rabbit secondary antibody conjugated with AlexaFluor 488 (1:1,000) or an anti-mouse secondary antibody conjugated with AlexaFluor 647 (1:1,000) for 2 h at RT. Cells were washed three times with PBS before coverslips were mounted in glycerol. Cells were scanned with a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) using a 40x oil immersion objective.

For regular fluorescence microscopy, confluent CRFK cells or Fcwf-4 cells in a 12 or 24-well plate were infected with mock (medium), FIPV-1146 or FECV-1683 at a MOI of 0.1 or 2. Cells were incubated at 37°C for 24 h prior to fixation with cold methanol. Confluent FWCF-4 cells in 24-well plates were infected with Mock-medium or 0.5 MOI FIPV-Black and incubated at 37°C for 50 h and fixed with cold methanol. Fixed cells were incubated with anti-FIPV GP polyclonal antibody diluted in a 1:200 ratio for 1 hour, followed by anti-GP-Ab-FITC at a 1:100 dilution for 1 hour. Images were acquired at 40x magnification through a Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany).

### **3.3.6 Western blot analysis**

Western Blot analysis was performed to assess the FCoV replication or PD-L1 expression. Confluent CRFK cells were infected with FIPV-1146 or FECV-1683 at an MOI of 2, and cell lysates were prepared at 24 hpi for Western blot analysis. For expression of hPD-L1 or fPD-L1, one-day old semi-confluent HEK-293T cells were transfected with pCMV6-hPD-L1 or pCVM6-fPD-L1, and cell lysates were prepared after incubation for 24 h. Proteins were resolved in a 10% Novex Bis-Glycine gel (Thermo Fisher, Waltham MA) and transferred to a nitrocellulose membrane. The membrane was incubated with the GP antibody to FCoV at a 1:200 dilution overnight, followed by anti-GP-ab-HRP or FLAG-HRP at a 1:1000 dilution for 2 hours. Chemiluminescent signals were visualized using FOTO/Analyst Luminary/FX systems (Fotodyne Inc, Harland, WI).

### **3.3.7 Interferon treatment on PD-L1 expression**

The PD-L1 expressions in Fcwf-4 and CRFK cells were examined by real-time qRT-PCR following treatment with various concentrations of IFN- $\gamma$  or type-I IFN. Briefly, confluent Fcwf-4 or CRFK cells in a 12-well plate were incubated with mock (media), fIFN- $\gamma$  (20, 100, or 200 ng/mL) or type-I IFN (20, 50, or 200 unit/ml). After 4 or 24 h, cell lysates were prepared for total RNA extraction and RT-qPCR.

### **3.3.8 The expression of PD-L1 on T cell activation**

To assess whether expression of hPD-L1 or fPD-L1 can attenuate T cell activation, Jurkat cells (immortalized human T-cells) were electroporated with a reporter plasmid under NFAT control (pNFAT-luc) using Neon electroporation system (Thermo Fisher, Chicago, IL) following the manufacture's instruction. The plasmids pCMV6-hPD-L1 or pCMV6-fPD-L1 were introduced into THP-1 using the Neon electroporation system or into HEK-293T cells via Lipofectamine 2000 transfection (Thermo Fisher, Waltham, MA). Jurkat cells were then co-cultured with THP-1 or HEK-293T cells for 24 h, followed by the addition of anti-CD3/CD28 mAbs to activate T cells and induce luciferase expression. The co-cultured cells were further incubated for 8 h, after which cell lysates were prepared for luciferase assay. The firefly luciferase activity was determined using the luciferase reporter assay system (Promega, Madison, WI) on a luminometer (GloMax® 20/20 Luminometer, Promega, Madison, WI) following the manufacturer's direction. To examine the effect of FIPV-induced fPD-L1 on T cell activation, similar co-culture assays were performed. Briefly, Jurkat cells electroporated with pNFAT-luc were co-cultured with Fcwf-4 or CRFK cells that had been treated with fIFN- $\gamma$  or infected with

FIPV-1146 or FECV-1683. T cell activation and the luciferase assay was performed as described above.

### **3.3.9 Cell signaling profiling to assess PD-L1 expression induced by FIPV-1146**

To assess potential mechanisms by which FIPV-1146 induces PD-L1 expression, CRFK cells were transfected with reporter plasmids targeting various cellular signaling pathways prior to virus infection. The plasmids included pIFN $\beta$ -luc, pISRE-luc, pNF- $\kappa$ B-luc, pSTAT3-luc, and pMYC-luc. Briefly, one-day old CRFK cells in a 12-well plate were transfected with each plasmid with lipofectamine 2000 and incubated for 24 h before infection with FIPV-1146 or FECV-1683 at an MOI of 0.1 or 2. Sendai virus was also inoculated at 1 or 10 units per well as a control. Virus infected cells were further incubated for 8 h, and cell lysates were prepared for the luciferase assay. To evaluate the expression of fIFN- $\alpha$  or fIFN- $\beta$  in CRFK cells after infection with FIPV-1146 or FECV-1683, confluent cells in a 12-well plate were inoculated with Mock-medium or each virus at an MOI of 1. After 24 h, cell lysates were prepared for total RNA extraction and RT-qPCR for each gene was performed with primers listed in Appendix table 1.

### **3.3.10 Statistical analysis**

A lognormal ordinary one-way ANOVA with Šídák's multiple comparisons test was performed to assess the significance of differences between unpaired sample means. P values <0.05 were considered significant. All experiments were repeated at least two or three times independently.

### **3.4 Results**

#### **3.4.1 Growth kinetics of both FIPV-1146 and FECV-1683 in Fcwf-4 and CRFK cells**

When the growth kinetics of both FIPV-1146 and FECV-1683 in Fcwf-4 cells and CRFK cells were compared up to 24 hours post infection (hpi), FECV-1683 initially replicated more slowly than FIPV-1146 in both cells but reached comparable levels by 24 hpi (Fig. 3.1A). Fluorescent antibody (FA) staining (Fig. 3.1B) and Western blot (Fig 3.1C) using an FCoV-specific antibody also demonstrated comparable replication of both viruses in CRFK cells at 24 hpi.

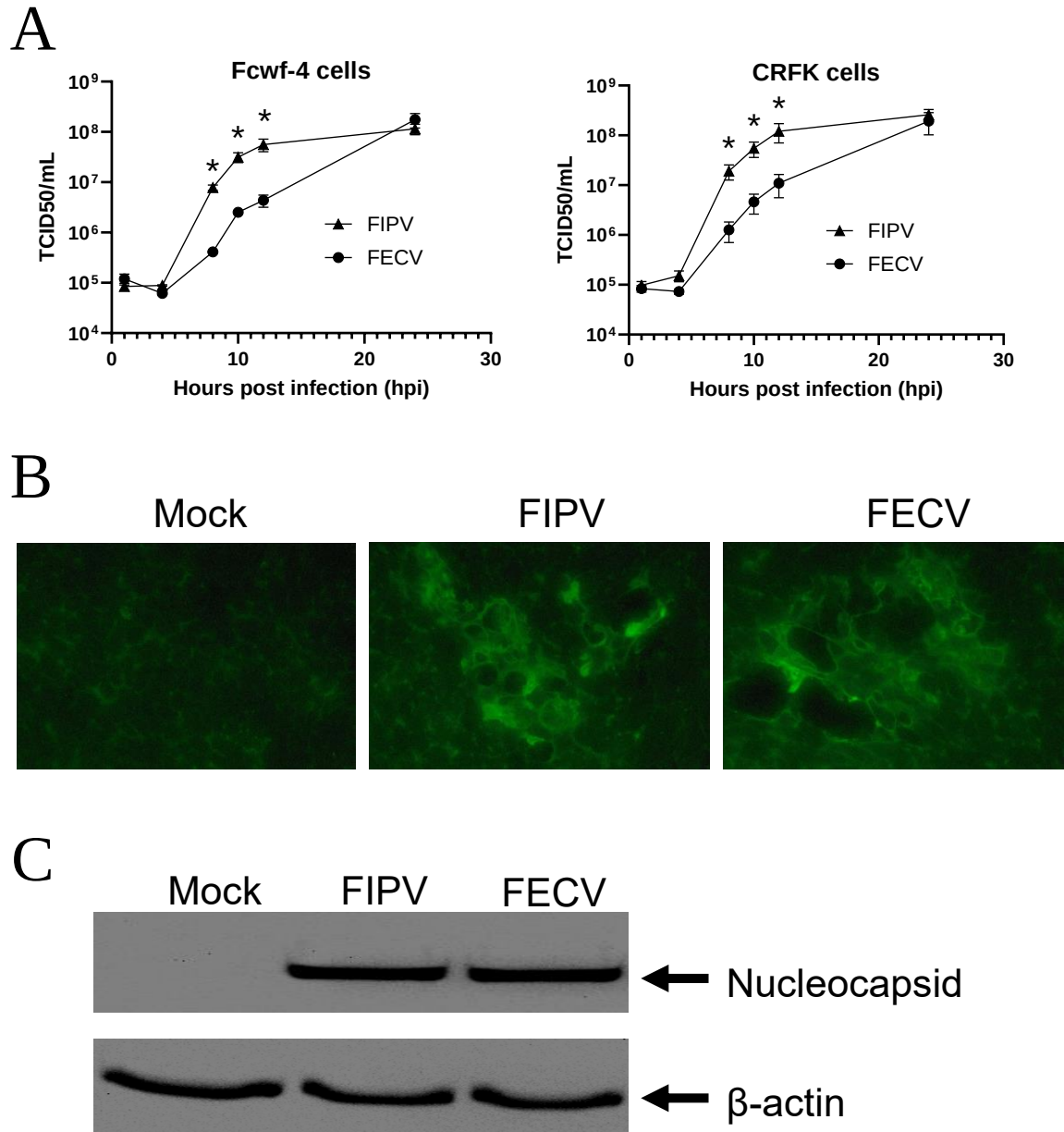


**Figure 3.1 Growth kinetics of FIPV-1146 and FECV-1683 in Fcwf-4 and CRFK cells**

(A) Growth kinetics of FIPV-1146 and FECV-1683 in Fcwf-4 and CRFK cells following viral infection at an MOI of 1 for up to 24 h. TCID50/mL values were calculated based on a standard curve generated from serial dilutions of the viral stock. (B and C) Fcwf-4 cells were infected with FIPV-1146 or FECV-1683 at an MOI of 1 and incubated for 24 h for IFA staining (B) or Western Blot analysis of nucleocapsid protein expression (C).  $\beta$ -Actin was used as a loading control. Statistical differences in TCID50/mL between FIPV-1146 and FECV-1683 are indicated with an asterisk (\*,  $P < 0.05$ ).

### **3.4.2 FIPV-1146 upregulates PD-L1 and PD-L2 expression in Fcwf-4 and CRFK cells**

PD-L1 expression induced by FIPV-1146 or FECV-1683 was determined in Fcwf-4 cells and CRFK cells at 4 and 24 hpi. At 4 hpi, Fcwf-4 cells infected with FIPV-1146 at a multiplicity of infection (MOI) of 2 exhibited a statistically significant increase (by approximately 2-fold) in PD-L1 mRNA expression compared to mock-infected controls at 4 hpi (Fig. 3.2A). However, no significant change in PD-L1 mRNA expression was observed at in Fcwf-4 cells infected with FIPV-1146 (0.1 MOI), FECV-1683 (0.1 MOI), or FECV-1683 (2 MOI) when compared to mock-infected controls at 4 dpi.

At 24 hpi, Fcwf-4 cells infected with FIPV-1146 at an MOI of 0.1 or 2 induced a marked increase (39-fold and 53-fold, respectively) compared to mock-infected cells. Notably, FECV-1683 infection did not result in significant changes in PD-L1 expression relative to mock-infected controls, despite comparable viral RNA levels between FIPV-1146 and FECV-1683 at each time point and MOI (Fig. 3.2C). This was also observed in CRFK cells infected with FIP-1146 or FECV-1683 (Fig. 3.2D and C). There was a slight increase (without significance) of PD-L1 mRNA expression in CRFK cells infected with FIPV-1146 (1.7-fold and 1.9-fold for 0.1 and 2 MOI, respectively) at 4 hpi (Fig. 3.2D). At 24 hpi, there were significant changes, approximately a 10-fold and 39-fold increase of PD-L1 mRNA levels in cells infected with FIPV-1146 at a MOI of 0.1 or 2 over mock-infected cells, respectively. As seen in Fcwf-4 cells, infection with FECV-1683 in CRFK cells did not result in significant changes of PD-L1 mRNA expression compared to mock-infected cells, despite comparable viral mRNA levels (Fig. 3.2E). The expression of PD-L2 in response to FIPV-1146 or FECV-1683 infection in Fcwf-4 cells was also assessed. Cells infected with FIPV-1146 at an MOI of 0.1 and 2 showed a significant increase in PD-L2

expression by approximately 1.3- and 1.6-fold, respectively, at 4 hpi when compared to the mock-infected control (Fig. 3.2B). A significant 1.5-fold increase in PD-L2 mRNA expression was also observed 24 hpi in cells infected with 2 MOI FIPV-1146 when compared to the mock-infected control. However, there was no significant change seen in PD-L2 expression in cells infected with FIPV-1146 at 0.1 MOI in 24 hpi, or in cells infected with FECV-1683 at a 0.1 MOI or 2 MOI in 4 and 24 hpi. The viral RNA levels of FIPV-1146 or FECV-1683 infections in Fcwf-4 cells were comparable at the measured time points (Fig. 3.2C).

