



Regulation of host cellular environment during coronavirus infection

Master thesis by

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This work was done by my many.

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Abstract

Despite the unprecedented worldwide effort employed to study COVID-19, there are many aspects of SARS-CoV-2 infection that remain unknown. Here, a comparative analysis between SARS-CoV-2, SARS-CoV-1 and MERS-CoV was performed to better understand the modulation of the host cellular environment during coronavirus infection. In particular, the interplay between the innate immune response to SARS-CoV-2 infection and its mechanisms of viral escape was investigated. First, a global proteomics study of pre-existing data of infected Huh-7 cells was performed. Pathway enrichment analysis identified commonly altered pathways among all the datasets, and a differential regulation of protein expression was noted during SARS-CoV-2, SARS-CoV-1 and MERS-CoV infection. Viral interference with the host immune signalling, translation machinery, complement and coagulation cascades was observed. Furthermore, several *in vitro* assays were employed to determine the mechanism of action of the coronaviral Papain-like protease (PLpro) and assess its role on inhibition of interferon (IFN) signalling. SARS-CoV-2 PLpro showed a diminished deubiquitinating but an increased deISGylating activity when compared to its counterparts in SARS-CoV-1 and MERS-CoV. Our findings suggest that the specific interaction between SARS-CoV-2 PLpro and ISG15 leads to blocking of IFN production. Together, these results show that, while several mechanisms of infection are conserved among coronavirus, proteins from different strains act in varying ways to modulate the host immune response. This knowledge may be a useful tool for the development of effective therapies to fight viral diseases and to prevent future outbreaks.

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Introduction

From the order *Nidovirales*, *Coronaviridae* family and *Coronavirinae* subfamily, several Coronaviruses (CoVs) that cause diseases in mammals and birds have been identified to date [1]. In total, seven strains of human pathogenic CoVs (hCoVs) are known and have been found to cause respiratory infections, ranging from the common cold to deadly diseases. Most notably, Severe acute respiratory syndrome coronavirus (SARS-CoV-1) was behind an outbreak in 2003 [2], Middle East respiratory syndrome coronavirus (MERS-CoV) caused another outbreak in 2012 [3], and, most recently SARS-CoV-2, identified as the causative agent of Coronavirus disease 2019 (COVID-19), caused a pandemic that amounted almost 4 million deaths worldwide, as per the writing of this text [4]. Even though these 3 viruses are structurally and genetically similar, the epidemiology, demographics, and symptomology of the viral diseases they cause varies significantly (Table 1). This suggests that each virus employs a distinct mechanism of viral infection causing different host cellular responses and disease progression.

Table 1. Comparison between genomic similarity, virology and corresponding disease epidemiology of SARS-CoV-2, SARS-CoV-1 and MERS-CoV.

	SARS-COV-2	SARS-COV-1	MERS-COV
Overall genomic similarity compared to SARS-CoV-2	100%	79% ^[5]	50% ^[5]
Host cell receptor	Angiotensin-converting enzyme 2 (ACE2) ^[6]	Angiotensin-converting enzyme 2 (ACE2) ^[7]	Dipeptidyl peptidase 4 (DPP4) ^[8]
Disease	COVID-19	SARS	MERS
Date of emergence in human population	2019	2003 ^[2]	2012 ^[3]
Geographical spread of the outbreak	Worldwide ^[9]	29 countries ^[10]	27 countries (80% of cases in Saudi Arabia) ^[3]
Transmissibility - reproductive number (R_0)	2-6.4 ^{a[11, 12]}	1.7-1.9 ^[13]	<1 ^[13]
Total number of confirmed cases	177 105 723 ^{b[9]}	8 096 ^[10]	2 574 ^[14]
Deaths	3 834 951 ^{b[9]}	774 ^[10]	886 ^[14]
Case fatality rate	2.2% ^[9]	9.6% ^[10]	34.4% ^[14]

^a Initially estimated at 2-2.5 in Wuhan, China [11] and later 3.5-6.4 in the US and in the eight most affected countries in Europe during March 2020 before broad control measures were implemented [12].

^b Ongoing. Information as of 17.06.2021.

Even though the mortality of COVID-19 is lower than SARS and MERS [15], a high proportion of asymptomatic patients (approximately 80% [15]) that are also contagious makes this a more rapidly and easily transmissible disease [16]. In fact, the COVID-19 pandemic caused a massive number of cases and deaths, and had a social, economic, and environmental impact far greater than the previous coronavirus outbreaks. Given their vast distribution, zoonotic behaviour and the frequent recombining ability of their genome, novel human pathogenic coronaviruses are expected to emerge

periodically [17]. It is, therefore, important to study the hCoVs identified to date and the differences and similarities between them, in order to improve care for the current patients and start preparing to prevent future pandemics.

Coronavirus genomic structure and mechanism of infection

Coronaviruses are enveloped positive-sense single-stranded RNA (+ssRNA) viruses which were subdivided in four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* [18, 19]. The human pathogenic coronaviruses SARS-CoV-2, SARS-CoV-1 and MERS-CoV are classified as a *Betacoronavirus* and share a similar overall organisation of their genome: a 5'-terminal portion that encodes non-structural proteins, and a 3'-terminal portion encoding the structural and accessory proteins (Figure 1) [18].

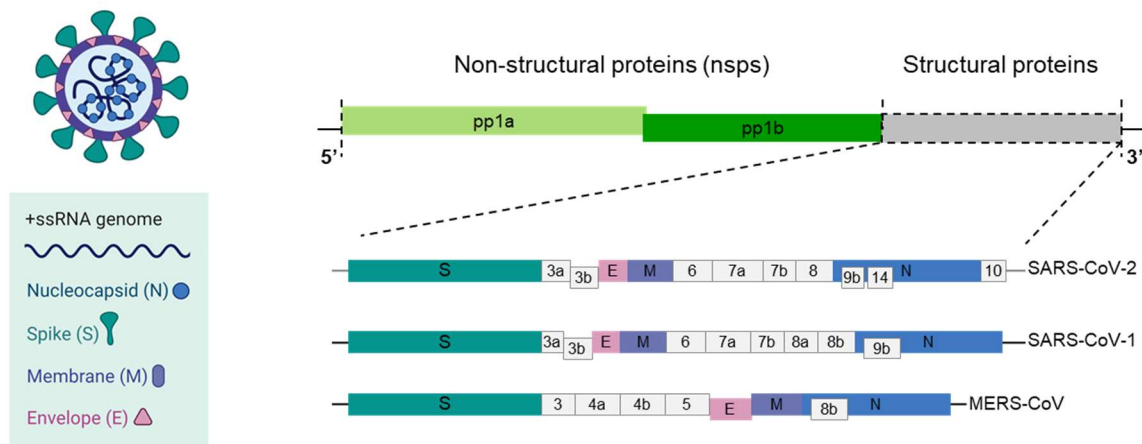


Figure 1. Coronavirus genome structure comparison between SARS-CoV-2, SARS-CoV-1 and MERS-CoV [18,19].

The structural proteins – Spike (S), membrane (M), envelop (E) and nucleocapsid (N) – make up the viral capsid while the non-structural proteins (nsps) are required for the assembly of new viral particles and are also involved in mechanisms of host immune evasion and modulation (Table 2) [5]. The two open reading frames (ORFs) found in the 5'-terminal portion of the genome are ORF1a, which encodes polyprotein (pp) 1a containing Nsp1-11, and ORF1b, which, together with ORF1a, produces pp1ab containing Nsp1-16 [18]. Different nsp3 are released by cleavage of the polyproteins by 3C-like protease (3CLpro) and Papain-like protease (PLpro), which are part of the of pp1ab/pp1a themselves [20].

Table 2. The nsps of Coronavirus. Based on several sources [1, 19, 21].

nsps	Functions
nsp1	Cellular mRNA degradation, IFN antagonist (not present in all coronaviruses)
nsp2	Unknown
nsp3	PLpro and other protein interaction domains, polyprotein processing, blocking the host innate immune response, promoting cytokine expression
nsp4	Transmembrane scaffold, involved in membrane remodelling, DMV formation
nsp5	3CLpro/Mpro, polyprotein processing, inhibiting IFN signalling
nsp6	Transmembrane scaffold, involved in membrane remodelling, restricting autophagosome expansion, DMV formation

nsp7	Forms a complex with nsp8 which is a cofactor of nsp12
nsp8	Cofactor with nsp7 and nsp12, primase
nsp9	Forms a dimer capable of binding nucleic acids in a nonsequence-specific manner, with a preference for ssRNA
nsp10	Interacts with itself, nsp1 and nsp7, scaffold protein for nsp14 and nsp16, regulation of polyprotein processing, involved in RNA capping
nsp11	Unknown
nsp12	Primer dependent RdRp domain, interacts with the nsp7-nsp8 complex, nsp13 and nsp14
nsp13	Helicase, involved in viral RNA synthesis, may also target host DNA, 5' triphosphatase involved in RNA capping
nsp14	Exoribonuclease, N7-Methyl Transferase, involved in RNA capping
nsp15	Endoribonuclease, evasion of dsRNA sensors
nsp16	2'-O- Methyl Transferase, involved in RNA capping, avoiding MDA5 recognition, inhibition of innate immunity

mRNA - messenger RNA; IFN - interferon; PLpro - Papain-like Protease; DMV - double-membrane vesicle; 3CLpro - 3-chymotrypsin-like protease also known as Mpro - main protease; RdRp - RNA-dependent RNA polymerase; dsRNA - double-stranded RNA viruses; ssRNA - single-stranded RNA; MDA5 - melanoma differentiation-associated protein 5.