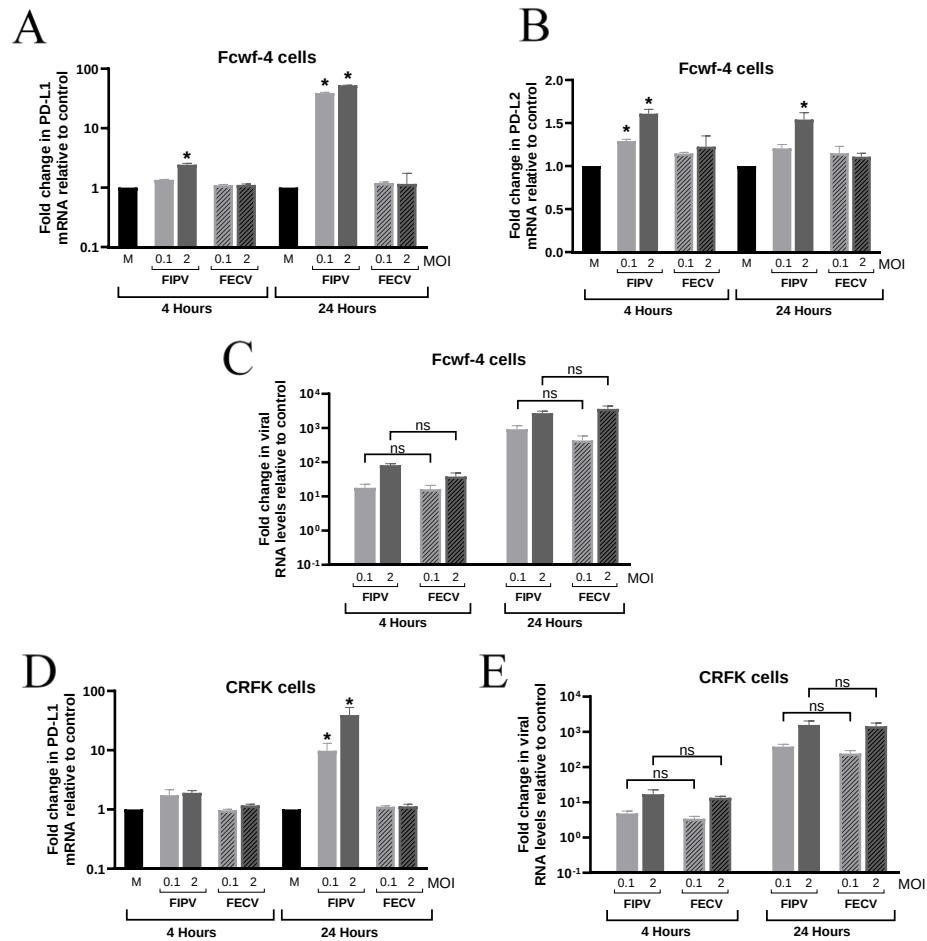


**Figure 3.2 Induction of PD-L1 in Fcwf-4 or CRFK after infection with FIPV-1146 or FECV-1683**

(A) Fold change of PD-L1 expression in Fcwf-4 cells infected with FIPV-1146 or FECV-1683 at 4 hpi and 24 hpi compared to uninfected mock. (B) Fold change in PD-L2 mRNA expression at 4 hpi and 24 hpi in cells infected with FIPV-1146 or FECV-1683 compared to the uninfected mock. (C) Fold change of FCoV mRNA in Fcwf-4 cells infected with FIPV-1146 and FECV-1683 4 hpi and 24 hpi compared to 1 hpi. (D) Fold change of PD-L1 expression in CRFK cells infected with FIPV-1146 and FECV-1683 4 hpi and 24 hpi compared to uninfected mock. (E) Fold change of FCoV mRNA in CRFK cells infected with FIPV-1146 and FECV-1683 4 hpi and 24 hpi compared to 1 hpi. Each bar indicates the mean and the standard error of the means. PD-L1 mRNA levels in FIPV-1146 and FECV-1683 infected cells were compared to mock-infected cells. FCoV mRNA levels were compared between FIPV-1146 and FECV-1683 for each MOI and time-point group. Statistical differences are indicated with an asterisk (\*,  $P < 0.05$ ) and “ns” indicates no significant difference.

### **3.4.3 Immunofluorescence confocal microscopy of PD-L1 expression in Fcwf-4 and CRFK cells infected with FIPV-1146 and FECV-1683**

To confirm the expression of PD-L1 in Fcwf-4 cells and CRFK cells infected with FIPV-1146 or FECV-1683, immunofluorescence confocal microscopy was performed. Infection with FIPV-1146 in Fcwf-4 cells or CRFK cells resulted in strong staining for both FCoV (red) and PD-L1 (green). When the images were merged, the two signals were well co-localized within the same cells (Fig. 3.3A and B). However, while infection of FECV-1683 resulted in strong staining for FCoV (red), PD-L1 (green) expression was minimal in both cells (Fig. 3.3A and B). Mock-infected cells showed no staining for FCoV (red) and only faint staining for PD-L1 (green) in Fcwf-4 cells or CRFK cells (Fig. 3.3A and B Top rows).

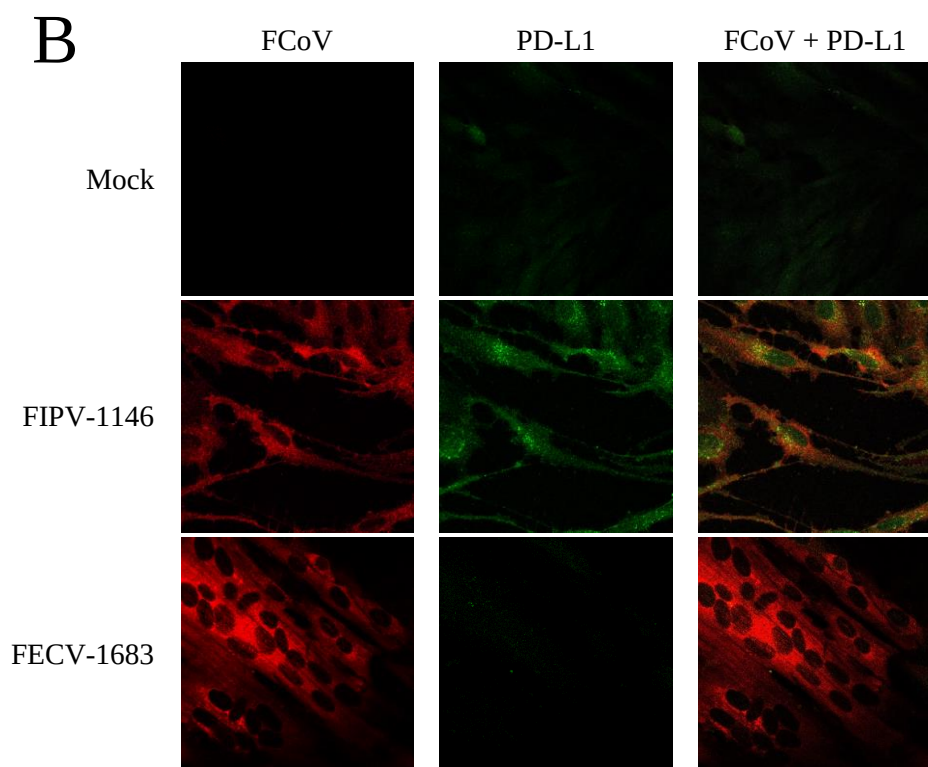
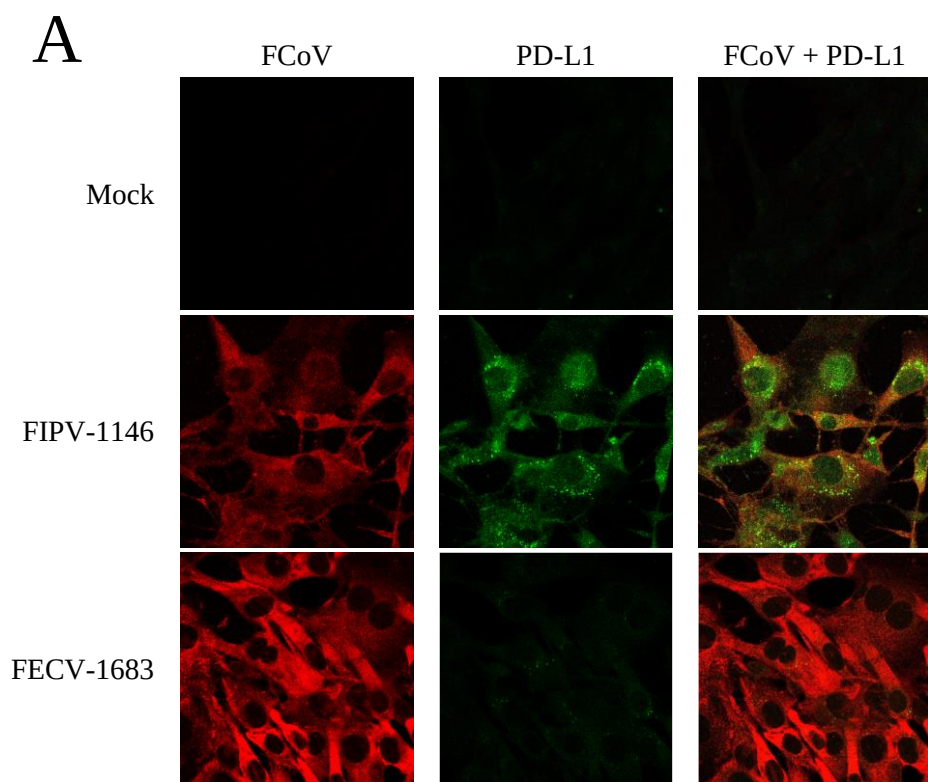


**Figure 3.3 Immunofluorescence confocal microscopy of Fcwf-4 (A) or CRFK (B) cells infected with FIPV-1146 or FECV-1683.**

(A and B) Cells were infected with FIPV-1146 or FECV-1683 at an MOI of 1 and incubated for 24 h. Cells were then incubated with anti-FCoV and anti-PD-L1 primary antibodies, followed by secondary antibodies conjugated with Alexa Fluor 647 (red) and Alexa Fluor 488 (green), respectively. Top row shows cells infected with mock (medium), middle row with FIPV-1146, and bottom row with FECV-1683. Left column shows cells stained anti-FCoV antibody; middle column with anti-PD-L1 antibody; right column displays merged images. Co-localization of FCoV (red) and PD-L1 (green) appears as yellow in the merged images. All images were acquired at 40x magnification.

**3.4.4 Induction of PD-L1 expression in Fcwf-4 cells infected with serotype 1 FIPV-Black strain**

Expression of PD-L1 in Fcwf-4 cells infected with 0.05, 0.2 or 0.5 MOI of the serotype 1 FIPV-Black strain was evaluated at 50 hpi. Cells infected with FIPV-Black showed significant, dose-dependent increases in PD-L1 mRNA levels compared to the mock control (Fig. 3.4A, left panel) with approximately 8.3-, 13.5-, and 17.8-fold increases following 0.05, 0.2, and 0.5 MOI, respectively. Replication of FIPV-Black strain in Fcwf-4 cells was confirmed by real-time qRT-PCR (Fig. 3.4A, right panel) and FA staining for FCoV (Fig. 3.4B).

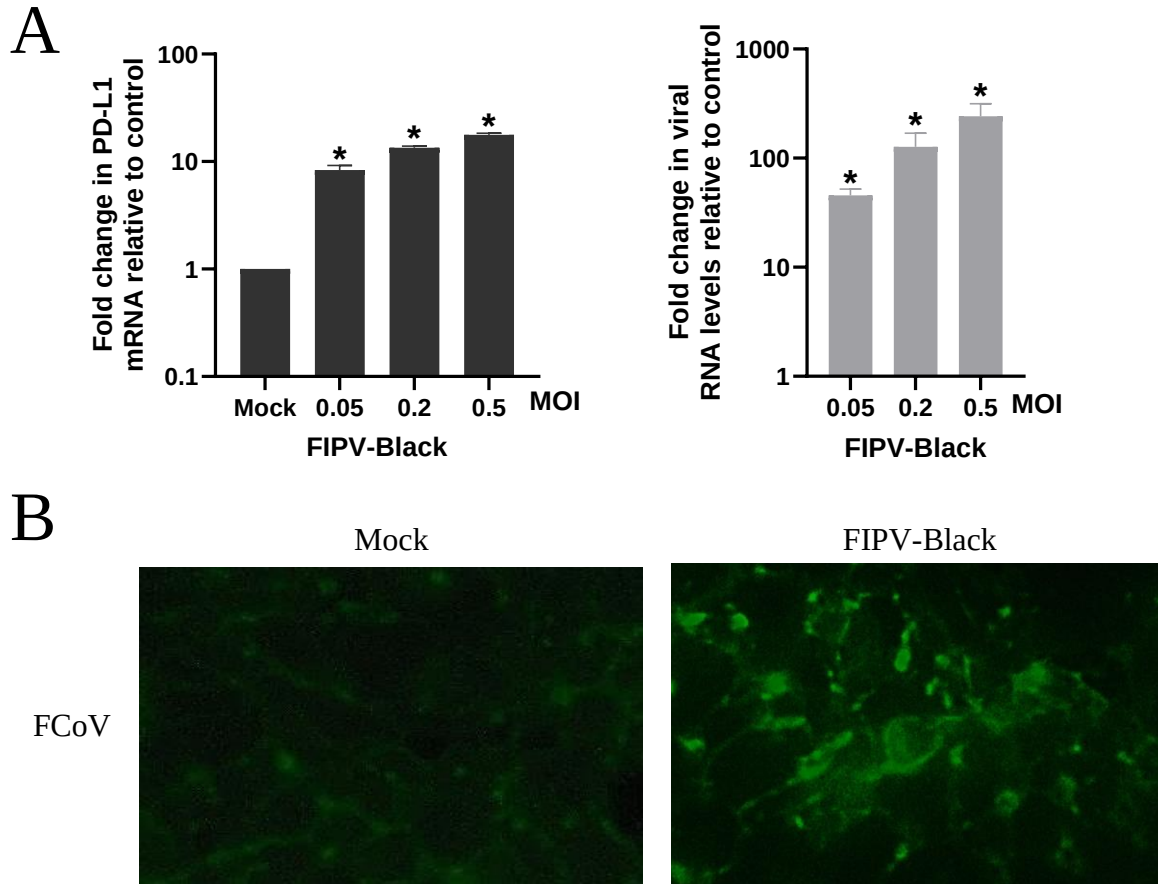


**Figure 3.4 Induction of PD-L1 expression in Fcwf-4 cells infected with FIPV-Black strain.**

Left panel shows fold changes in PD-L1 mRNA expression in Fcwf-4 cells infected with 0.05, 0.2, or 0.5 MOI of FIPV-Black, compared to the mock control. Right panel shows fold changes in FIPV-Black RNA levels in Fcwf-4 cells at 50 hpi compared to 1 hpi. Each bar indicates the mean and the standard error of the means. PD-L1 mRNA levels in cells infected with 0.05, 0.2, or 0.5 MOI FIPV-Black were compared to mock-infected cells. FCoV mRNA levels at 50 hpi were compared to FCoV mRNA levels at 1 hpi. Statistical differences are indicated with an asterisk (\*,  $P < 0.05$ ). (B) IFA staining of Fcwf-4 cells infected with FIPV-Black was performed using an anti-FCoV antibody at 50 hpi.

### **3.4.5 Induction of PD-L1 expression in Fcwf-4 cells or CRFK cells treated with fIFN- $\gamma$ or type-I IFN**

As human IFN- $\gamma$ , a type-II interferon, is a strong inducer of PD-L1 expression, the effects of fIFN- $\gamma$  on PD-L1 mRNA levels were measured in Fcwf-4 cells or CRFK cells at 4 or 24 h following treatment with various concentrations (20, 100, and 200 ng/mL) of fIFN- $\gamma$ . All tested concentrations of fIFN- $\gamma$  significantly increased PD-L1 expression in Fcwf-4 cells by approximately 25-40-fold compared untreated cells at 4 or 24 h (Fig. 3.5A). In CRFK cells, the same treatment led to a significantly increased PD-L1 expression by approximately 5-9-fold at 4 or 24 h (Fig. 3.5B). Various concentrations (20, 50, and 200 units) of type-I IFNs also significantly increased expression of PD-L1 mRNA in both cell lines. The fold increases of PD-L1 mRNA levels were approximately 20-40-fold and 5-16-fold at 4 h and in Fcwf-4 cells, respectively (Fig. 3.5C). The mRNA levels of PD-L1 in CRFK cells increased by approximately 3-4-fold at 4 h compared to untreated cells (Fig. 3.5D). However, there was no significant change in PD-L1 expression at 24 h in CRFK cells.

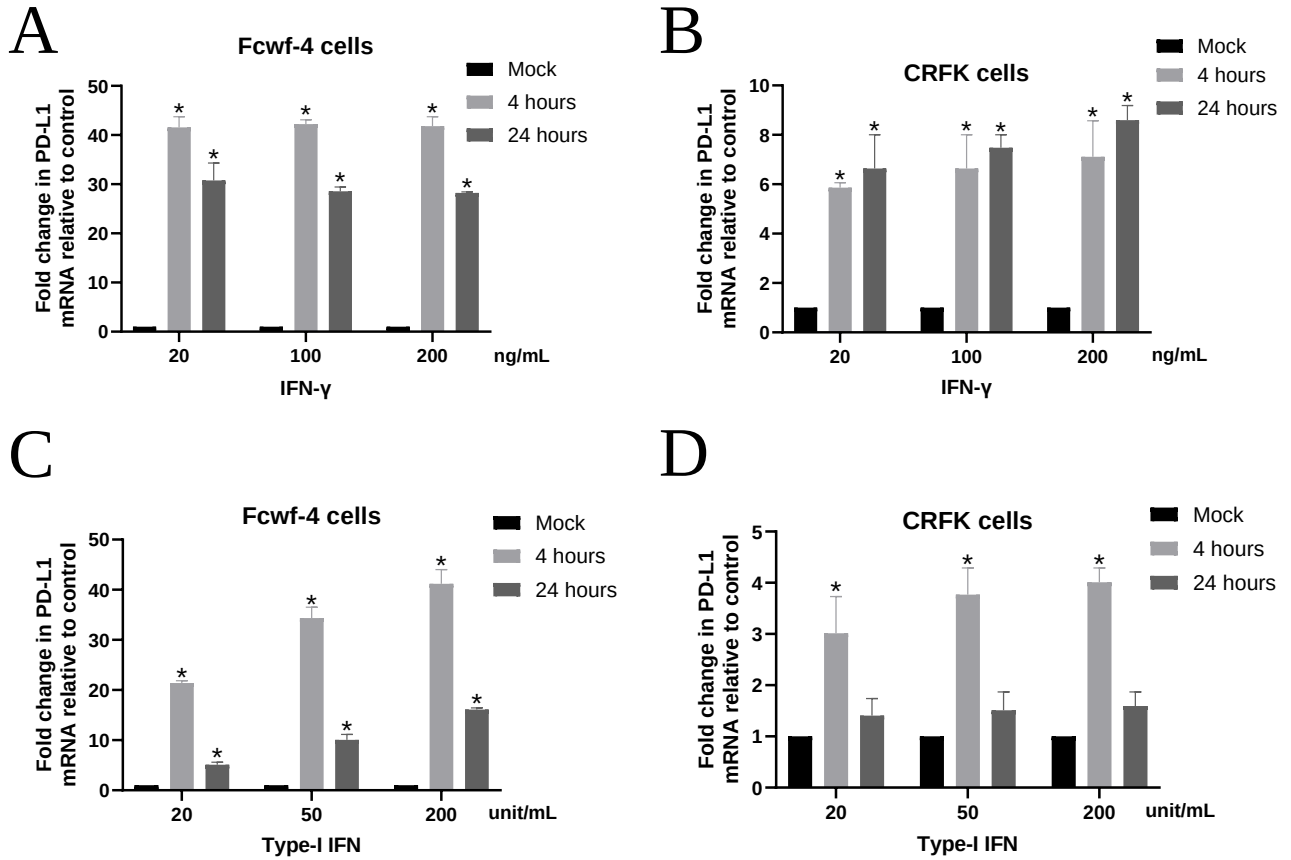


**Figure 3.5 Induction of PD-L1 expression in Fcwf-4 and CRFK cells treated with fIFN-γ or type-I IFN.**

(A and B) Fold change in PD-L1 mRNA expression in Fcwf-4 (A) or CRFK cells (B) at 4 and 24 hpi following fIFN-γ treatment compared to the untreated mock. (C and D) Fold change in PD-L1 mRNA expression in Fcwf-4 (C) or CRFK (B) cells at 4 and 24 hpi following type-I IFN treatment compared to the untreated mock. Each bar indicates the mean and the standard error of the means. Statistical differences of interferon treatment groups compared to the untreated mock at each time point are indicated with an asterisk (\*,  $P < 0.05$ ).

### **3.4.6 Effects of expression of human or feline PD-L1 on T cell activation (Jurkat cells)**

To evaluate whether PD-L1 expression can attenuate T cell activation, Jurkat cells (an immortalized human T-cell line) electroporated with pNFAT-Luc was used in the study. Luciferase expression in Jurkat cells were significantly reduced when co-cultured with THP-1 cells exogenously expressing PD-L1 when compared to Jurkat cells co-cultured with untransfected THP-1 cells. Specifically, hPD-L1 reduced luciferase activity by approximately 75%, while fPD-L1 led to a reduction of about 25% (Fig. 3.6A). However, no significant reduction in luciferase expression was observed when Jurkat cells were co-cultured with HEK-293T cells exogenously expressing either hPD-L1 or fPD-L1 (Fig. 3.6A). Expression of hPD-L1 or fPD-L1 in HEK-293T cells was confirmed by Western blot analysis using an anti-FLAG antibody (Fig. 3.6B).

Luciferase expression in Jurkat cells co-cultured with Fwcf-4 cells either treated with fIFN- $\gamma$  or infected with FIPV-1146, was significantly reduced by approximately 20% or 23%, respectively, compared to Jurkat cells co-cultured with untreated/uninfected cells (mock) (Fig. 3.6C). However, there was no significant change in luciferase expression in Jurkat cells co-cultured with Fcwf-4 cells infected with FECV-1683 compared to Jurkat cells co-cultured with untreated/uninfected cells (mock) (Fig. 3.6C). In Jurkat cells co-cultured with CRFK cells, no significant differences in luciferase expression were observed regardless of fIFN- $\gamma$  treatment or infection with FIPV-1146 or FECV-1683, compared to Jurkat cells co-cultured with untreated/uninfected cells (mock) (Fig. 3.6C).

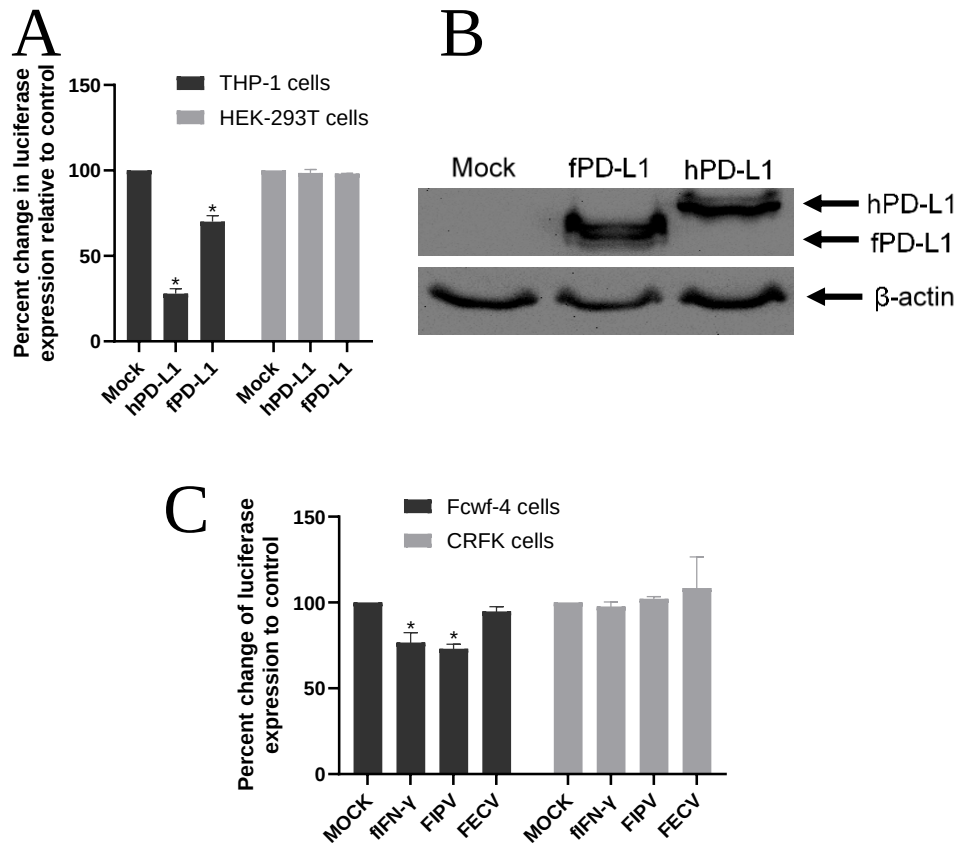


**Figure 3.6 Effects of hPD-L1 or fPD-L1 expression on T cell activation in Jurkat cells.**

(A and B) Jurkat cells electroporated with pNFAT-Luc were co-cultured with THP-1 or HEK-293T that had been transfected with pCMV6-hPD-L1 or pCMV6-fPD-L1. To activate Jurkat cells and induce luciferase expression, co-cultures were treated with anti-CD3/CD28 mAbs. (A) Fold change in luciferase expression in activated Jurkat cells co-cultured with hPD-L1 or fPD-L1 expressing THP-1 cells or HEK-293T cells relative to Jurkat cells cultured with untransfected (mock) THP-1 or HEK-293T cells, respectively. (B) Western blot analysis confirming the expression of hPD-L1 or fPD-L1 in transfected HEK-293T cells. (C) Jurkat cells electroporated with pNFAT-Luc were co-cultured with Fcwf-4 or CRFK cells that had been pre-treated with IFN- $\gamma$  or infected with FIPV-1146 or FECV-1683. Jurkat cells were activated by anti-CD3/CD28 mAbs. (C) Fold change in luciferase expression in activated Jurkat cells co-cultured with Fcwf-4 cells or CRFK cells treated with IFN- $\gamma$  or infected with FIPV-1146 or FECV-1683 compared to activated Jurkat cells co-cultured with untreated/uninfected (mock) Fcwf-4 cells or CRFK cells. Each bar indicates the mean and the standard error of the means. Statistical differences are indicated with an asterisk (\*,  $P < 0.05$ ).

### **3.4.7 Cell signal profiling assays in CRFK cells infected with FIPV-1146, FECV-1683, or Sendai virus**

Changes in PD-L1 expression following viral infection were measured in CRFK cells transfected with reporter plasmids targeting various cellular signaling pathways. Luciferase expression was significantly increased in cells transfected with pIFN $\beta$ -luc and infected with FIPV-1146, showing approximately 5- and 12-fold increases at MOI 0.1 and 2, respectively (Fig. 3.7). In contrast, FECV-1683 infection at either MOI did not induce luciferase expression in pIFN $\beta$ -luc-transfected cells (Fig. 3.7). However, no significant differences in luciferase expression were observed in cells transfected with pISRE-luc, pNF- $\kappa$ B-luc, pSTAT3-luc, or pMYC-luc and infected with FIPV-1146 or FECV-1683 (Fig. 3.7). Sendai virus (SeV), used as a positive control, significantly increased luciferase expression in cells transfected with pIFN $\beta$ -luc, pISRE-luc, or pNF- $\kappa$ B-luc at 1 or 10 units/well, but had no effect with pSTAT3-luc or pMYC-luc. When the mRNA of fIFN- $\alpha$  or fIFN- $\beta$  was monitored by infection with FIPV-1146 or FECV-1683 at 24hpi, cells infected with FIPV-1146 resulted in approximately 50- and 150-fold increases at MOI of 1, respectively (data not shown). However, there were no changes of the mRNA of fIFN- $\alpha$  or fIFN- $\beta$  in cells infected with FECV-1683.