When SARS-CoV-1 was first discovered and characterized, many of its proteins showed novel functional domains when compared to previous hCoVs [17]. When it comes to SARS-CoV-2, despite substantial homology to SARS-CoV-1 (79%), it contains additional gene regions and shows limited similarity between the amino acid sequences of some its proteins when compared to their counterparts in SARS-CoV-1. The distinctiveness of coronavirus proteins greatly influences the immune response to viral infection and explain the differential mechanisms of disease progression of SARS, MERS and COVID-19 [5].

Coronavirus infection and viral cell entry

The coronavirus replication cycle comprises 5 steps: attachment, penetration, biosynthesis, maturation and release (Figure 2) [1, 16]. Infection starts with the binding of viral proteins to cell surface receptors (attachment), enabling the viral particle to enter the cell through the endosomal pathway (penetration). Cell entry occurs via receptor mediated endocytosis or fusion with the host membrane, and once inside the cell, the viral RNA is delivered to the cytoplasm where its replication and transcription occurs (biosynthesis) [22]. After assembly and maturation, the new virions are then released from the host cell.

To initiate viral internalization, the MERS-CoV S protein has been shown to bind to the DiPeptidyl Peptidase 4 (DPP4) receptor and, in the same way as SARS-CoV-1, SARS-CoV-2 S can bind to the Angiotensin-Converting Enzyme 2 (ACE2) membrane receptor [6-8] (Figure 2). Moreover, recent studies reported that the S protein of SARS-CoV-2 can also interact with other cell surface receptors, such as neuropilin-1 (NRP1) and CD147, thus widening the spectrum of cells and tissues susceptible to infection and increasing viral pathogenicity [23, 24].

Activation of the S protein through cleavage by host proteases is necessary to expose its two functional subunits: S1, that binds to the host cell receptor, and S2, which is thought to be responsible for the fusion of the viral and cellular membranes [6, 16]. Transmembrane protease serine 2 (TMPRSS2) has

been shown to cleave the S protein of the 3 viruses (Figure 2) but priming of the S protein can be performed by other soluble proteases, further increasing the number of potential cell targets for coronavirus infection [8, 16, 25, 26]. Particularly, in the case of MERS-CoV, it has been reported that, if cell-surface proteases are not present, the virus can be endocytosed and endosomal proteases such as cathepsin L can cleave S to complete viral entry [27]. Also, in the absence of TMPRSS2, delayed infection occurs when SARS-CoV-2 is endocytosed and sorted into endolysosomes, later entering the cytosol via pH-activated cathepsin L protease [28].

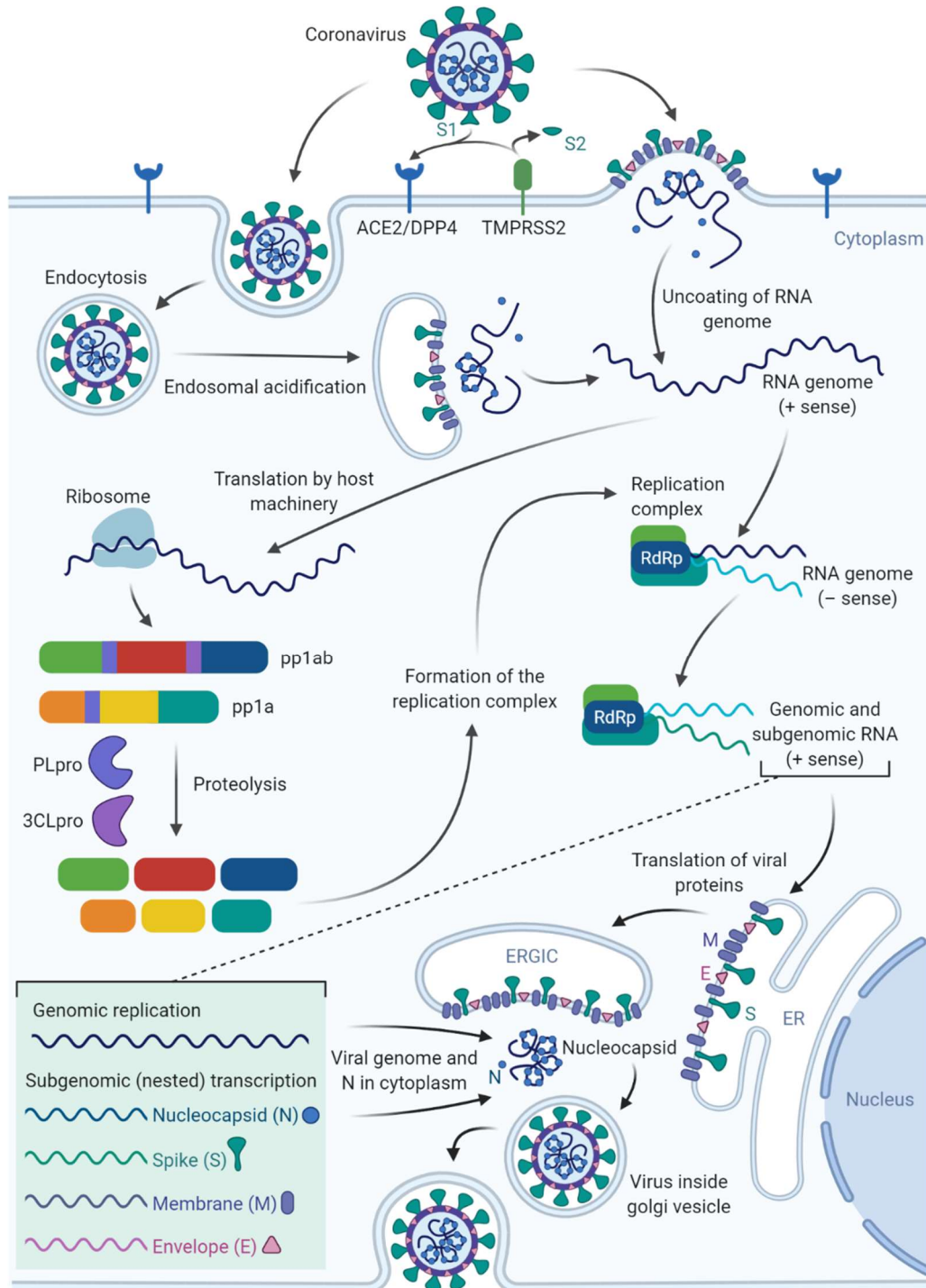


Figure 2. SARS-CoV-2 infection. S1/2 - subunits of the Spike protein; ACE2 - Angiotensin-Converting Enzyme 2; DPP4 - DiPeptidyl Peptidase 4; TMPRSS2 - Transmembrane protease serine 2; pp1ab/a - polyprotein 1ab/a; RdRp - RNA-dependent RNA polymerase; ER - Endoplasmic Reticulum; ERGIC - ER-Golgi Intermediate compartment. Figure based on several sources [8, 21, 29] and created with BioRender.com.

Furthermore, it is interesting to note that the increased transmissibility of SARS-CoV-2, when compared to SARS-CoV-1, might be partially explained by a higher binding affinity of its S protein to the ACE2 receptor [30, 31]. The ACE2 receptor is widely present on endothelial cells, being expressed in multiple organs including the lung, heart, kidney, and intestine [32]. Consistently, patients with severe cases of COVID-19, in addition to respiratory problems, also presented liver injury and cardiovascular complications [32-34]. In the airway, ACE2 expression is abundant, being the highest in the nose epithelium and decreasing throughout the lower respiratory tract [22, 35]. Notably, co-expression of ACE2 and TMPRSS2 was found within nasal goblet secretory cells and type II pneumocytes [26]. Initial infection likely happens in the upper respiratory track and subsequent aspiration allows the virus to infect the lung, where most of the COVID-19 pathology is observed [35].

Another aspect of SARS-CoV-1 and 2 viral pathogenicity lies on the dysregulation of the renin-angiotensin system (RAS) [36, 37], in which ACE2 is an important intervenient. Angiotensin I is cleaved into angiotensin II by the angiotensin-converting enzyme (ACE). Angiotensin II binds to its type 1 receptor (AT1R), resulting in vasoconstriction and increased blood pressure. In turn, ACE2, inactivates angiotensin II, hydrolysing it into angiotensin 1-7, which is a potent vasodilator. Binding of the viral S protein to ACE2 leads to its downregulation and drastically reduces the angiotensin II conversion rate. Excessive angiotensin II stimulation of AT1R increases pulmonary vascular permeability, thereby contributing to lung damage [38]. Additionally, endothelial cells also express the ACE2 receptor and there's evidence of their direct viral infection, which can be linked with the endothelial inflammation that has been observed in COVID-19 and SARS patients [32]. It is well established that endothelial dysfunction results in platelet activation and subsequent coagulation, increasing the chance of circulatory problems [39, 40]. As such, endotheliitis, which results in systemic impairment of microcirculatory function [32], in tandem with the RAS dysregulation caused by viral infection, might be the driving forces behind the emergence of cardiovascular complications associated with severe disease. Despite there being no evidence of MERS-CoV interacting with ACE2, coagulation disorders were also reported in patients with MERS [41]. This suggests that coronavirus infection might affect the coagulation cascade through several mechanisms.

Innate immune response to coronavirus infection

Even though innate immunity is crucial for the control of viral infection, the development of an exacerbated acute inflammatory response can contribute to the severity of the disease, increasing morbidity and mortality [42]. The host's ability to launch an appropriate response to the virus is, thus, indicative of disease outcome.

As the respiratory tract constitutes the interface where coronavirus infection usually begins, physical barriers, such as the mucus and lung epithelial layers, are at the forefront of antiviral defence [43]. Epithelial cells are among the first affected by virus infection and, besides functioning as a mechanical defence barrier, they are also very important players in the mounting of the immune response [44].

On the one hand, the cytopathogenicity caused by viral infection results in the release of damage-associated molecular patterns (DAMPs) that are promptly detected, triggering both humoral and cellular general immune responses. Particularly, activation of the complement system, which is a potent catalyst of inflammation, has been reported in patients with severe cases of COVID-19 [45]. Complement overactivation has been previously associated to immunopathogenesis in SARS and MERS, as well [46, 47].

On the other hand, infected cells directly respond to viral infection and secrete signalling molecules that further stimulate and sustain cascades of inflammatory response. After viral cell entry, several pathogen-associated molecular patterns (PAMPs) are recognised by host pattern recognition receptors (PRRs) triggering signalling cascades that culminate in the production of acute inflammatory response proteins, pro-inflammatory cytokines and chemokines, as well as interferons (IFNs) which together prompt a cell-intrinsic state of antiviral response [42].

The IFN signalling pathway and cytokine release

The IFN response is essential for the launching of an effective response against RNA viruses [48]. Particularly, Type I (mainly IFN- α and IFN- β) and Type III (IFN- λ) IFNs are the key players against coronavirus infection [5, 42, 48].

After uncoating of the incoming viral particle, the coronavirus genome itself and the replication intermediates are sensed by cytosolic RIG-I-like receptors (RLRs) – retinoic-acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) – and endosomal RNA receptors – particularly Toll-like receptor (TLR) 3, TLR7 and TLR8 (Figure 3) [48]. The majority of cells express RLRs but TLRs are mainly present in innate immune cells [48], and the PRR that senses the viral RNA is dependent on the virus and on the type of host cell [49]. The sensing of virus-specific RNA structures initiates signal transduction pathways that lead to the translocation of transcription factors into the cell nucleus, namely interferon-regulatory factors (IRFs) 3 and 7, and Nuclear Factor (NF) κ B. Here, these proteins stimulate the expression and subsequent release of IFN-I and IFN-III, as well as other pro-inflammatory cytokines and chemokines that, in turn, activate important antiviral mechanisms [5, 42, 43, 50].

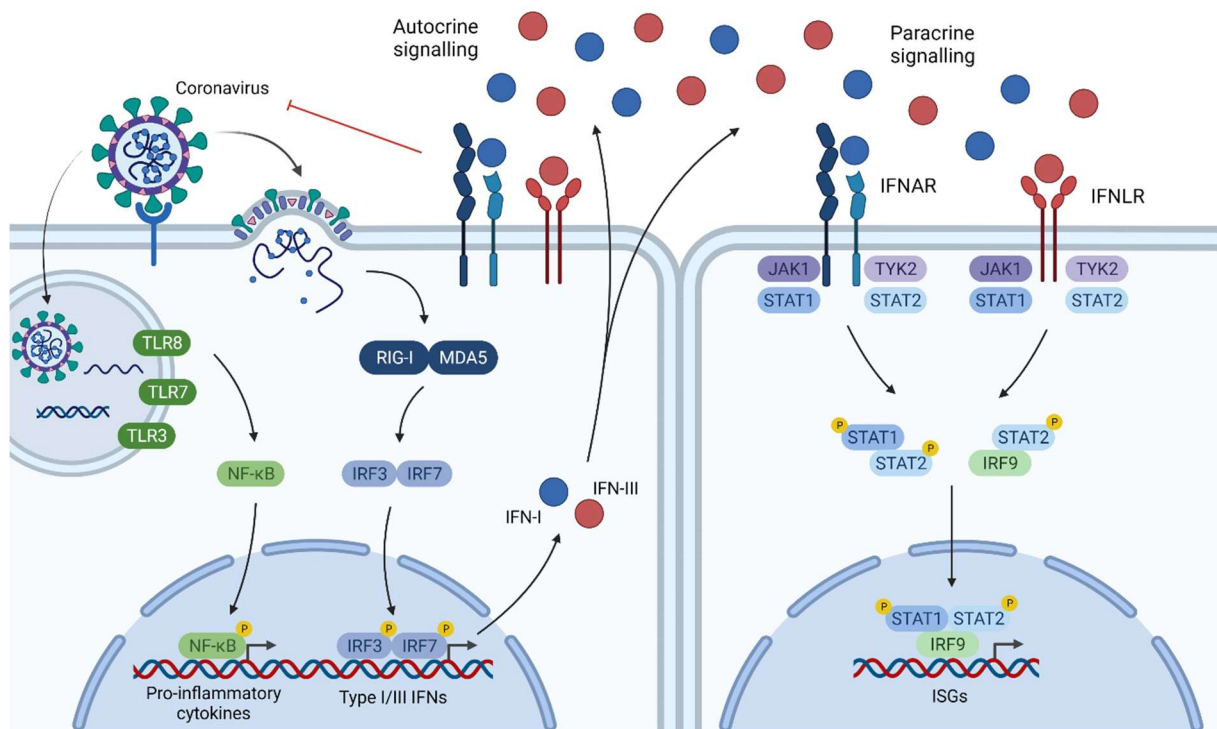


Figure 3. Innate immune recognition and interferon signalling upon coronavirus infection. Figure based on information from several sources [48, 51, 52] and created with BioRender.com.

Once secreted, IFN-I binds to its ubiquitously expressed receptor, IFNAR, and IFN-III binds to IFNLR, expressed on epithelial and certain myeloid cells [48]. IFNs can act in an autocrine or paracrine manner, and receptor stimulation activates the downstream JAK-STAT signalling pathway. The kinases JAK1 and TYK2 then phosphorylate STAT1 and STAT2 that, together with IRF9, form the IFN-stimulated gene factor 3 (ISGF3) complex. After nuclear translocation, this complex binds to IFN-stimulated response elements (ISREs) in promoters of interferon-stimulated genes (ISGs), culminating in their upregulation.

ISG products are intrinsically involved in inflammation, signalling and immunomodulation, comprising PRRs that further sensitize the cell to pathogens, several antiviral factors that inhibit specific steps of the viral replication cycle and also proteins which decrease membrane fluidity, preventing viral egress or membrane fusion. Furthermore, they activate several antiviral mechanisms such as slowing down the cell metabolism to impair viral replication or inducing the secretion of cytokines that promote the activation of cellular immunity [53].

Even though IFN-I and IFN-III induce a similar ISG signature, the former leads to a faster induction and consequent decline of ISG expression [48]. The IFN response not only helps to control viral replication in an early stage, but it is also important for the later development of an effective adaptive immune response required to fully suppress the infection [5].

Interestingly, ACE2 has been identified as an ISG meaning that IFNs stimulate its expression [26]. Though it might seem counter intuitive that the host innate immune response could increase the amount of cellular targets for viral infection, ACE2 upregulation actually has a protective effect against severe acute lung failure [38]. IFN-induced expression of ACE2 counterweighs the downregulation caused by the viral S protein, re-establishing the angiotensin II conversion rate, and thus minimizing the RAS dysregulation caused by SARS-CoV-2 and SARS-CoV-1 infection.

Parallel to IFN, the pro-inflammatory cytokines secreted in response to viral infection stimulate cellular immunity by mediating the recruitment and coordination of specific subsets of leukocytes [54]. Infected pulmonary epithelial cells secrete interleukin-6 (IL-6), IL-8 and gamma interferon (IFN- γ)-inducible protein 10 (IP-10) which are potent modulators of various functions of the immune cells present on site [44]. Sensing of PAMPs and DAMPs by certain cytosolic PRRs also leads to inflammasome activation with subsequent maturation and release of IL-1 β that effectively drives inflammation [55]. Particularly, SARS-CoV-2 encoded proteins E, ORF3a and ORF8b share some similarities with their counterparts in SARS-CoV-1 that have been previously identified as activators of the NOD-like receptor protein 3 (NLRP3) mediated inflammasome [56].

Recruitment and activation of immune cells

Together with epithelial cells, alveolar macrophages and dendritic cells (DCs) are the first responders to respiratory infections. DCs reside underneath the epithelium while macrophages are located in the lumen of the alveoli [16]. Once exposed to the various DAMPs, PAMPs and the cocktail of pro-inflammatory cytokines on site, these immune cells are activated and promptly start phagocytosis as well as antigen processing and presentation. Activated macrophages and DCs produce even more cytokines that recruit circulating immune cells. Granulocytes, namely neutrophils, rapidly arrive on site, thus mediating the acute inflammatory response.

Immune dysfunction and cytokine storm

Since coronaviruses employ several mechanisms of viral evasion of innate immunity, the host response is often delayed and diminished during the early phases of infection. Consequentially, the virus starts to propagate uncontrollably and, if the host cannot launch an efficient immune response rapidly enough to counteract this, massive destruction of the affected tissues occurs. The damaged cells release pro-inflammatory cytokines that induce inflammation which is largely mediated by the alveolar macrophages and DCs present on site. These activated immune cells, in turn, produce more pro-inflammatory cytokines which further stimulate the acute inflammatory response.