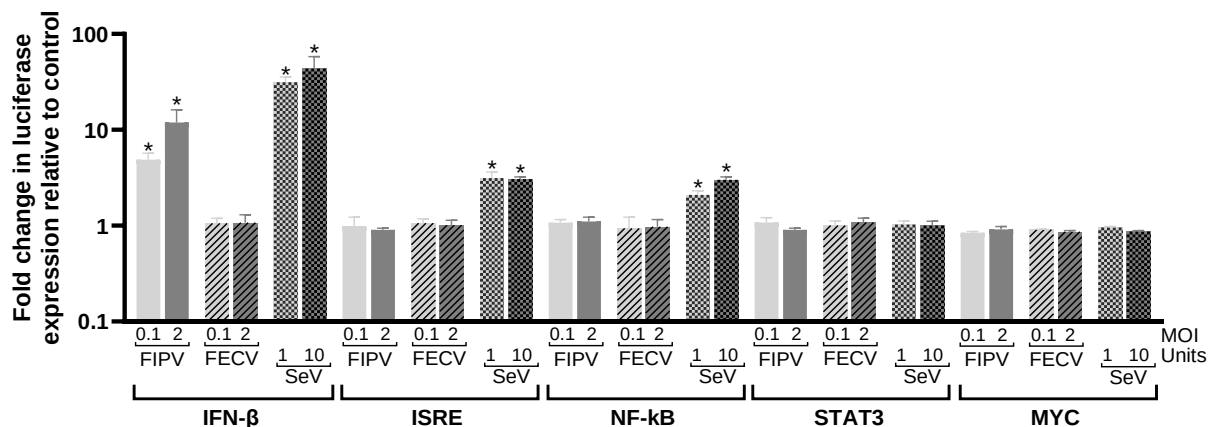


**Figure 3.7 Cell signal profiling assays in CRFK cells infected with FIPV-1146, FECV-1683, or Sendai virus (SeV).**

CRFK cells were transfected with luciferase reporter plasmids targeting various cellular signaling pathways (pIFN $\beta$ -luc, pISRE-luc, pNF- $\kappa$ B-luc, pSTAT3-luc, or pMYC-luc) and incubated for 24 h prior to infection with FIPV-1146, FECV-1683, or Sendai virus (SeV). Cell lysates were prepared 24 h after virus infection for luciferase assay. Each bar indicates the mean and the standard error of the means. Statistical differences of luciferase expression between virus-infected cells and mock-infected cells are indicated with an asterisk (\*,  $P < 0.05$ ).

### 3.5 Discussion

FIPV-1146 and FECV-1683, both serotype II FCoV, are well-established prototype strains representing FIPV and FECV, respectively. Although they exhibit high overall RNA sequence homology with 95.3% in the S protein, 95% in the ORF 3c accessory protein, and 82.5% in the 7b accessory protein, they cause distinct diseases in cats. Inoculation of FECV-1683 in specific pathogen free (SPF) kittens causes unapparent or mild enteritis and does not lead to FIP (274, 289). On the other hand, FIPV-1146 is highly virulent, inducing FIP and causing over 90% mortality in SPF kittens, even after serial passages in cultured cells (158). In addition to mutations that shift cell tropism from enterocytes to macrophages, impaired T cell immunity plays a critical

role in the development of FIP (162, 290, 291). Although T cell immunity has been implicated in the development of FIP, the mechanisms by which mutated viruses persist within macrophages to drive disease progression remain largely unknown. Cell-mediated immunity, primarily driven by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), plays crucial roles in the elimination of virus-infected cells and malignant cells. Upon recognizing virus-infected cells or cancer cells, NK cells or CTLs release cytotoxic granules containing perforin and granzymes, which kill the target cells. Additionally, these immune cells can induce cell death via death receptor pathways, such as Fas/FasL and TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL-R, providing complementary mechanisms for immune defense (292). While NK cells are key components of the innate immunity, CTLs belong to the adaptive immunity and recognize virus-infected or malignant cells through peptides presented by major histocompatibility complex class I (MHC-I) molecules, a process known as MHC restriction (293). The cytotoxic activity of NK cells and CTLs against virus-infected and malignant cells can be severely impaired by the expression of PD-L1 (and/or PD-L2) on target cells (195). PD-1 with its ligands (PD-L1 and PD-L2) function as a major immune checkpoint system by down-regulating T cell activities (187, 294). PD-1 is expressed on most T cells (including antigen specific T cells), and mediates inhibitory effects by binding to its ligands PD-L1 and PD-L2 (188). The expression of PD-1 is upregulated during T cell activation as a part of negative feedback mechanism that limits excessive immune responses and maintains immune homeostasis (188, 295). Through engagement with its ligands PD-L1 and PD-L2, PD-1 inhibits T cell proliferation, cytokine production, and cytotoxic activity (188). PD-L1 is broadly expressed across a wide range of cell types (278). In contrast, PD-L2 expression is restricted to macrophage-type cells (187). The expression of PD-L1 and PD-L2 is relatively low in normal physiological conditions, but

their expression levels can be upregulated by various stimuli, particularly inflammatory cytokines such as IFN- $\gamma$ , IL-6, and TNF- $\alpha$  (194). Due to its crucial role in regulating T cell activity and cell-mediated immunity, the PD-1 axis has become a major target for immune evasion strategies employed by cancer cells, and extensive research has established the PD-1 axis as an excellent target for cancer immunotherapy (296). Several mAbs targeting PD-1 and PD-L1 have been approved by the FDA for the treatment of various cancers. PD-1 inhibitors include nivolumab, pembrolizumab, and cemiplimab, while PD-L1 inhibitors include atezolizumab, durvalumab, and avelumab (297).

Infection with several viruses have been shown to induce PD-L1 (and/or PD-L2) *in vitro* or *in vivo* as a mechanism to evade host immune responses. These viruses include LCMV, HIV, SIV, influenza virus, herpes virus, RSV, HBV, hCMV, Hantavirus and SARS-CoV-2 (195-197). HIV infection has been shown to upregulate PD-L1 expression on dendritic cells through the action of the viral Tat protein, which induces TNF- $\alpha$  and activates TLR4-mediated signaling (199). The HCV core protein upregulates PD-L1 expression on primary human Kupffer cells and monocytes through a TLR2- and PI3K-dependent signaling pathway *in vitro* (298, 299). The Hantavirus N protein has been shown to upregulate PD-L1 and PD-L2 expression via TLR3-mediated signaling (207). The latency-associated transcripts (LATs) of HSV-1 have been shown to upregulate PD-L1 expression on mouse neuroblastoma cells (300).

Similarly, FIPV has also been shown to induce PD-L1 expression. Transcriptional profiling analysis revealed that infection of CRFK cells with FIPV-1146 led to the upregulation of 44 genes, including PD-1 and PD-L1 (285). Among the upregulated genes, three genes, A3H, PD-1 and PD-L1, were identified as relevant to FIP, and real-time RT-PCR analysis of PBMCs from seven cats with confirmed FIP revealed upregulation of all three (285). To investigate the

potential role of PD-L1 in modulating T cell responses during FIPV infection, we examined its differential expression in CRFK and Fcwf-4 cells following infection of cells with FIPV-1146 and FECV-1683. Our results showed that infection of CRFK cells with FIPV-1146 induced a robust upregulation of PD-L1 expression, reaching up to a 39-fold increase at 24 hpi compared to mock-treated cells (Fig. 3.2D). The induction of PD-L1 by FIPV-1146 was also observed in Fcwf-4 cells, a feline macrophage cell line, where expression increased by a 53-fold at 24 hpi (Fig. 3.2A). Notably, PD-L1 induction was not observed in both cell lines following infection with FECV-1683, despite comparable levels of viral replication to FIPV-1146 (Fig. 3.2C and E). Prior studies have shown that both FIPV-1146 and FECV-1683 replicate efficiently in CRFK and Fcwf-4 cells, a finding supported by our results, which demonstrate comparable growth kinetics in both cell types (Fig. 3.1). Infection of Fcwf-4 cells with FIPV-1146 also led to an increase in PD-L2 RNA levels, although the induction was considerably lower compared to that of PD-L1 (Fig. 3.2A and B). Induced expression of PD-L1 in virus-infected CRFK and Fcwf-4 cells was demonstrated by confocal immunofluorescence microscopy (Fig. 3.3A and B). The serotype I FIPV strain, FIPV-Black, was shown to induce PD-L1 mRNA expression in Fcwf-4 cells (Fig. 3.4). Although serotype I FIPV strains are predominant in the field, they generally do not grow well in cell culture; however, the FIPV-Black strain, a field isolate of serotype I, demonstrates limited growth in cell culture (301-306) (FIPV-Black was kindly provided by Drs. Whittaker and Diel at Cornell University). As macrophages, the natural target cells of FIPV, express both PD-L1 and PD-L2, our findings of increased expression of these ligands following infection with FIPV-Black, as well as FIPV-1146, suggest a biologically relevant mechanism by which FIPV may potentially reduce CTL-mediated attack on virus-infected macrophage.

PD-L1 expression has been shown to be regulated through multiple mechanisms, including both transcriptional and post-transcriptional pathways, with transcriptional regulation being predominant (307). Key signaling pathways involved include type-I (IFN- $\alpha$ , IFN- $\beta$ ) and type-II interferons (IFN- $\gamma$ ), which activate transcription factors such as STATs and JAKs to upregulate PD-L1 expression (192). Additional factors, including HIF-1, STAT3, NF- $\kappa$ B, and AP-1, have also shown to regulate PD-L1 expression (308). Inflammatory cytokines, including TNF- $\alpha$  and IL-4, can induce PD-L1 expression through activation of the NF- $\kappa$ B pathway (308, 309). Oncogenic signaling pathways such as RAS/MAPK and PI3K/AKT also contribute to PD-L1 upregulation via AP-1, and MYC (310). In addition to transcriptional control, post-transcriptional and post-translational modifications further regulate PD-L1 stability and activity, depending on the cellular context and microenvironment (307). To determine whether PD-L1 upregulation was mediated by IFN- $\gamma$  and type I IFN in the cell lines used in this study, we treated CRFK and Fcwf-4 cells with IFN- $\gamma$  or type I IFN in the absence of viral infection. Both treatments induced PD-L1 expression in both cell lines, with a markedly stronger induction observed in Fcwf-4 cells compared to CRFK cells (Fig. 3.5). Interestingly, the increase in PD-L1 expression by IFN- $\gamma$  and type I IFN treatment in Fcwf-4 cells were relatively comparable to the levels observed following FIPV-1146 infection. In contrast, CRFK cells exhibited a much more subdued PD-L1 response to IFN treatment compared to viral infection (Fig. 3.5).

To evaluate whether FIPV-induced upregulation of PD-L1 can attenuate T cell activation, we used Jurkat cells that were transfected with reporter plasmids driven by NFAT-responsive elements as a model for T lymphocytes. Because of limitations on feline reagents including mAbs against feline PD-L1 as well as mAbs to induce feline T cell activation, we utilized human Jurkat cells and T cell activation with anti-human CD3/CD28 mAbs. To validate the relevance of hPD-

1 × fPD-L1 interaction, we used a co-culture of Jurkat cells and THP-1 or HEK-293T cells expressing either hPD-L1 or fPD-L1. Upon co-culture with THP-1 or HEK-293T cells expressing either human or feline PD-L1, the Jurkat cells exhibited increased NFAT-driven luciferase activity in THP-1 cells (Fig. 3.6A and B), indicating that both forms of PD-L1 can modulate T cell signaling. Notably, hPD-L1 resulted in a greater reduction of luciferase activity compared to fPD-L1, which suggests stronger inhibitory effects on T cell activation, potentially influenced by the shared origin of T cells and macrophage-derived cells (Fig. 3.6A). However, co-culture with HEK-293T cells expressing either hPD-L1 or fPD-L1 did not induce any reduction in luciferase activity, suggesting a lack of functional PD-L1-mediated inhibition of Jurkat cell activation (Fig. 3.6A). Similar results were observed when Jurkat cells were co-cultured with Fcwf-4 cells infected with FIPV-1146: T cell activation was reduced when co-cultured with Fcwf-4 cells either infected with FIPV-1146 or treated with fIFN- $\gamma$ , but not with Fcwf-4 cells infected with FECV-1683 or with CRFK cells (Fig. 3.6C), regardless of treatment. This finding suggests that FIPV-induced PD-L1 expression in macrophage-derived cells, the primary target cells for FIPV, contributes to the suppression of T cell activity.

Then we performed cell signaling profiling assays to investigate the mechanisms underlying FIPV-1146-induced PD-L1 expression in CRFK cells. Although CRFK cells did not mediate a reduction in T cell activation, upregulation of PD-L1 was robustly induced by FIPV-1146, but not by FECV-1683, resembling the phenomenon observed in Fcwf-4 cells (Fig. 3.2D). Among the tested reporter plasmids targeting various signaling pathways, IFN- $\beta$  activity was significantly increased following FIPV-1146 infection, but not FECV-1683, compared to the uninfected control (Fig. 3.7). Our findings suggest that the IFN- $\beta$  pathway may contribute to PD-L1 induction following FIPV-1146 infection, although additional studies using macrophage-

derived cell lines could help further clarify this mechanism. Several signaling pathways are known to regulate PD-L1 expressions, including IFN- $\beta$ , NF- $\kappa$ B, STAT3, and MYC (308, 309). IFN- $\beta$  mediated PD-L1 expression has been observed in both rabies virus and influenza A infection (206, 311). In rabies virus infections, PD-L1 upregulation has been shown to depend on IFN- $\beta$  production triggered by Toll-like receptor 3 (TLR3) signaling (206). Similarly, moderate knockdown of IFN- $\beta$  significantly reduces PD-L1 expression in influenza-infected monocyte-derived macrophages (MDMs) (311). PD-L1 upregulation has also been observed in lung macrophages and MDMs infected with Influenza A virus (311). In LCMV infections, PD-L1 expression appears to be linked to type-I IFN production, and the use of type I IFN-neutralizing antibodies has been shown to promote viral clearance in persistent infections (198, 312, 313).

While FIPV-1146 activates the IFN- $\beta$  reporter in correlation with PD-L1 induction, the exact pathway of between IFN- $\beta$  and PD-L1 expression is unclear. When the expression of fIFN- $\alpha$  or fIFN- $\beta$  was monitored, there were significantly increased mRNA in cells infected with only FIPV-1146 but not FECV-1683, which was consistent with cell signaling profiling assay with IFN- $\beta$ -luc. Interestingly, there was no induction of ISRE pathway after infection with either FIPV-1146 or FECV-1683 in CRFK cells. It was well documented that coronaviruses have multiple mechanisms to evade innate IFN pathways including both inhibiting IFN induction and IFN responses (314). For FIPV, Nsp5 and ORF 7a accessory protein were shown to inhibit type I IFN production (315, 316). It is possible that while there were differences in IFN induction between FIPV-1146 and FECV-1683, both maintain the mechanisms to inhibit IFN responses as shown in our results. In addition, because the infection studies and the pathway assay system with the reporter expression measures in short timelines, the results might reflect a timing issue as well as the differential modulation on the IFN induction by FIPV-1146 and FECV-1683. We are

currently working on these possibilities with wider ranges of MOIs and longer incubations as well as detection of functional IFN proteins after FIPV-1146 infections.

Our findings suggest that FIPV, but not FECV, suppresses T cell activity by inducing the expression of immune checkpoint molecules PD-L1 and PD-L2, which appears to be mediated, at least in part, by IFN- $\beta$ . Similar instances of T cell suppression linked to systemic infection have been reported in other coronaviruses, underscoring the importance of T cell immunity in disease progression. Murine hepatitis virus (MHV), a murine coronavirus, has two biotypes based on their primary tissue tropism: Enterotropic strains and polytropic strains (317, 318). Infection with MHV-Y, an enterotropic strain, remains confined to the gastrointestinal tract in immunocompetent or B cell-deficient mice (319). However, T cell-deficient mice infected with MHV-Y develop a multisystemic lethal disease with peritonitis, closely resembling FIP in cats (162, 319). Ferret systemic coronavirus infection resembles FIP with it is believed to arise from mutation of ferret enteric coronavirus and involved in dysregulation of cellular immunity (320). Field strains of FECV and type I FIPV typically do not grow in cell culture, with the exception of the virus strains used in this study. Currently, we are evaluating PD-L1 expressions in other strains of FIPV and FECV in cell culture and/or FCoV-positive tissues obtained from naturally infected cats, as well as quantitative analysis using the flow cytometry to further support FIPV-mediated PD-L1 induction. Nevertheless, these findings provide valuable insight into the interplay between coronaviral infection of macrophages and T cell immunity, and how their interaction contributes to disease development. These findings also suggest the possibility of utilizing PD-1/PD-L1 axis blockade as a therapeutic strategy in treating FIP. mAbs targeting PD-1 and/or PD-L1 have demonstrated efficacy in enhancing viral clearance *in vivo* in several chronic viral infections, including LCMV (209, 210), SIV (211), HIV(321), and HSV (212) *in vivo*.

Because FIPV strains directly induce PD-L1 and PD-L2 expression, inhibiting this pathway may help restore T cell function and improve viral control when used in conjunction with antiviral drugs (322-325). This approach may be especially beneficial in cats with relapsing FIP following antiviral treatment, where residual viral infection persists despite initial therapeutic response. In such cases, immune checkpoint blockade may serve as a valuable adjunct to currently available antivirals, potentially enhancing viral clearance and reducing the likelihood of disease recurrence.

## **Chapter 4 - PD-L1 expression in tissues of cats with feline infectious peritonitis**

### **4.1 Abstract**

Feline infectious peritonitis (FIP) is a fatal, systemic infection in cats caused by virulent feline infectious peritonitis virus (FIPV). Feline coronavirus (FCoV) typically circulates as feline enteric coronavirus (FECV), which is ubiquitous among cat populations, and causes asymptomatic to mild enteric disease. However, viral mutations in combination with impaired host immune response may lead to FIP in a small number of cats. Programmed death-1 (PD-1) is a major immune-checkpoint protein expressed on activated T cells, and its engagement by the ligands PD-L1 and PD-L2 on target cells leads to suppression of T-cell activity. Previously, we found that infection with FIPV, but not FECV, upregulated the expression of PD-L1 in feline cells, which can attenuate the T cell activities. We also demonstrated that feline interferons (IFNs), particularly IFN- $\gamma$ , are potent inducers of PD-L1 in the cells. In this study, we assessed the expression of PD-L1 in association with FIPV in the tissues of four cats with naturally occurring FIP using histopathology, real-time RT-qPCR and confocal microscopy with specific antibodies. Using confocal microscopy, we found that FIPV and PD-L1 co-localized in the tissues, although the relative levels of PD-L1 RNA and viral RNA varied among tissues and between individual cats. The cells expressing PD-L1 were located adjacent to FIPV positive cells, which is probably due to inflammatory cytokines such as IFN- $\gamma$ . These findings support our previous findings of a close association between PD-L1 and FIPV and further underscore the need to investigate the roles of PD-L1 and T cell responses in the development of FIP in cats.

## 4.2 Introduction

There are two biotypes of feline coronavirus (FCoV), the feline infectious peritonitis virus (FIPV) and the feline enteric coronavirus (FECV). FECV circulates in feline populations and commonly presents as enteric infection (274). However, in a small population of cats, this enteric infection can give rise to a fatal systemic disease known as feline infectious peritonitis (FIP). Clinically, cats with FIP commonly exhibit various signs such as pyrexia, lethargy, weight loss, fluid accumulation in body cavities, and neurologic or ocular signs, depending on the form of the disease (271-273). Without antiviral treatment, FIP is invariably fatal, and the exact mechanism underlying its development remains unknown.

It is widely accepted that FIP develops in individual cats as a consequence of viral mutations combined with an inadequate cellular immune response following FECV infection. The hallmark distinction between FECV and FIPV is the shift in viral tropism from enterocytes to macrophages. Currently, mutations in the ORF 3c gene and the spike (S) gene have been associated with this tropism change and FIP development (32, 51, 163, 168, 275-277). T cell immunity plays a vital role in the control of virus-infected cells, and resistance to FIP development has been associated with a robust FCoV-specific T cell response (175, 326). The importance of effective T cell immunity in the course of coronavirus infections has also been demonstrated in studies of murine hepatitis virus (MHV), a murine coronavirus. In these studies, T cell-deficient mice infected with an enterotropic strain of MHV have been shown to develop a fatal systemic condition resembling FIP (162, 317-319). Many viruses have mechanisms to circumvent T cell immunity, and the programmed cell death protein 1 (PD-1) axis is a well-established target for viral evasion strategies. PD-1 is a major checkpoint protein expressed on activated T cells, B cells, and myeloid cells. It regulates overactive T cells responses by inhibiting

downstream signal cascades (185-188). PD-1 has two ligands: PD-L1, which is expressed by a wide variety of cell types, and PD-L2, which is primarily expressed on hematopoietic cells (278-284). Inflammatory cytokines, such as interferon (IFN)- $\gamma$ , are well-known inducers of PD-L1 and PD-L2 expression. Many viruses have also been shown to upregulate PD-L1 expression as an immune evasion strategy (195-208).

Previously, we reported that PD-L1 is upregulated in Crandell-Rees Feline Kidney (CRFK) cells and Fcwf-4 cells following infection the serotype II FIPV-1146 strain and serotype I FIPV Black strain, whereas infection with serotype II FECV-1683 strain did not induce PD-L1 expression (193). In addition, PD-L1 expression induced by FIPV suppressed T cell activation in a co-culture assay with Jurkat cells. Furthermore, we found that feline IFNs, particularly IFN- $\gamma$ , are potent inducers of PD-L1 expression in these cells. These findings suggest that FIPV exploits host immune pathways to suppress T cell activity, thereby facilitating survival of FIPV.

Given these findings, we sought to investigate PD-L1 expression in the tissues of cats with naturally occurring FIP. Using post-mortem tissue samples, we assessed the PD-L1 expression and FCoV using real-time RT-qPCR, and examined the co-localization of PD-L1 and FCoV using confocal microscopy. The results revealed clustered regions of co-localized PD-L1 and FCoV. These findings support our previous findings and warrant further studies on the role of PD-L1 and T cells in FIP development in cates.

## **4.3 Materials and Methods**

### **4.3.1 Sample collection and preparation**

Frozen and fixed tissue samples were obtained from three deceased cats (designated as A, B, and C) diagnosed with FIP by immunohistochemistry (IHC) at Kansas State University

Veterinary Diagnostic Lab. Frozen tissues were initially stored at -20°C before being transferred to -80°C. For histology, IHC, and confocal microscopy, tissue samples were fixed in 10% formalin (StatLab, McKinney, Texas) for a minimum of 24 hours prior to paraffin embedding. Tissues were dehydrated in increasing concentrations of ethanol (70-100%) 6 times for 240 minutes at 40°C in a Sakura Tissue-Tek VIP 6 Vacuum Infiltration Processor (Sakura Finetek USA, Inc., Torrance, California). Tissues were embedded in paraffin (Leica Biosystems, Nussloch Germany), heated to 61°C, and sectioned into 4 µm thick using a Leica RM2235 rotary microtome (Leica Biosystems, Nussloch, Germany). Tissues were transferred to positively charged histological slides until further preparation.

#### **4.3.2 Histology and immunohistochemistry**

Histopathology slides were processed using a Leica BOND-MAX automatic stainer (Leica Microsystems, Nussloch Germany) with the Bond Polymer Refine Detection kit and DAB chromogen (Leica Biosystems, Nussloch Germany). Pretreatment antigen retrieval was performed with BOND Epitope Retrieval Solution 2 (Leica Biosystems, Newcastle, United Kingdom), an EDTA based pH 9.0 solution, for 20 minutes at 100°C. Primary incubation of the mAb FIPV3-70 clonal antibody (Custom Monoclonals International, Sacramento, California) at a dilution of 1:1000 was performed for 15 minutes at RT. Secondary incubation with the PowerVision Poly-HRP  $\alpha$ -mouse IgG polymer (Leica Biosystems, Newcastle, United Kingdom) was then performed for 25 minutes at RT. The slide was then counterstained with Hematoxylin from the Bond polymer Refine Detection kit according to the manufacturer's protocol.

### **4.3.3 RNA extraction from tissues**

For RNA extraction, 60 mg of each tissue was homogenized with an Omni Bead Ruptor 24 (Kennesaw, GA) homogenizer using 1.4mm ceramic beads. Homogenates were centrifuged at 13,000 RPM for 3 minutes prior to aliquoting for RNA extraction. Total RNA of cell lysates was extracted using Qiagen RNeasy Mini purification kit (Germantown, MD).

### **4.3.4 Sequencing and genetic analysis of 3CLpro**

Complimentary DNA was generated using the Promega GoTaq 2-step RT-qPCR System (Madison, WI) with a reverse primer targeting the FCoV 3C-like protease (3CLpro) on samples with the highest viral load from each cat (liver from Cat A, spleen from Cat B, and small intestine from Cat C), following the manufacturer's protocol. PCR amplification was performed with an initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec), and extension (72°C for 1.5 minutes). PCR products were purified using the Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI) and sequenced by Genewiz (South Plainfield, NJ) using the 3CLpro primers listed in Appendix Table 1. The phylogenetic tree was generated using Clustal Omega.

### **4.3.5 Real-time qRT-PCR for PD-L1 and FIPV**

RT-qPCR was performed using the SuperScript One-Step RT-PCR system with Taq DNA Polymerase (Thermo Fisher) for feline PD-L1 (fPD-L1), FIPV, and  $\beta$ -actin as a control. For cDNA synthesis, samples were incubated at 50 °C for 30 minutes, then pre-denatured at 95 °C for 2 minutes. Amplification was conducted over 40 cycles, with denaturation at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds. Appendix

Table 1 contains the sequences of primers and probes used for real-time RT-qPCR. Relative mRNA levels of PD-L1 and FCoV RNA, normalized to  $\beta$ -actin, were calculated using the  $2^{-\Delta ct}$  method.

#### **4.3.6 Immunofluorescence confocal microscopy**

Sections of the small intestine and mesenteric lymph nodes from Cat C were examined using the confocal microscopy. Slides for immunofluorescence confocal microscopy were manually prepared and processed for immunofluorescence staining. Paraffin-embedded tissue slides were treated with XS-3 for three times for three minutes each. Slides were rehydrated using decreasing concentrations of ethanol (100%, 95%, 70% and 50%) for 5 minutes each. Slides were rinsed with deionized distilled water before treatment with PBS with 0.05% Tween20 for 10 minutes. Antigen retrieval was performed by heat induced antigen retrieval BOND Epitope Retrieval Solution 2 (Leica Biosystems, Newcastle, United Kingdom), an EDTA based pH 9.0 solution, for 20 minutes at 100°C, and washed with deionized distilled water once cooled. Slides were permeabilized with 0.1% Triton-x100 (Fisher Scientific, Pittsburgh, PA) in PBS 10 min at RT. After three washes with PBS, the slides were incubated with a blocking buffer of 4% nonfat dried milk in PBS with 0.05% Tween20 for 1 hr at RT. Slides were then treated with rabbit anti-PD-L1 antibodies 28-8 and RM1012 (Abcam, Waltham, MA), and/or E1L3N rabbit mAb (Cell Signaling Technology, Danvers, MA), and mouse anti-FCoV antibodies (MyBioSource, San Diego, CA) diluted in blocking buffer at 4°C overnight. Anti-FCoV antibodies were diluted in a 1:250 ratio, anti-PD-L1 clone 28-8 was diluted at a 1:100 ratio, and anti-PD-L1 RM1012 clone and E1L3N clone were diluted at a 1:200 ratio. Cells were washed three times with PBS for 5 min before incubation with an anti-rabbit secondary antibody conjugated with AlexaFluor 488

(1:1,000) or an anti-mouse secondary antibody conjugated with AlexaFluor 647 (1:1,000) for 2 h at RT. Cells were washed three times with PBS before coverslips were mounted in Prolong Gold Antifade Mountant with DNA Stain DAPI (Invitrogen, Waltham, MA). Cells were scanned with a Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany). An unstained slide was used to calibrate the scope and remove autofluorescence signals. Fiji software was used to process images, generate the Costes' mask, and calculate the Manders' Coefficients using Costes' automatic threshold.