Additionally, it has been reported that SARS-CoV-2, but not SARS-CoV-1 or MERS-CoV, induces endotheliitis [57, 58] which exacerbates immune cell recruitment [32, 40]. This, in combination with the increased pulmonary vascular permeability caused by RAS dysregulation [37], facilitates the rapid establishment of a sizeable immune infiltrate of neutrophils, monocytes and macrophages in the lung tissue which constitute additional sources of pro-inflammatory cytokines [5, 32, 59].

Facing an extremely high viral load, the host is unable to control the infection and enters a vicious cycle of immune cell recruiting and cytokine production. Serum levels of IL-1 β , IL-8 and IL-6 are elevated and a “cytokine storm” is established in the lung. The high concentration of pro-inflammatory cytokines halts the ability of DCs to prime naïve T cells, blocking the development of an adaptive immune response [44] while inducing a hyperinflammatory state on the infiltrated myeloid cells. This immune dysfunction causes massive tissue damage and lung immunopathology, as the patient starts to develop acute respiratory distress syndrome (ARDS) [5]. At this point, even though the viral load starts to decrease, tissue damage and disease severity constantly increase [60].

In addition to immunopathogenesis, the cytokine storm directly induces cell death by pyroptosis [56]. The dying cells keep releasing cytokines and various DAMPs that perpetuate the inflammatory cascade in the affected tissue.

Furthermore, inflammatory cytokines are strong inducers of hyaluronan-synthase-2 which is expressed by endothelial and lung alveolar epithelial cells as well as fibroblasts. The cytokine storm therefore causes an overproduction of hyaluronan (HA) which results in the jellification of the pulmonary surfactant, impairing ventilation and ultimately accelerating organ failure [59].

It is, therefore, crucial to understand how the viruses modulate the host immune response to infection in order to develop effective therapeutics and preventive strategies to control coronavirus related diseases.

Viral modulation of the innate immune response

As previously mentioned, the pathogenicity of coronaviruses is tightly linked to the plethora of mechanisms that these viruses employ to impair the host immune system. Notably, the fact that even asymptomatic infected patients contribute substantially to SARS-CoV-2 transmission is precisely indicative of a delayed antiviral innate immune response [5].

Failure to launch a robust IFN antiviral response allows for a rapid increase of the viral load and sustained viral replication as the host cannot control infection and starts to progress into severe disease (Figure 4) [54]. In fact, severe and critically ill patients show a common phenotype of profound

impairment of the interferon response with low IFN-I and IFN-III production and limited ISG response, with a parallel exacerbated induction of chemokines and pro-inflammatory cytokines [48, 61].

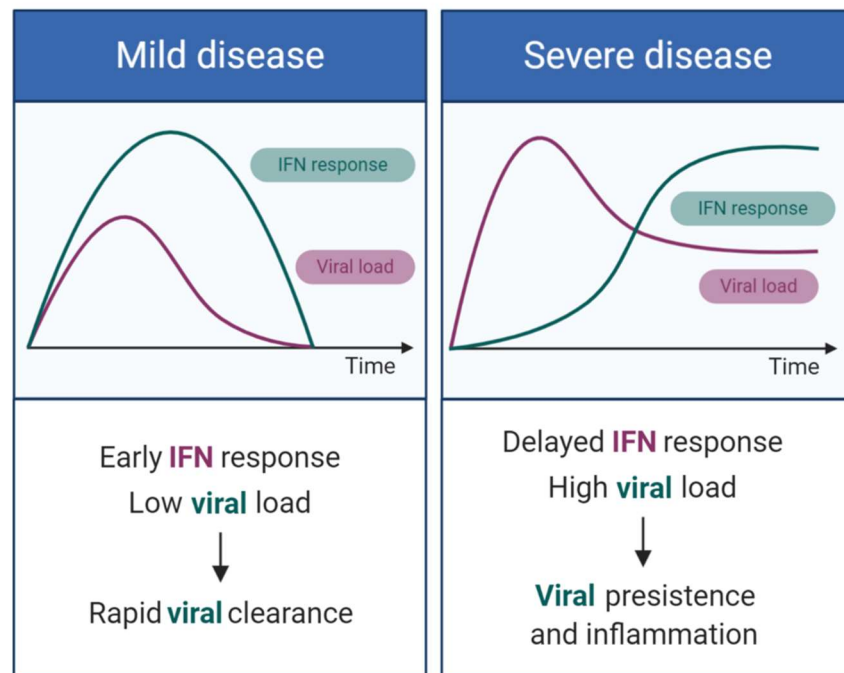


Figure 4. Interplay between viral load and IFN response after coronavirus infection in mild and severe disease. Figure adapted from Park and Iwasaki (2020) and created with BioRender.com.

Coronaviruses can interfere with the innate antiviral immune response at several levels: innate viral sensing, IFN production, IFN signalling, and ISG effector function [48, 62, 63].

Avoiding PRR detection

Viruses can circumvent or minimize sensing by host PRRs through numerous tactics, such as by hiding the viral genome and replication intermediates, directly interacting with the PRRs, targeting adapter proteins, etc. [63]. Coronaviruses replicate in virus-induced double membrane vesicles lacking in PRRs, thereby avoiding host detection and also preventing activation of RLRs by viral dsRNA replication intermediates [48]. SARS-CoV-1 employs the viral nsp14 and the nsp10-nsp16 complex to cap the viral RNAs in a way that mimics the host's mRNAs preventing their recognition by MDA5 [60]. A recent study showed that SARS-CoV-2 RNAs are also capped at the 5'-end, thus escaping recognition from PRRs [64, 65]. Though mRNA capping has not been reported for MERS-CoV, the structure of its nsp10-nsp16 complex counterpart suggests a similar mechanism to avoid host recognition [60]. Moreover, SARS-CoV-1 N protein has been associated with IFN inhibition early in the signalling cascade and MERS-CoV ORF4a blocks MDA5 activation [60]. Poor induction of RLR-dependent antiviral signalling has also been reported in early SARS-CoV-2 infection [66].

Inactivation of transcription factors

As previously mentioned, transcription factors are integral parts of the IFN signalling pathway. Viruses can block IFN production by interfering with transcription factors at several levels: blocking downstream signalling of PRRs to inhibit their activation; sequestering them and impeding nuclear translocation; or inhibiting their binding to ISREs, thus blocking the expression of ISGs [63]. Even if all

their mechanisms of action are not yet fully understood, many coronaviruses encoded IFN antagonists have been reported to date.

MERS-CoV ORF4a, ORF4b, ORF5 and M proteins have been shown to inhibit nuclear trafficking of IRF3 and activation of the IFN- β promoter [60]. ORF4b and the M protein, particularly, inhibit the IRF3 phosphorylation [60, 67]. Accessory protein ORF8b also suppresses MDA5 and NF- κ B signalling [67]. The SARS-CoV-1 M protein inhibits activation of the IRF3/IRF7 transcription factors [68]. SARS-CoV-2 nsp13, nsp14, nsp15 and ORF6 inhibit nuclear translocation of IRF3 [69]. Particularly, ORF6 not only suppresses IFN-I and IFN-III production, but also blocks ISG induction after RIG-I activation or IFN- β treatment of infected cells [70]. Together with ORF6, SARS-CoV-2 ORF8 inhibits expression from ISRE promoters. Also, ORF8 and the N protein significantly suppress mRNA expression of IFN-I and ISGs [54]. Interestingly, SARS-CoV-2 ORF3b, which is a truncated version of its SARS-CoV-1 encoded counterpart due to the presence of premature stop codons in its gene, suppresses the induction of type I interferon more efficiently. Interestingly, a natural variant of SARS-CoV-2 which was isolated from two patients with severe disease, shows partial reversion of the stop codons and produces an even more efficient ORF3b. This suggests that the C-terminus of SARS-CoV-1 ORF3b impairs its immune evasion activity [71].

Antagonizing IFN Induced JAK-STAT Signalling

Downstream of IFN production and release, the JAK-STAT Signalling pathway is essential to prompt ISG expression and achieve the antiviral effect of IFNs. Viruses can act to antagonize the JAK-STAT signalling pathway through mechanisms including targeting and degradation of IFN receptors and STATs through the proteasome system, retention of STATs in the cytoplasm, inhibition of their activation, etc. [63]. Particularly, one study found that SARS-CoV-1 nsp1 inhibits STAT1 phosphorylation causing viral-induced attenuation of the IFN response in infected macrophages and DCs [69]. Also, Orf6 of SARS-CoV-1 and SARS-CoV-2, blocks STAT1 nuclear translocation to inhibit IFN signalling [72].

Antagonizing ISGs

Since an impaired or delayed ISG production is advantageous for viral replication and spread into host tissues, viruses exploit several methods to antagonize the host ISG response [63]. A study found that, even though SARS-CoV-1 infection stimulates a robust ISG production, the ISG response is delayed for 24 to 48h, allowing for the establishment of peak viral titers prior to ISG production. Also, MERS-CoV not only delays ISG induction but induces repressive histone modifications, resulting in a condensed chromatin structure that likely excludes transcription factors and reduce RNA expression of a subset of ISGs [73]. Moreover, MERS-CoV NS4b proteins cause enzymatic degradation of the host's OAS-RNase L, which is responsible for cleaving viral and host ssRNA and leads to translational arrest and subsequent cell death, preventing viral replication and spread [74].

Hijacking the host translation machinery

Coronaviruses also use a combination of ways to achieve host shut-off, which refers to the blocking of all cellular protein expression, not only effectively suppressing the cellular antiviral responses, but simultaneously enabling the virus to take full advantage of the host translation machinery for its own use [75]. SARS-CoV-1 nsp1 binds the 40S subunit of ribosomes inhibiting all mRNA translation while also causing the degradation of host mRNA by activating the cellular mRNA degradation machinery

[43]. SARS-CoV-2 nsp1 also binds to the 40S subunit in ribosomal complexes and to 18S ribosomal RNA, leading to global inhibition of mRNA translation [76, 77]. Its nsp16 binds to the mRNA recognition domains of the U1 and U2 splicing RNAs to suppress global mRNA splicing [76]. Additionally, ORF6 disrupts the bidirectional nucleocytoplasmic transport and blocks the expression of newly transcribed mRNAs [78].

The role of PLpro as antagonist of IFN signalling

The nsp3 of coronaviruses encodes a papain-like protease (PLpro) which plays many roles in the viral replication cycle and infection. Besides being responsible for releasing nsp1, nsp2, and nsp3 from the N-terminal region of polyproteins 1a/1ab, PLpro also acts as a deubiquitinating (DUB) and deISGylating enzyme [18].

Ubiquitination is a reversible post-translational modification in which the small regulatory protein ubiquitin is covalently bound to a protein substrate [79]. The most prominent function of ubiquitin conjugation is to tag proteins for degradation by the proteasome system [80]. Similarly, ISG15 is a ubiquitin-like protein, and also an interferon stimulated gene, which can be conjugated with several targets – ISGylation [81]. Conjugation and deconjugation of ubiquitin and ubiquitin-like proteins regulate almost all cellular processes, by affecting protein degradation, localization, function and complex formation [82]. It is crucial for cell survival and differentiation, regulating transcription, signal transduction, membrane trafficking, clearing of misfolded proteins and more, also greatly influencing innate and adaptive immunity, and the antiviral response [79, 80].

Host cells employ ubiquitination and the ubiquitin-proteasome system to counteract viral infection at several levels. Viral proteins can be ubiquitinated and directly targeted for degradation, or ubiquitination of host and viral proteins blocks their function, effectively halting viral replication [80]. Ubiquitin also regulates immune signalling. Particularly, K63-linked ubiquitin chains have been shown to contribute to effective RIG-I oligomerization and signal transduction, thus mediating IFN production [83]. Additionally, ubiquitination generates target structures recognized by T cells, and mediates the proteasomal degradation of I κ B, which normally sequesters NF- κ B in the cytoplasm, leading to the release of NF- κ B, which activates the expression of pro-inflammatory cytokines [80]. Likewise, ISGylation also has important antiviral effects. ISG15 conjugation interferes with the function of viral proteins and blocks viral entry, inhibits protein synthesis and virus replication and can also prevent the formation, budding and release of new viral particles [81]. Specifically within the IFN signalling pathway, ISGylation of the caspase activation and recruitment domains of MDA5 promotes its oligomerization, and thereby regulates the immune cellular responses to viral infection [84]. Interestingly, ISG15-KO macrophages display reduced activation and phagocytic activity [81].

Coronaviruses employ their PLpro domain to counteract the antiviral action of ubiquitin and ISG15, blocking the host innate immune response to infection and even enhancing their own replication. In particular, this viral protease has been found to disrupt the IFN pathway at several levels, acting as a key interferon antagonist during coronavirus infection [43].

Importantly, SARS-CoV-1 PLpro has been found to deubiquitinate RIG-I, leading to inhibition of IFN production [86]. Moreover, it inhibits the TLR7-mediated IFN-I response and the NF- κ B pathway by removing the Lys63-linked polyubiquitin chain from TRAF3 and TRAF6, which are upstream regulators of IRF3 and NF- κ B [85]. SARS-CoV-1 PLpro has also been shown to physically interact directly with IRF3, and TBK1, IKK ϵ , and STING, disrupting the assembly of the STING-TRAF3-TBK1 complex [86]. This

interaction further disrupts the phosphorylation and dimerization of IRF3, ultimately blocking its nuclear translocation.

Even though, SARS-CoV-1 and SARS-CoV-2 PLpro share 83% sequence identity, they exhibit different host substrate preferences and mechanisms of action [70, 87]. SARS-CoV-2 PLpro has a reduced deubiquitylating activity but binds to ISG15 with higher affinity and predominantly acts a deISGylating enzyme [87]. Indeed, SARS-CoV-2 PLpro has been shown to directly interact and deISGylate MDA5, therefore preventing its activation and blocking this signalling pathway [84]. Further studying can unveil novel mechanisms of action of SARS-CoV-2 PLpro.

Due to its strong IFN antagonising activity, coronaviral PLpro has been widely regarded as an important target for the developing antiviral drugs [18, 88]. In general, a good understanding of the strategies employed by coronaviruses to subvert the host immune response is essential for the development of new vaccines and therapies [72].

The utility of proteomics for the study of Coronaviruses

Since proteins are central players in most biological processes, proteomics-based strategies can provide important insights for the study of pathogens and their mechanisms of infection, as well as characterization of the host response to infection, disease progression and ultimately the identification of antiviral targets [89]. Several proteomics methods have been applied to study different aspects of coronavirus infection: from identifying the virus receptor and other host-virus interactions, to discovering disease biomarkers for improved diagnostics and prediction of antiviral drug targets [90-92]. Technological advances in techniques such as antibody-based affinity purification (AP) and Mass spectrometry (MS) have been especially vital to provide the capacity for precise, sensitive identification and high-throughput analysis of proteins on a large scale, as is required for proteomics studies [89, 90].

Receptor identification

Identifying the host receptor targets with which the viruses interact to initiate infection is essential to understand host cell susceptibility to viral infection. Notably, approaches based on AP followed by MS (AP-MS) have been employed to first identify the interaction between the S protein of MERS-CoV and DPP4 [8] and of SARS-CoV-1 with ACE2 [7]. Due to the high degree of similarity between SARS-CoV-2 and its predecessor, the usage of the ACE2 receptor by SARS-CoV-2 was also quickly confirmed [6, 93].

Host-virus interactions

Proteomics studies can help identify and quantify cellular alterations induced by virus infection [90] and elucidate how the virus hijacks the host cellular machinery for its own replication, providing valuable insights for the understanding of viral pathogenicity [94]. A recent study using AP-MS combined with statistical modelling, profiled the interactome of SARS-CoV-1 and SARS-CoV-2 and characterized their influence on the transcriptome, proteome, ubiquitinome and phospho-proteome of a lung-derived human cell line [95]. Stukalov et al. reported that both viruses target specific protein complexes involved in innate immunity, stress response components and DNA-damage response mediators, interfering with the same cellular processes at multiple levels, though with distinct temporal patterns [17]. Another recent proteomics study revealed that SARS-CoV-2 infection alters central cellular pathways such as carbon and nucleic acid metabolism, protein homeostasis,

translation and splicing [96]. The authors also found that inhibitors that target these pathways prevented viral replication, suggesting approaches for targeted therapies for coronavirus related diseases.

Proteomics for detection and diagnosis

Proteomics can also be a useful tool to identify disease biomarkers, develop new approaches for early detection of viral infection and to predict the cases which might progress to clinically severe conditions. In the search for biomarkers of MERS-CoV infection, only antibody-based technologies were used to measure the levels of selected cytokines in patient samples however, in the case of SARS-CoV-2, a mix of both immunoassays and MS has been applied to characterize COVID-19 [17]. In one study, the plasma concentration of 48 cytokines was systematically measured and analysed in combination with clinical data from a total of 50 COVID-19 cases, ranging from moderate to critically ill [97]. The levels of 14 of those cytokines were found to be elevated in COVID-19 and, particularly, the concentration of IFN- γ -induced protein 10 (IP-10, also called CXCL10), monocyte chemotactic protein-3 (MCP-3), and the combination between these two cytokines, were considered the most reliable biomarkers for the prediction of disease progression [97]. Another study found that high serum levels of cancer biomarkers positively correlate with COVID-19 disease severity [98]. Screening of specific IgG antibodies of 13 recombinant proteins associated with four structural proteins (S, E, N, and M) and five putative uncharacterized proteins of SARS-CoV-1 (3a, 3b, 6, 7a and 9b), suggested that anti-S and anti-N antibodies are reliable diagnostic markers [99]. The association of machine learning strategies with MS analysis of patient samples has also been explored. A study performed proteomic and metabolic profiling of patient plasma and built a machine learning model that had an 93.5% accuracy in identifying patients with severe disease [100]. Another team of researchers proposed a machine learning-based model for identification of COVID-19-positive patients after AP-MS analysis of nasopharyngeal swab samples. This method achieved 97.8% sensitivity and 100% specificity, comparable to RT-PCR-based molecular testing [101].

Drug Repurposing

When new viral diseases emerge, drug repurposing is an advantageous strategy to quickly find effective antiviral drugs. Repurposing previously approved drugs allows for lower overall costs of development and shorter timelines until a therapy is introduced in the market [102]. The search for potential candidates for drug repurposing is challenging, but proteomics tools can be employed to rapidly identify possible viral targets and match that information with databases of already approved drugs [103].