## **4.4 Results**

### **4.4.1 Gross and histological lesions of Cat A, B, and C**

The primary gross findings in Cat A (3-year-old, male neutered, domestic short hair) included ascites, fibrin deposits on the serosa, and multifocal to coalescing hepatic nodules (Fig. 4.1A, Table 5). Histologically, the liver showed severe, multifocal, pyogranulomatous to lymphocytic, histiocytic, and neutrophilic necrotizing hepatitis (Fig 4.2A). The spleen showed mixed inflammatory cells and fibrin accumulation on the serosal surface. Few changes were noted in the kidney histologically. Severe, chronic serositis and chronic pyogranulomatous lymphadenitis were also observed histologically. Diagnostic IHC for FCoV performed on the liver and spleen showed strong positive staining within inflammatory foci in the liver and along the serosal surface of the liver and spleen (Fig. 4.2B).

Bicavitary effusion was noted on Cat B (11-year-old, female spayed, domestic shorthair) with fibrinous and granulomatous peritonitis and pleuritic (Table 5). The whole body was moderately to severely icteric. The capsules of the spleen and liver had multifocal to coalescing granulomatous lesions and fibrin deposits (Fig. 4.1B). There was fibrinous and granulomatous

serositis with diffuse inflammation of the mucosal surface of the small intestine. Severe lymphadomegaly with congestion was observed in the mesenteric and colonic lymph nodes. Lymph nodes and multifocal hepatic areas exhibited strong FCoV IHC staining (Fig. 4.2D).

Gross necropsy of Cat C (8-month-old, female spayed, Bengal) showed severe ascites, along with adhesions of the omentum to the large intestine and peritoneum (Fig. 4.1C, Table 5). Off-white to brown nodules and plaques diffusely covered the abdominal serosa and visceral surfaces, along with the serosal surface of the small intestine. Lymphadenopathy of the mesenteric lymph nodes and hepatic lipidosis with a reticular pattern was observed. The kidneys were unremarkable outside of a focal nodule. Limited histology was performed on the spleen, lymph node, and small intestine. The spleen showed severe fibrinosuppurative and necrotizing splenitis with lymphoid depletion and capsular fibrin deposition. Severe, acute, fibrinosuppurative and necrotizing lymphadenitis with lymphoid depletion was observed in the lymph nodes (Fig. 4.3C). The small intestine had severe, acute, fibrinosuppurative serositis/peritonitis with extension into the tunica muscularis (Fig. 4.3A). Diagnostic IHC for FCoV showed strong positive staining in the lymph nodes (Fig. 4.3D), multifocal areas of strong staining in the intestinal serosa, strong positive staining in the lumen of the intestine (Fig. 4.3B), and mild staining in the liver.

**Table 5 Summary of clinical signs and histopathological lesions of three cats with FIP**

|                                                               | <b>Cat A</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | <b>Cat B</b>                                                                                                                                                                                                                                                                                                          | <b>Cat C</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
|---------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Signalment</b><br>(Age, Sex, Breed)                        | 3 year old<br>MN<br>DSH                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | 11 year old<br>FS<br>DSH                                                                                                                                                                                                                                                                                              | 8 month old<br>FS<br>Bengal                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| <b>Clinical Signs/Given History</b>                           | Lethargy<br>Pyrexia<br>Inappetence<br>Ascites<br>Leukocytosis<br>Lymphadenopathy                                                                                                                                                                                                                                                                                                                                                                                                                         | Unable to thermoregulate<br>Thrombocytopenia<br>Lymphocytosis<br>Monocytosis<br>Hyperphosphatemia<br>Hypocalcemia<br>Hypochloremia<br>Hypoalbuminemia<br>Hyperglobulinemia<br>Hyperbilirubinemia                                                                                                                      | Self-resolving hematochezia (1 month prior)<br>Found deceased                                                                                                                                                                                                                                                                                                                                                                                                 |
| <b>Form</b>                                                   | Wet                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | Mixed                                                                                                                                                                                                                                                                                                                 | Mixed                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| <b>Effusion</b>                                               | Abdominal                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Abdominal, thoracic                                                                                                                                                                                                                                                                                                   | Abdominal, Pericardial                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <b>Primary Lesions</b>                                        | <p><u>Whole Body:</u> Abdominal effusion</p> <p><u>Peritoneum:</u> Severe, chronic, fibrinous and pleocellular serositis</p> <p><u>Liver:</u> Severe multifocal pyogranulomatous to lymphocytic, histiocytic, and neutrophilic necrotizing hepatitis</p> <p><u>Lung:</u> Mild to moderate interstitial pneumonia with diffuse pulmonary congestion and edema</p> <p><u>Lymph Nodes:</u> Moderate lymphoid hyperplasia, sinus histiocytosis, and multifocal to chronic pyogranulomatous lymphadenitis</p> | <p><u>Whole body:</u> Moderate to severe icterus, abdominal and thoracic effusion</p> <p><u>Liver:</u> Severe, chronic granulomatous hepatitis with fibrinous capsulitis</p> <p><u>Lymph Nodes:</u> Severe, chronic granulomatous lymphadenitis</p> <p><u>Spleen:</u> Capsulitis</p> <p><u>Kidney:</u> unreported</p> | <p><u>Whole body:</u> Abdominal and pericardial effusion</p> <p><u>Liver:</u> Moderate lipidosis</p> <p><u>Lung:</u> Moderate congestion</p> <p><u>Lymph Nodes:</u> Severe, acute, fibrinosuppurative and necrotizing lymphadenitis with lymphoid depletion and intralesional bacteria</p> <p><u>Spleen:</u> Severe, acute, fibrinosuppurative and necrotizing splenitis</p> <p><u>Intestine:</u> Severe, acute, fibrinosuppurative serositis/peritonitis</p> |
| MN= male neutered, DSH= domestic shorthair, FS= female spayed |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                               |

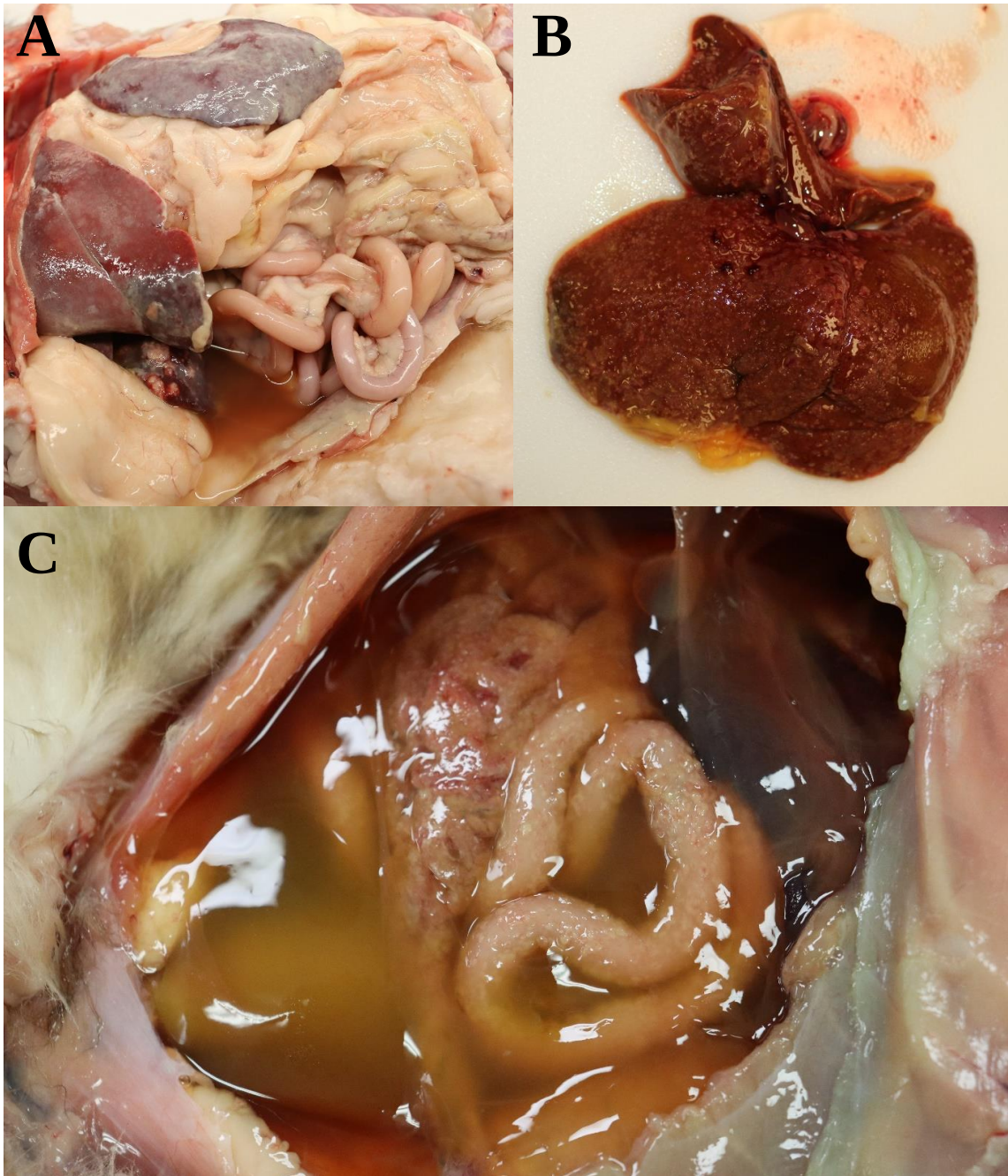


**Figure 4.1 Gross Lesions**

(A) Gross appearance of abdomen of Cat A. Opaque, fibrinous plaques are observed on the serosal surface of the spleen and liver. There is an accumulation of viscous fluid in the abdominal cavity. (B) Gross lesions on liver of Cat B. Fibrin deposits and granulomatous inflammation are present on the serosal surface of the liver. (C) Gross appearance of the abdomen of Cat C. The abdominal cavity displays fibrinous peritonitis and contains a large volume of pale orange effusion. Serosal plaques can be observed on the small intestine.

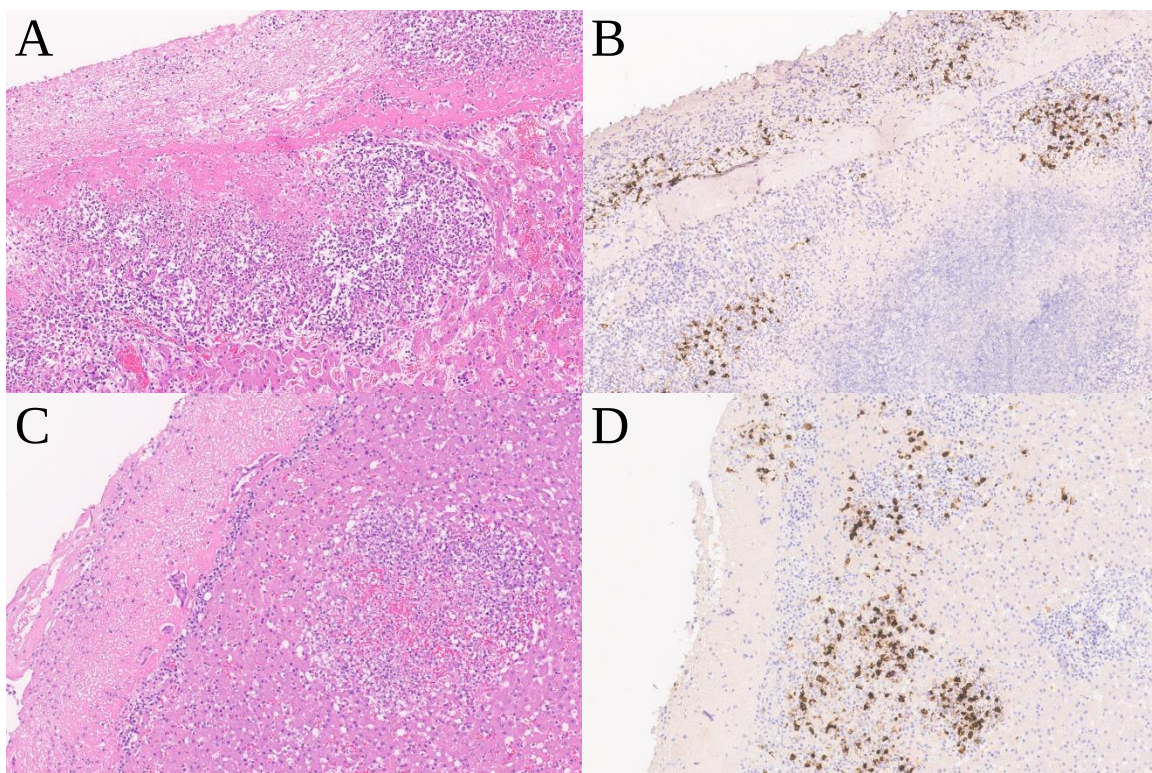


**Figure 4.2 Histological lesions of Cat A (A and B) and Cat B (C and D).**

(A) Cat A, liver, H&E stain, 10x magnification. Severe multifocal pyogranulomatous to lymphocytic histiocytic, and neutrophilic necrotizing hepatitis. (B) Cat A, liver, FCoV IHC, 10x magnification. Strong positive in inflammatory foci in the liver and throughout the capsule. (C) Cat B, liver, H&E, 10x magnification. Severe, chronic multifocal hepatitis with fibrinous capsulitis. (D) Cat B, liver, FCoV IHC, 10x magnification. Multifocal areas of strong staining throughout the capsule and parenchyma in inflammatory foci.