A pilot study identified 332 high-confidence protein–protein interactions between SARS-CoV-2 and human proteins, being that 66 of the later can be targeted by 69 compounds that are either already approved by the FDA, are in clinical trials or under preclinical testing [104]. Additionally, the previously mentioned study that identified core cellular pathways affected by SARS-CoV-2 infection, reported viral inhibition by two known drugs: ribavirin, an inhibitor of nucleotide synthesis, and 2-deoxy-d-glucose, an inhibitor of carbon metabolism and an anti-cancer drug [96]. Both of these drugs are currently undergoing clinical trials as therapies against COVID-19 [105, 106].

As of the writing of this text, a database curated by the Milken Institute, lists a total of 223 drugs previously submitted through clinical trials for other diseases and many of them already FDA

approved, that have now been repurposed in the search of a therapy against COVID-19 [107]. Of these, only one is listed as FDA approved for the treatment of COVID-19 - Remdesivir, a nucleotide analogue prodrug that inhibits viral RNA polymerases, which had been previously employed to treat Ebola [108].

Aim of the work

The overall aim of this thesis was to perform a comparative analysis between SARS-CoV-2, SARS-CoV-1 and MERS-CoV to better understand the modulation of the host cellular environment during coronavirus infection. In particular, the interplay between the host immune response to SARS-CoV-2 infection and its mechanisms of viral escape was investigated. This work comprises a bioinformatics and a wet lab component with two distinct, yet complementary aims:

Aim 1: Global proteomics analysis of pre-existing data to evaluate the differences in the cellular response to SARS-CoV-2 infection, when compared to the previous pathogenic human coronaviruses.

Aim 2: *In vitro* study to determine the mechanism of action of the coronaviral Papain-like protease and assess its role in inhibition of interferon signalling.

Materials and Methods

Cell lines

HeLa cells (CCL-2) and Calu-3 cells (HTB-55) were obtained from ATCC. Caco2 cells were obtained from CLS cell line services, GmbH, Germany (#300137). The Human hepatocyte-derived cellular carcinoma Huh-7 cell line was obtained from Marburg Virology Lab, Germany [109], Huh-7 ISG15 knockout and control cell lines were a kind gift from Connor Bamford (Queens university, Belfast). The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Sweden) supplemented with 10% fetal bovine serum (FBS, ThermoFisher, USA) and 20 units/mL Penicillin combined with 20 µg/mL Streptomycin (Sigma, USA) (Pen-Strep) and incubated in 5% CO₂ at 37 °C.

Virus and infection

Lysates from cells infected with the first Swedish isolate of SARS-CoV-2 (SWE/01/2020) at a multiplicity of infection (MOI) of 1 and 0.1, were obtained as described in previous studies from our lab [94, 110]. Briefly, the SARS-CoV-2 virus was isolated from a nasopharyngeal sample of a patient at Public Health Agency (Sweden), was confirmed by sequencing (Genbank accession number MT093571) and titrated. Infection was performed by incubating Vero-E6 (#CRL-1586, ATCC), Huh-7, 293FT (Invitrogen, #10769564 obtained from Matti Sällberg, Karolinska Institutet, Sweden), A549 (#CCL-185, ATCC), Calu-3 (#HTB-55, ATCC), 16HBE (obtained from Lena Palmberg, Karolinska Institutet, Sweden), and Caco2 cells with 100µL of DMEM supplemented with 5% heat-inactivated FBS containing SARS-CoV-2 either at MOI 1 or 0.1 for 1h at 37°C. Then, the medium was removed and replenished with fresh medium. The cells were incubated for 120 h post-infection (hpi). The virus production was measured by performing qPCR targeting the viral N-gene RNA in the cell culture supernatant starting at 3 hpi and then every 24h, followed-up to 120 hpi. The viral cytotoxicity was measured by using the Viral-ToxGlo assay (Promega).

Reagents and antibodies

The stocks of 50M Tris-Cl pH 7.4, 5M NaCl and 0.5M EDTA at pH 8.0 were obtained from the Karolinska Institutet substrate department. NP-40 (IGEPAL CA-630, I3021) DL-Dithiothreitol (DTT, D0632), Sucrose (S8501), MgCl₂ (M8266) and ATP (A2383) were purchased from Sigma Aldrich. The cOmplete™ Protease Inhibitor Cocktail (04693116001) was purchased from Roche. Low Molecular Weight Polyinosinic:polycytidylic acid (LMW Poly (I:C))/LyoVec (tlrl-picwlv) was purchased lyophilized from InvivoGen (France) and resuspended in sterile endotoxin-free LAL water provided by the manufacturer at the final concentrations specified. Interferon-α 2a (IFN-α; #11100-1) and Interferon-β 1a (IFN-β; #11415-1) were purchased from PBL assay science (USA).

The antibodies used for western blotting (Table 3) were prepared in 5% non-fat milk in 1X Tris-Buffered Saline (VWR, J640-4L) with 0.1% Tween-20 (20%, Karolinska Institutet Substrate Department) (TBS-T) and incubated for 1 h at room temperature or overnight at 4 °C.

Table 3. Antibodies used for western-blotting.

Antibody	Dilution	Manufacturer	Catalog number
Mouse anti-FLAG	1:4000	Sigma-Aldrich	F3165
Mouse anti-V5 (C-9)	1:5000	Santa Cruz Biotechnology	sc-271944
Rabbit anti-ISG15 (7H29L24)	1:1000	Invitrogen	#703131
Mouse anti-ISG15 (F-9)	1:1000	Santa Cruz Biotechnology	sc-166755
Mouse Anti- β -Tubulin (TUB 2.1)	1:1000	Sigma-Aldrich	T5201
Rabbit Anti-Mouse Immunoglobulins	1:4000	Agilent Technologies	P0260
Goat Anti-Rabbit Immunoglobulins	1:4000	Agilent Technologies	P0448

Plasmid constructs and plasmid amplification

The aminoacidic sequence of SARS-CoV-2, SARS-CoV-1 and MERS-CoV encoded PLpro as well as an Empty construct containing an N-terminal 3xFLAG-tag (3x DYKDDDDK-tag) was codon optimised, synthesized and subcloned into a pcDNA3.1 vector in Geneuniversal (Newark, DE, US). SARS-CoV-2 WT, SARS-CoV-2 C111A catalytic mutant, and Empty C-terminal V5 tagged plasmids were a gift from Prof. Michaela Gack (Cleveland Clinic, FL, US) and C-terminal V5-tagged SARS-CoV-1 and MERS-CoV encoded PLpro were kindly gifted by Prof. Susan Baker (Loyola University Chicago, IL, US).

For plasmid amplification, One Shot™ Stbl3™ Chemically Competent *E. coli* (Invitrogen, C737303) and One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen, C404003) were transformed with the FLAG and V5 tagged plasmids, respectively. Briefly, competent cells were thawed on ice and 1 μ l of Plasmid solution was added to the cells that were kept on ice for 30 min before proceeding to Heat-shock for 45 seconds in a water bath at exactly 42 °C. Tubes were immediately placed on ice for 2 minutes and 900 μ l of SOC medium (Sigma Aldrich, S1797) at 4 °C was added to each transformation reaction. Tubes were incubated for 60 minutes at 37 °C with 225 rpm shaking. Afterwards, the transformation reaction was inoculated in previously warmed Luria-Bertani agar (LA) 9cm plates with Ampicillin 100 μ g/ml, pH 7.5 (Karolinska Institutet Substrate Department) at 37 °C and incubated overnight at the same temperature. The following day, single colonies were inoculated on a start-up culture of 2 ml of Luria-Bertani broth (LB) with Ampicillin 100 μ g/ml, pH 7.5 (Karolinska Institutet Substrate Department) that was incubated for 6-8 h at 37 °C with 225 rpm shaking. Then, 200 to 400 μ l (1:1000 to 1:500 dilution) were inoculated in a 200 ml LB medium maxiprep culture that was incubated overnight at 37 °C with 225 rpm shaking. 30% glycerol stocks were prepared using 50% sterile Glycerol (Karolinska Institutet substrate department) and LB broth and stored at -80 °C. Plasmids were isolated using QIAGEN Plasmid Maxi Kit (QIAGEN, 12162) according to the manufacturer's instructions and were finally diluted in 400-600 μ l EB buffer (QIAGEN, 19086). The correct sequence of all constructs was confirmed by DNA sequencing through Eurofins Genomics TubeSeq Service (Ebersberg, Germany).

DUB probing

For DUB probing, cells were seeded in a 6 well plate at a density of 3×10^5 cells per well. The following day, the cells were transfected with the indicated plasmids using FuGENE® HD Transfection Reagent (Promega, #E2311) following the protocol provided by the manufacturer using a Reagent:DNA ratio of 3:1 at 3.0 μ g DNA per well. After 24 h, cells were collected and lysed on ice with lysis buffer containing 50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 supplemented with 1 mM DTT and 1X cComplete™ Protease Inhibitor Cocktail, freshly added. Protein concentration was measured with the

DC protein Assay Kit (Bio-Rad Laboratories, 5000112). For the probing reaction 0.5 µg HA-Ub-VME (Ubiqbio, UbiQ-035) and 20 µg of protein lysate were incubated at 37 °C for 1 h in a total of 40 µl in reaction buffer with 250 mM Sucrose, 50 mM Tris-Cl pH 7.4, 5 mM MgCl₂, and freshly added 1 mM DTT and 2 mM ATP. The reaction was terminated by adding 1X LDS sample buffer (Life Technologies Corporation, NP0008), 1X Reducing Agent (Thermo Fisher Scientific, NP0009) and incubated for 10 min at 100 °C.