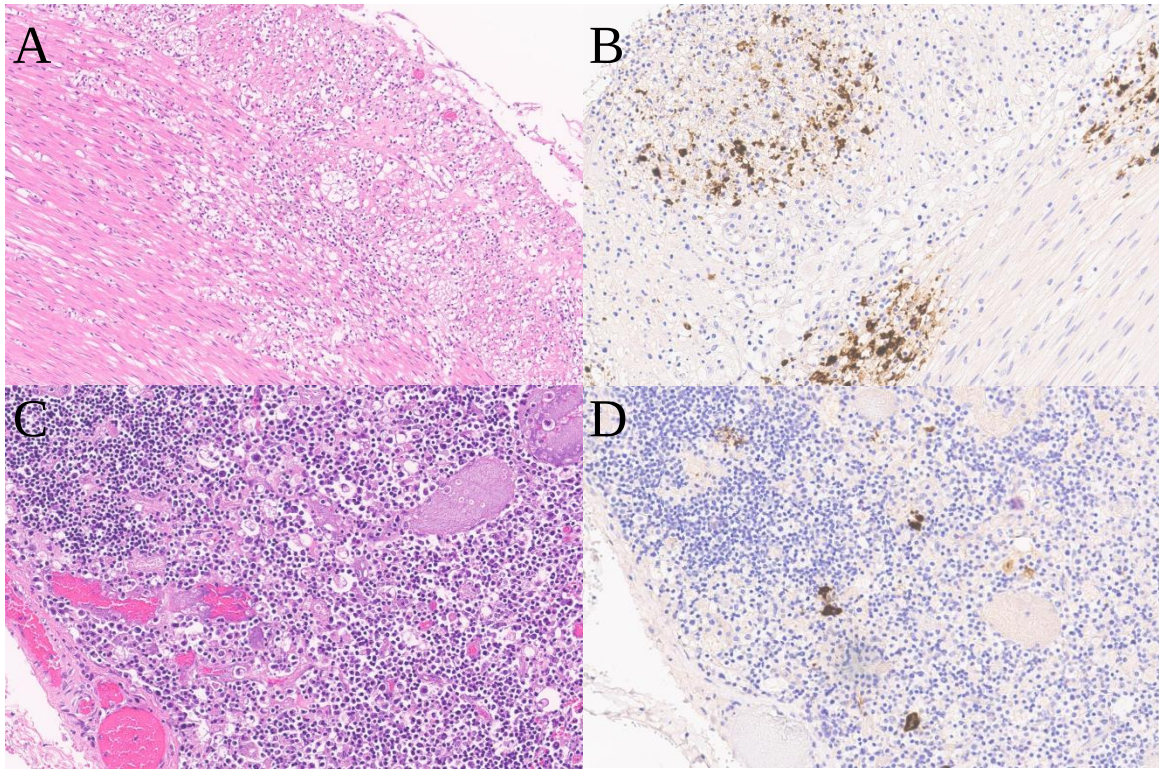


**Figure 4.3 Histological lesions of Cat C.**

(A) Small intestine serosal surface, H&E stain, 10x magnification. Severe, acute, fibrinosuppurative serositis/peritonitis. (B) Small intestine, FCoV IHC staining, 20x. Multifocal regions of strong staining in inflammatory foci in the serosa and tunica muscularis. (C) Lymph node, H&E, 20x. Marked lymphoid depletion with poorly developed follicles. Large numbers of degenerate neutrophils mixed with bacterial colonies. (D) Lymph node, FCoV IHC staining, 20x. Multifocal areas of strong FCoV staining were seen throughout the node.

#### 4.4.2 Phylogenetic analysis of 3CLpros from each cat.

The FIPV 3CLpro sequences from each cat were sequenced and the deduced amino acid sequence was aligned with those from 15 other coronaviruses available in GenBank, including serotype II FIPV-1146 and FECV-1683, as well as serotype I FIPV-Black. Phylogenetic analysis demonstrated that the FIPV 3CLpros from the three cats were not closely related, each segregating onto a distinct branch of the tree (Fig. 4.4).

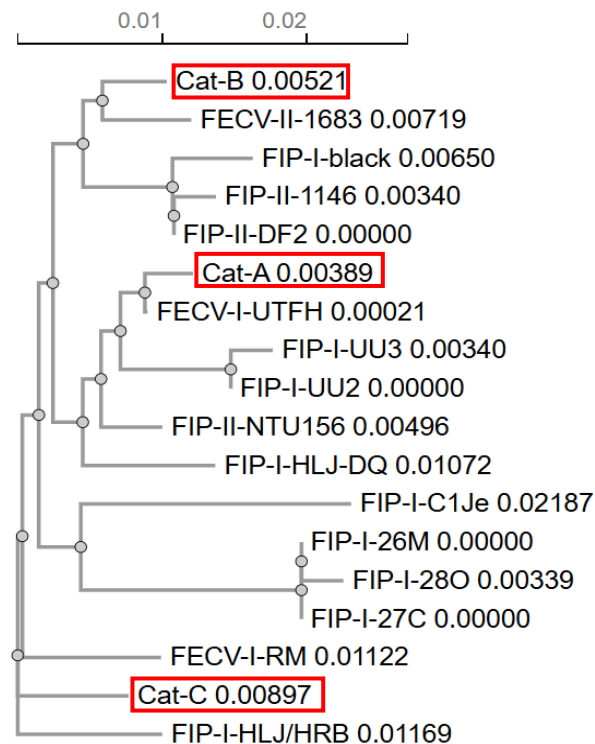


**Figure 4.4 Phylogenetic analysis of FCoV 3CLpro from Cat A, B and C**

#### 4.4.3 Viral load and PD-L1 mRNA expression

Relative PD L1 expression in relation to viral load was variable among cats (Fig. 4.5A-D). Cat A demonstrated elevated PD L1 expression alongside increasing FCoV load, with the liver showing the greatest values. (Fig. 4.3A), whereas Cats B and C showed no clear relationship between viral load and PD L1 expression (Fig. 4.5B and C).

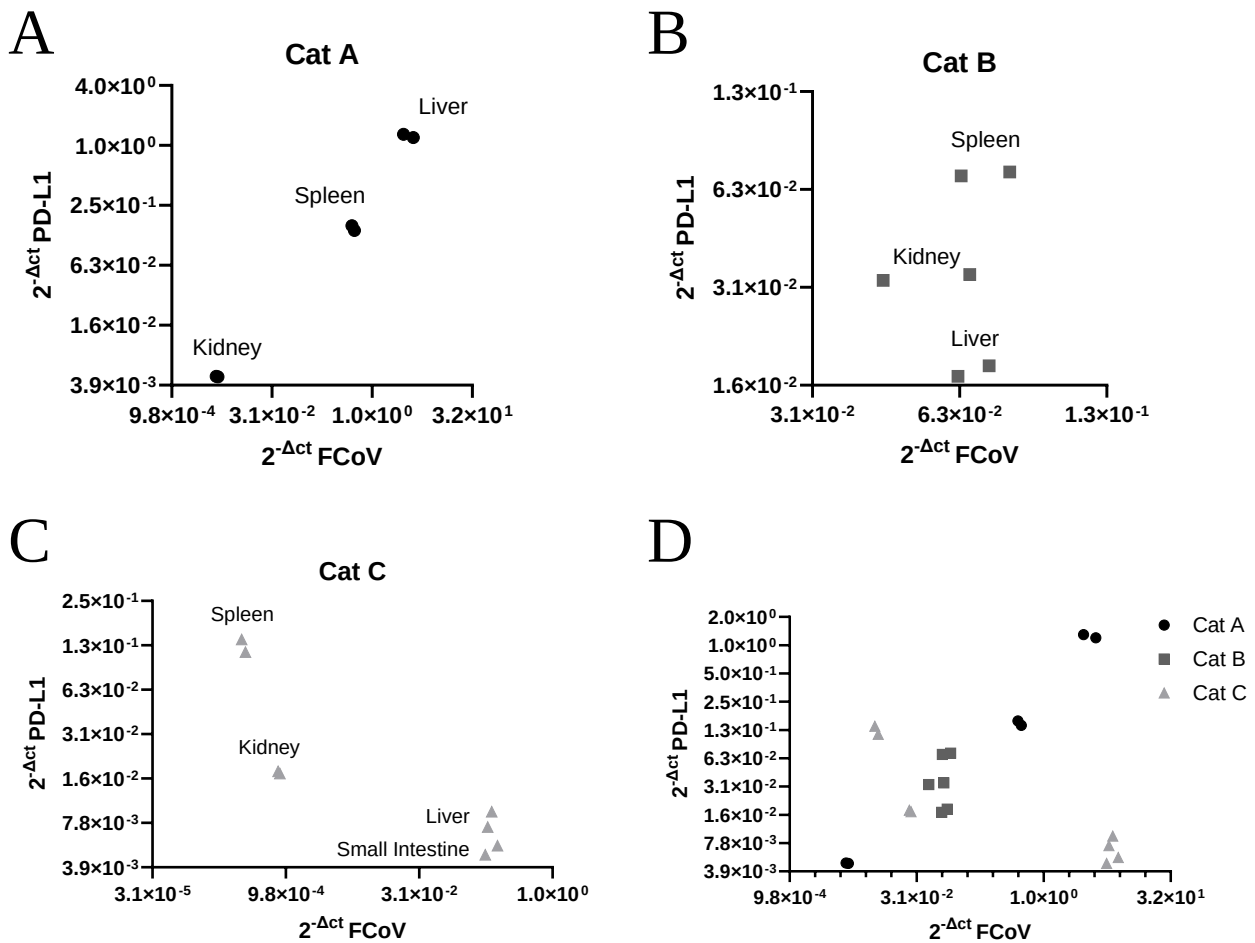


**Figure 4.5 The relative levels of PD-L1 mRNA and FIPV RNA**

Relative PD L1 mRNA (Y-axis) and FCoV RNA (X-axis), normalized to  $\beta$  actin, were plotted as  $2^{-\Delta Ct}$  values for Cat A (A), B (B), and C (C). The values from all cats are plotted together in D.

#### **4.4.4 Confocal microscopy of PD-L1 expression in the small intestines and lymph nodes from Cat C**

The lymph nodes were strongly positive for FCoV, with multifocal staining distributed across both cortical and medullary regions (Fig. 4.6A). The subscapular and medullary sinuses were strongly positive for PD-L1, and mild to moderate PD-L1 expression was observed within regions of strong FCoV staining (Fig. 4.6A). In the small intestine, multifocal areas of strong FIPV and PD-L1 staining were observed in the serosal layer (Fig. 4.6B). There was very mild PD-L1 staining throughout the serosa where no virus was detected (Fig. 4.6B and C). Manders' coefficient analysis revealed that approximately 97% of PD L1 expression overlapped with FCoV, while 63% of FCoV overlapped with PD L1 expression in the lymph node section (Fig. 4.2A). In the small intestine section, approximately 99% of PD-L1 expression overlapped with FCoV and approximately 90% of FCoV overlapped with PD L1 (Fig. 4.2C).

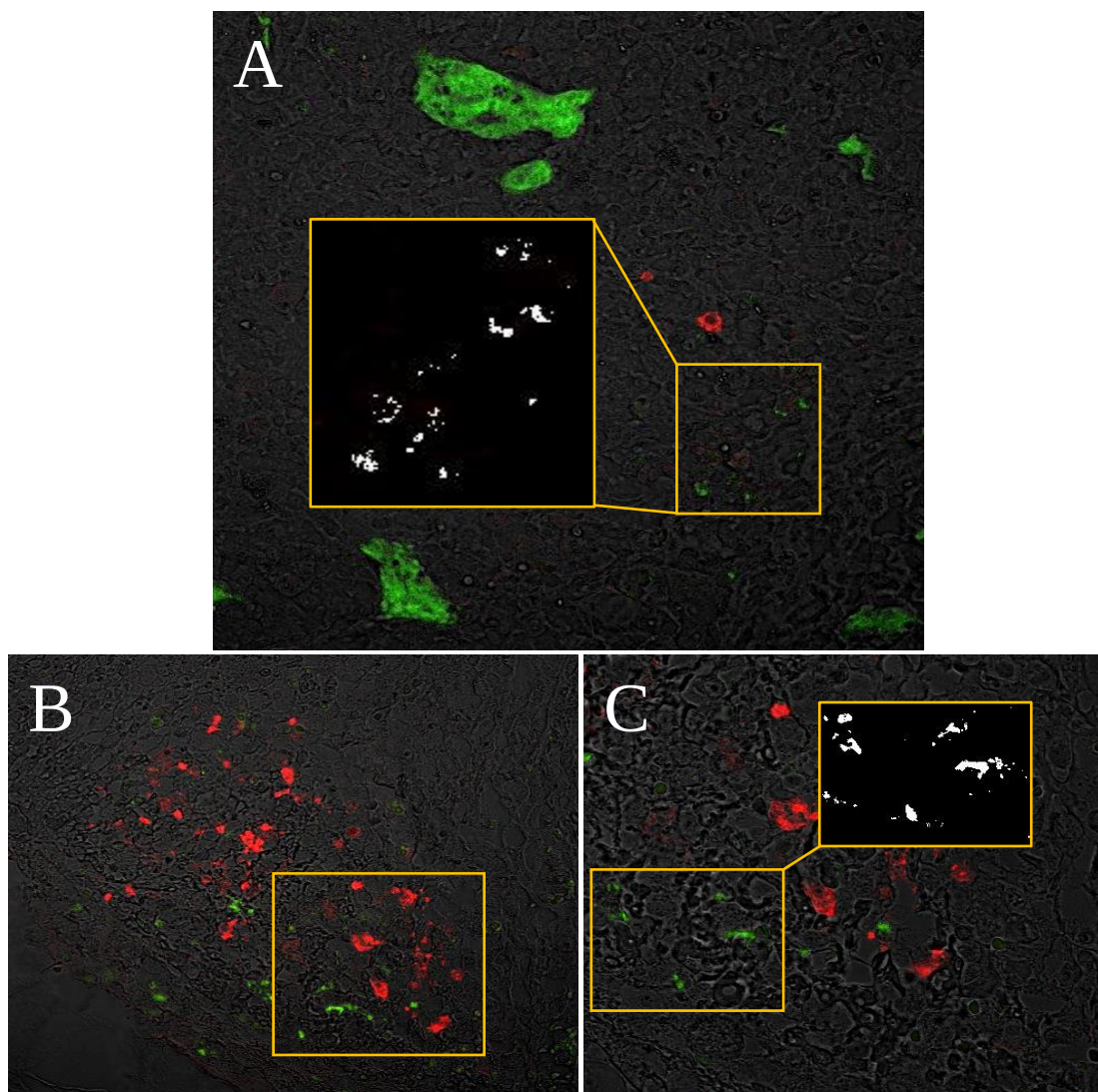


**Figure 4.6 Confocal images of the lymph node and small intestine of Cat C**

(A) The lymph node of Cat C at 20X magnification with Costes' mask (white) showing colocalization of PD-L1 (green) and FIPV (red). (B) Serosa of the small intestine at 20X magnification with strong PD-L1 and FCoV expression. The yellow box indicates the area visualized at 40X. (C) Serosa of the small intestine at 40X magnification with Costes' mask on the selected region showing colocalization.