Poly(I:C) and Interferon treatment

Cells were seeded in 6 well plates in 2 ml DMEM supplemented with 10% heat-inactivated FBS and 0.2% Pen-Strep, at a density of 3x10⁵ cells per well. The following day, cells were transfected with the indicated plasmids using FuGENE® HD Transfection Reagent (Promega, #E2311) following the protocol provided by the manufacturer using a Reagent:DNA ratio of 3:1 at 3.0 µg DNA per well. After 24 h, the cells were treated with 1 or 2 µg/mL LMW poly(I:C)/Iyovec or 1 000 U/ml IFN-α/β in 2 ml supplemented DMEM for 6 or 16 h. Cells were then scraped and collected in 1 ml of 1X Phosphate-buffered saline (PBS) (Karolinska Institutet substrate department). Of this cell suspension, 600 µl were separated for western-blotting and 400 µl for RNA extraction. After centrifugation at 5000 rpm for 10 min, the supernatant was discarded, and the cell pellet were stored at -80 °C until further processing.

Western-blotting

The cells were lysed on ice for 30 min with 50 to 100 µl, according to cell pallet size, of NP-40 lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl; 1 mM EDTA, 0.5% NP40; 1 mM DTT and 1X cComplete™ Protease Inhibitor Cocktail, freshly added), pipetting up and down every 10 min. Protein lysates were obtained by centrifugation at 13000 rpm for 20 mins at 4 °C and collecting the supernatant. The protein concentration was evaluated by DC Protein Assay. Evaluation of protein expression was performed by running 20 µg of total protein lysate on NuPage Bis–Tris 4–12% gels (Invitrogen). Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad Laboratories, 1610373) or PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, 26619) were used as size standards. Proteins were transferred using iBlot dry transfer system (Invitrogen) and blocked for 1 h using 5% non-fat milk in 0.1% TBS-T. Subsequent antibody incubation was performed at 4 °C overnight or for 1 h at room temperature. Membranes were washed using 0.1% TBS-T and proteins were detected using ECL or ECL Select (GE Healthcare, USA) on ChemiDoc XRS + System (Bio-Rad Laboratories, USA). The Western blot analysis was performed by using antibodies previously mentioned in Table 3.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the Direct-zol™ RNA Miniprep Kit (Zymo Research, R2072) and RNA concentration was assessed using a spectrophotometer (NanoDrop UV Visible Spectrophotometer, Thermofisher, USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) with addition of RiboLock RNase Inhibitor (Thermo Scientific, EO0382) in 20 µl volume per reaction using 500 ng or 200 ng of extracted RNA according to the following conditions: 25 °C for 10 min, 30 °C for 120 min, 85 °C for 5 min. The generated cDNA was used for qRT-PCR. mRNA expression of IFNβ and β-actin transcripts was measured by qRT-PCR. The sequences of the qPCR primers pairs used are as follows: IFNβ_Fwd: 5'-TCCAAATTGCTCTCTGTTG-3', IFNβ_Rev: 5'-GCAGTATTCAAGCCTCCCAT-3' and β-actin_Fwd: 5'-AAAGCCTGCCGGTGACTAAC-3',

β -actin_Rev: 5'-AGGAAAAGCATCACCCGGAG-3'. Quantitative RT-PCR assays were set up using the Power SYBR Green PCR Master Mix (Applied Biosystems, UK) using 10 μ M of each primer with cycling conditions: initial denaturation 95 °C 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Melting curves were run by incubating the reaction mixtures at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, ramping from 60 °C to 95 °C in 1 °C/s, and finishing at 60 °C for 15 s. The values were normalized to endogenous β -actin. Fold change was calculated as fold change = $2^{-\Delta(\Delta C_t)}$ where $\Delta C_t = C_{t_{\text{target}}} - C_{t_{\text{housekeeping}}}$ and $\Delta(\Delta C_t) = \Delta C_{t_{\text{infected}}} - \Delta C_{t_{\text{nontreated}}}$, according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines.

Quantitative proteomics analysis

The proteomics data here analysed refers to infected Huh-7 cells at a MOI of 1, in triplicates, and 48 hpi for SARS-CoV-2 (SWE/01/2020) and SARS-CoV-1, as this was the timepoint that showed most significant changes, or 24 hpi for MERS-CoV, which was observed to be highly cytopathic and, by 48 hpi all the cells were dead, thus restricting our analysis to 24 hpi.

The proteomics workflow was performed as described previously [110, 111]. Briefly, proteins were extracted with SDS-based buffer, digestion was performed on S-Trap microcolumns (Protifi, Huntington, NY, USA), and resulting peptides were labeled with isobaric TMTpro™ reagents. Labeled peptides were fractionated by high pH reversed-phase chromatography, and each fraction was analyzed on an Ultimate 3000 UHPLC (ThermoFisher Scientific, USA) in a 120 min linear gradient. Data were acquired on an Orbitrap Fusion Lumos™ tribrid mass spectrometer (ThermoFisher Scientific, USA) in data-dependent acquisition mode, isolating precursors in 2 s cycle time with 120,000 mass resolution in the mass range of 375-1500 m/z, maximum injection time (IT) of 50 ms and dynamic exclusion of 45 s; precursor isolation width of 0.7 Th with high collision energy of 34%, resolution of 30,000 and maximum IT of 54 ms.

Proteins were searched against both SwissProt human and SARS-CoV-1/SARS-CoV-2 databases using the search engine Mascot Server v2.5.1 (MatrixScience Ltd., UK) in Proteome Discoverer v2.4 (ThermoFisher Scientific, USA) software allowing up to two missed cleavages. Oxidation of methionine, deamidation of asparagine and glutamine, TMTpro modification of lysine, and N-termini were set as variable modifications; while carbamidomethylation of cysteine was used as fixed modification. The false discovery rate (FDR) was set to 1%.

Bioinformatics analysis

The quantitative proteomics data was processed and normalised as described previously [111]. Briefly, differential abundance analysis was performed using R package *limma* [112] between mock-infected and 24 hpi for MERS-CoV and 48 hpi in SARS-CoV-2 and SARS-CoV-1 proteomics data. Pairwise comparisons were extracted and Benjamini–Hochberg adjustment was applied on p values. The subsequent analysis done here was performed using R version 4.0.2 [113]. First, genes with adjusted p values < 0.05 were selected. Pathway enrichment analysis was performed with Enrichr [114]. The KEGG 2021 library was selected since it is a manually curated database that offers detailed pathway information and has been recently updated to include the latest data on coronavirus infection [115]. The volcano, bar and dot plots were built using R package *ggplot2* [116]. The Venn diagram was created using R package *VennDiagram* [117]. The heatmap was generated using R package *gplots* [118]. Protein-protein interaction analysis was carried out with STRING using the default settings

(Network Type: full STRING network; Required score: medium confidence (0.400); FDR stringency: medium (5%)). The protein-protein interaction networks were exported to Cytoscape 3.8.2 [119], nodes were colour coded according to fold change and the size of the circle is proportional to significance (small = less significant, higher p-value to large = more significant, lower p-value).

Data availability

The raw mass spectrometric data was previously deposited in the ProteomeXchanger Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the dataset identifier PXD023450 [94, 110].

All the code, processed data and figures generated here are available in the GitHub repository at <https://github.com/beatrizsavinhas/Thesis>.

Results

1. Cellular response to pathogenic human coronaviruses

1.1. Susceptibility and cytotoxicity of SARS-CoV-2 in commonly used laboratory cell lines

A suitable cell line is essential for the *in vitro* study of SARS-CoV-2 infection. To find cell models that are susceptible to infection and can withstand the viral cytotoxicity, several commonly used laboratory cell lines were infected with the first Swedish isolate of SARS-CoV-2 virus (SWE/01/2020) at a MOI of 1 and 0.1, as described in a previous study from our lab [94]. Cell lysates of infected cell lines originating from African green monkey kidney cells (Vero-E6), human lung (Calu-3, A549, 16HBE), colon (Caco2), liver (Huh-7), and kidney (293FT), were analysed and protein expression was determined by Western blot (Figure 5). ACE2 and TMPRSS2 expression was determined as a correlation with the tropism, while Cell viability was used as a measure of cytotoxicity. Cell viability was assessed with the Viral-ToxGlo assay, and virus production was measured starting at 3 hpi and then every 24h, followed-up to 120 hpi (data not shown) [94].

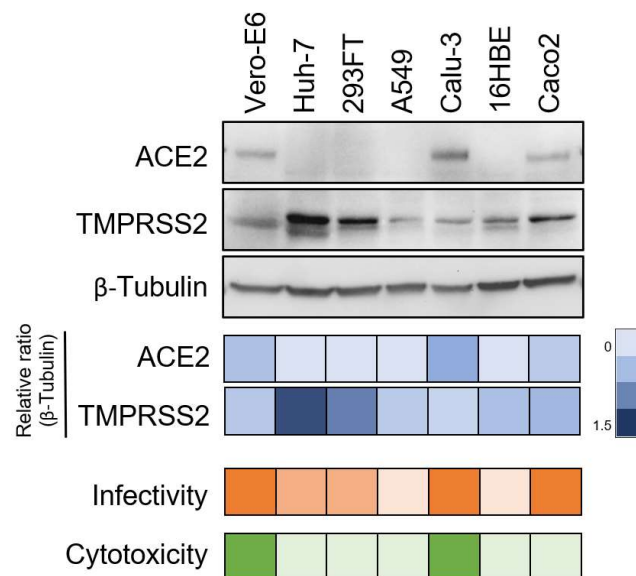


Figure 5. ACE2 and TMPRSS2 receptor expression in common laboratory cell lines. ACE2 and TMPRSS2 expression was determined by western blot. Heatmaps with relative protein quantification (blue), susceptibility (orange), and cytotoxicity (green) are shown. Reproduced from Saccon et al., 2021.