## 4.5 Discussion

Cytotoxic T cells and natural killer (NK) cells play a crucial role in eliminating virus-infected cells, and inhibition of these cells can promote viral survival. Viruses employ diverse strategies to manipulate cell-signaling pathways, thereby attenuating host immune responses. Some viruses such as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and human cytomegalovirus (HCMV) inhibit T cell activation by preventing the surface expression of the MHC-I-peptide complex (327-335). Another mechanism by which viruses evade cell-mediated cytotoxicity is through the upregulation of immune checkpoint proteins. The PD-1/PD-L1 axis is a key checkpoint for T cells, functioning to suppress activation signaling when the T cell receptor is engaged by antigen. Many cancers and viruses have been shown to exploit the PD-1/PD-L1 signaling pathway as a mechanism of immune evasion. Viruses including LCMV, HIV, SIV, influenza virus, herpes virus, respiratory syncytial virus, hepatitis B virus, HCMV, Hantavirus and SARS-CoV-2 have been found to upregulate the expression of PD-L1 (195-197).

Additionally, PD-L1 expression can be induced via inflammatory signals such as IFNs. In particular, IFN- $\gamma$  is a potent inducer of PD-L1, as demonstrated by us (193) and other groups (191, 192). IFN- $\gamma$ , primarily secreted by T cells and NK cells, is produced in response to various inflammatory cytokines such as IL-12, IL-15, IL-18, and type I IFNs (336). Previously, it was reported that FIPV-1146 infection upregulates PD-L1 in CRFK cells, and increased PD-L1 is seen in PBMCs from cats with FIP, implicating the PD-1/PD-L1 axis in immune evasion (285). However, to date, tissue-level association of PD-1 with FIPV has not been reported, highlighting a gap in understanding of checkpoint regulation and FIP. In cats with FIP, increased levels of IFN- $\gamma$  have been reported in the tissues, including necrotizing/fibrinous and granulomatous lesions (180-182, 337). Therefore, in this study, we evaluated the expression of PD-L1 in various

tissues from three cats with naturally occurring FIP (Figure 4.5). In Cat A, the liver showed severe necrotizing hepatitis with strong IHC staining for FCoV (Fig. 4.2A and B), and RT-qPCR confirmed the highest viral and PD-L1 RNA expression in this tissue (Fig. 4.5A). The PD-L1 and FCoV RNA levels were moderate in the spleen and lowest in the kidney (Fig. 4.5A), which reflects the severity of the histopathological changes observed in these tissues. In Cat B, inflammatory foci and the serosal surface of the liver stained very strongly on diagnostic FCoV IHC (Fig. 4.2D). Interestingly, while viral load was relatively similar across tissues, PD-L1 expression varied considerably between them (Fig. 4.5B). In Cat C, strong intestinal serosal staining (Fig. 4.3B) and mild hepatic staining were noted on diagnostic FCoV IHC, but PD-L1 expression again did not correspond to viral levels (Fig. 4.5C). This variability in PD-L1 expression and viral levels among different cats cannot be fully explained at present. However, potential contributing factors may include the timing of PD-L1 induction following viral infection, differences in viral strains, and tissue-specific immune responses, and requires further investigation.

The PD-1 levels observed in these cats are inclusive of induction with FIPV infection, as no uninfected control samples were available. However, when the small intestine and lymph node from Cat C were assessed for co-localization of FCoV and PD-L1 using confocal microscopy, cells stained positively for both FCoV and PD-L1 were observed throughout the tissues, with particularly strong staining in the intestinal serosa for both FCoV and PD-L1 (Fig. 4.6B and C). Notably, Manders' coefficient analysis showed that nearly all PD-L1 expression co-localizes with FCoV, whereas a smaller proportion of FCoV signal co-localizes with PD-L1. This observation may be explained by the possibility that cells with strong FCoV signal were undergoing necrosis, as the regions exhibiting intense staining were primarily composed of proteinaceous material and

fibrin (Fig. 4.3A). Additionally, cells expressing PD-L1 in the absence of detectable FCoV may represent non-virus-susceptible cell populations, with PD-L1 expression driven by inflammatory cytokines such as IFN- $\gamma$  rather than direct viral infection. Our previous work (193) showed that feline IFN- $\gamma$  is a strong inducer of PD-L1 in CRFK and Fcwf-4 cells, and inflammatory cytokines are likely abundant in tissues with necrosis, fibrosis and granulomatous inflammation (Figure 4.2 and 4.3).

There is strong evidence that T cells are largely responsible for the control of FCoV infection and the prevention of FIP (175, 326). As such, increased PD-L1 expression would be most advantageous to FIPV during the early stages of tropism change and systemic spread and establishment of FIP infection. As FIP progresses, however, lymphopenia often arises, rendering PD-L1 expression largely less efficacious in suppressing T cell responses. In end stage FIP, as examined here, the relationship of FIPV and PD-1/PD-L1 axis may be less evident, which underscores the need for investigations focused on the early stages of FIP in cats.

## **Chapter 5 - Discussion and future directions**

Coronaviruses continue to be major pathogens in both humans and animals. As has been shown through the last decades, novel animal-originated coronaviruses in humans with increased pathogenicity continue to emerge. In addition to the impacts on human health, they continue to lead to economic losses in the agriculture industry and emotional distress when companion animals succumb to severe coronavirus infections. As territories of humans and domestic animals expands, there is an increasing risk of novel coronavirus emergence as there is closer contact to reservoir species such as bats. Research has demonstrated the various SARS-like coronaviruses and other coronaviruses circulating in bat populations. With viral mutations and crossover events, the probability of a spillover event of bat coronaviruses to humans or domestic animals remains a constant threat. The knowledge gained from the SARS-CoV outbreak provided the framework for a rapid response in identification and containment efforts in the SARS-CoV-2 and MERS-CoV outbreaks. It is imperative to understand basic biology and pathogenicity of coronaviruses so we can continue to improve responses to emerging threats.

Coronaviruses utilize various host proteins to enhance their entry, replication, immune evasion, and pathogenicity. Because the coronavirus S is primarily responsible for attachment and entry, changes in S can lead to increased binding affinity to the functional receptor and increased processing by host proteases, subsequently improving entry efficiency, infectivity, zoonotic potential, and/or virulence. Once S is bound to its functional receptor, further processing occurs to expose the fusion domain and facilitate viral entry into the cell. Various host proteases have been found to process S from many coronaviruses, but the two major proteases directly involved in the entry process are TMPRSS2 and cathepsins. TMPRSS2 expression has been found to process the S2' cleavage site at the level of the cell membrane to expedite viral entry into the cell.

Cathepsins are implicated in processing S following endosomal entry to enable viral endosomal escape. However, the major protease processing of S typically occurs prior to viral release from the infected cell by furin. The processing of the S1/S2 site by furin is believed to be linked to improved receptor availability, receptor affinity, and improved accessibility of the S2' site to proteases such as TMPRSS2.

In our first study, we assessed the entry ability and protease processing of S from SARS-CoV, SARS-CoV-2 (parental and Omicron variants), and MERS-CoV. Using a lentivirus-based pseudotyped virus assay, we assessed the entry potential of SARS-CoV, SARS-CoV-2 parental strain and SARS-CoV-2 Omicron variants BA.1.1, BA.4/5, XBB.1, and JN.1 into cells stably expressing animal ACE2. It was found that all of the tested SARS-CoV and SARS-CoV-2 pseudoviruses efficiently entered cells expressing ACE2 from various species including dog, cat, hamster, and white-tailed deer. We found variable processing of the S1/S2 cleavage site by furin, and the presence of TMPRSS2 significantly increased the entry of all tested coronaviruses. Continued surveillance using the pseudotyped virus assay with protease susceptibility testing would provide crucial information on preparedness for potential threats to humans and animals by novel coronaviruses.

In addition to the utilization of host proteases to increase viral entry, coronaviruses have been shown to interact with proteins involved in immune signaling, subsequently promoting immune evasion. FIP is a fatal systemic disease that can occur in a minority of cats infected with ubiquitous FECV. While viral mutation shifting cell tropism from enterocytes (FECV) to macrophages (FIPV) has been shown to play a crucial role in FIP development, T cell responses are another major factor in disease development and progress. Our second study assesses the virus-mediated expression of PD-L1 in feline cells infected with FIPV and FECV. We found that

infection with FIPV-1146 or FIPV-Black significantly increased PD-L1 expression, while infection with FECV did not, despite comparable virus replication levels. We confirmed PD-L1 expression induced by viral infection could attenuate T cell activation using the co-culture assays with Jurkat cells. Furthermore, IFNs including IFN- $\gamma$  are potent inducers of PD-L1 expression in feline cells, confirming that IFNs as well as inflammatory cytokines are major modulators for the PD-1/PD-L1 axis. These novel findings suggest that FIPV modulates PD-L1 in target cells to evade T cell-mediated killings and promote persistent infection in cats.

Our third study assessed the PD-L1 expression in the post-mortem tissues of cats with naturally acquired late-stage FIP. We found that FIP and PD-L1 levels were variable among tissues and cats based on real time RT-qPCR. On confocal microscopy, we found that PD-L1 expression was colocalized with virus presence, and strong expression of FCoV and PD-L1 was typically found in severe inflammatory foci. It is likely that PD-L1 expression in late stage FIP is multifactorial and results from a combination of viral infection and IFN- $\gamma$  signaling from inflammation.

Our studies have demonstrated the complex interplay between coronavirus infection/replication and host proteins. The COVID-19 pandemic will likely not be the last novel coronavirus outbreak that poses a threat to public health, and methodologies for identifying potential factors of virulence, transmissibility, zoonotic potential, and rapid vaccine development will be critical to combat potential coronavirus outbreaks. There are still many unknowns about how various coronaviruses can evade the immune response, especially concerning the events that lead to the development of FIP. PD-L1 was first identified in its role in cancer immune evasion, and further studies identified many viruses that utilize PD-L1 to attenuate T cell response through various mechanisms. It was also shown that the neutralization of the PD-1/PD-L1 axis could clear

persistent virus infections *in vivo*. Further study of the role of PD-1/PD-L1 in the development of FIP as well as other coronavirus infections is crucial, and the immune checkpoint blockade may serve as a valuable adjunct to FIP antivirals that are currently available, potentially enhancing viral clearance and reducing the likelihood of disease recurrence.

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## Appendix A - Primers

**Appendix Table A-1 Primers**

| Primer/probe                            | Sequences                                                |
|-----------------------------------------|----------------------------------------------------------|
| <b>fPD-L1 Forward</b>                   | 5' - CTATGGCGGTGCTGACTATAAG - 3'                         |
| <b>fPD-L1 Reverse</b>                   | 5' - CCTCAGCCTGACACATTAGTT - 3'                          |
| <b>fPD-L1 Probe</b>                     | 5' - /56-FAM/TCTGTGGAT/ZEN/CCTGTACCTCTGAACA/3IABkFQ - 3' |
| <b>fPD-L2 Forward</b>                   | 5' - ACCCGAGGGATGTGGAATA - 3'                            |
| <b>fPD-L2 Reverse</b>                   | 5' - CAGTCTGCAGAAGGTGGAAA - 3'                           |
| <b>fPD-L2 Probe</b>                     | 5' - /56-FAM/CCACTTTGC/ZEN/TGGAGGAGCATCTGT/3IABkFQ/-3'   |
| <b>FCoV 3' UTR Forward</b>              | 5' - GATCCAGACGTTAGCTCTTCC - 3'                          |
| <b>FCoV 3' UTR Reverse</b>              | 5' - GGAGGTACAAGCAACCCTATT - 3'                          |
| <b>fIFN-<math>\alpha</math>-Forward</b> | 5' - GTCAGAAGGACAGGAATGACTT - 3'                         |
| <b>fIFN-<math>\alpha</math>-Reverse</b> | 5' - AGGCACAAGGGCTGTATTT - 3'                            |
| <b>fIFN-<math>\beta</math>-Forward</b>  | 5' - CATCCTCCAAATCGCTCTCTT - 3'                          |
| <b>fIFN-<math>\beta</math>-Reverse</b>  | 5' - CGTTTCCAGGTGTTCTTCT - 3'                            |
| <b>FCoV 3CLpro Forward</b>              | 5' - AATGAGTTGTCTAGTGTGCGTTTA - 3'                       |
| <b>FCoV 3CLpro Reverse</b>              | 5' - TAATACTAACAAATGTCGGGTAAAT - 3'                      |