Expression of the ACE2 receptor strongly correlated with the susceptibility of the Vero-E6, Calu-3 and Caco2 to SARS-CoV-2 suggesting a prominent role of ACE2 in virus infectivity. It is interesting to note that Huh-7 and 293FT, cell lines that lacked expression of ACE2 but have strong TMPRSS2 expression, were also moderately susceptible to the SARS-CoV-2. Earlier studies have shown that Huh-7 cells are susceptible to the SARS-CoV-1 and MERS-CoV and thus Huh-7 can be considered for comparative analysis between these three pathogenic human coronaviruses [120, 121].

1.2. Quantitative global proteomics of SARS-CoV-2, SARS-CoV-1 and MERS-CoV infected Huh-7 cells identifies a common Coronavirus disease pathway

A

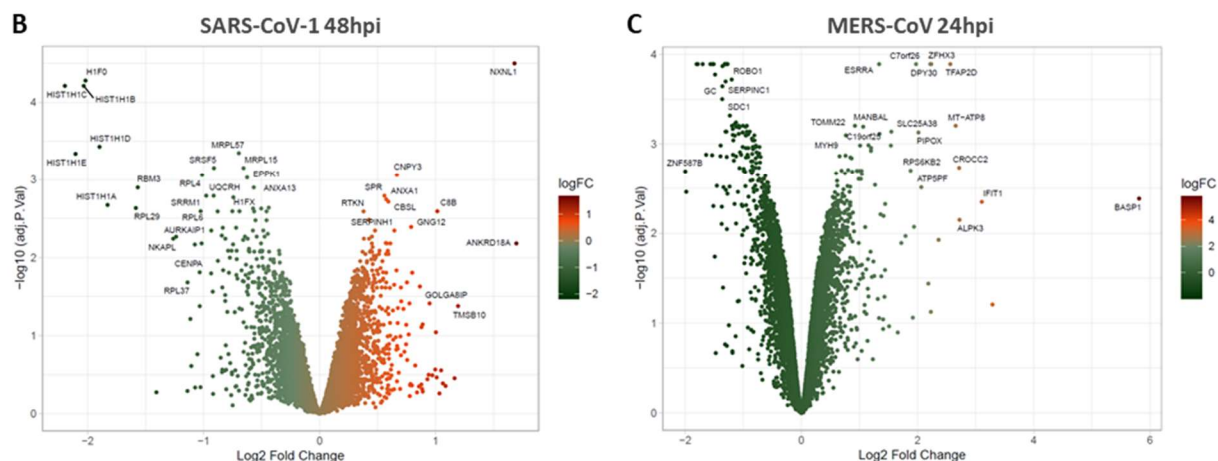
SARS-CoV-2 48hpi

$-\log_{10}(\text{adj. P.Val})$

Log2 Fold Change

logFC

2
1
0
-1
-2



21

The significantly differentially abundant proteins ($p < 0.05$) were submitted to pathway enrichment analysis, and the top 20 most significant pathways found in the KEGG 2021 database for the SARS-CoV-2 dataset are represented as a bar plot (Figure 7.A). Interestingly, comparative analysis of the pathways found for SARS-CoV-2, SARS-CoV-1 and MERS-CoV showed an overlap of 3 common significantly altered pathways (Figure 7.B) given that the one of these is identified in the database as the Coronavirus disease – COVID-19 pathway (Pathway: hsa05171) (Figure 7.C).

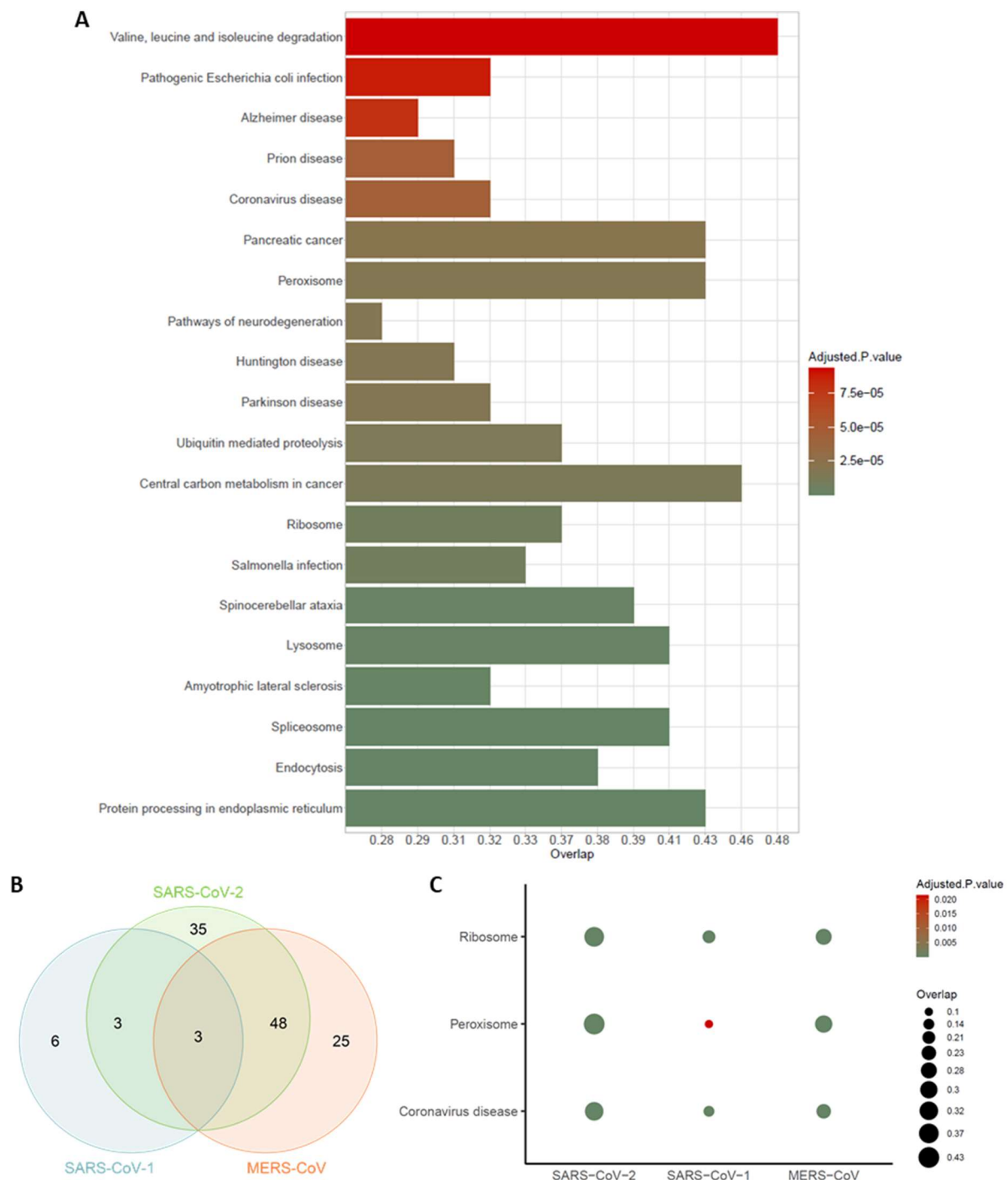


Figure 7. Pathway enrichment analysis. A. Bar plot of the top 20 most significant pathways identified in the KEGG 2021 library for the SARS-CoV-2 dataset. Bars are coloured according to the adjusted p-value computed by Enrichr (low = green to high = red) and overlap refers to the proportion of proteins from the pathway that was differentially expressed in the dataset. B. Venn diagram of the common pathways found amongst the viral strains. C. Dot plot of the 3 common pathways identified for all viruses. Dots were colour coded according to the adjusted p-value computed by Enrichr (low = green to high = red) and their size was computed according to protein overlap in the pathway.

1.3. SARS-CoV-2, SARS-CoV-1 and MERS-CoV display differential patterns of protein expression

To analyse the differences in protein abundance within the Coronavirus disease pathway in the 3 viruses considered, a heatmap containing the proteins that showed differential abundance in at least one of the datasets was generated (Figure 8). Notably, all viruses showed different patterns of protein expression, suggesting differential regulation of the host cellular environment by these viruses.



Figure 8. Heatmap of differentially abundant proteins ($p < 0.05$) of the Coronavirus disease pathway (KEGG 2021 Pathway: hsa05171) found on the SARS-CoV-2, SARS-CoV-1 and MERS-CoV datasets. The colour code indicates the fold change in abundance of the protein in the corresponding dataset and the grey colour refers to non-significant values.

1.4. Protein-protein interaction analysis of the coronavirus disease pathway

Protein-protein interactions between the differentially abundant proteins from the SARS-CoV-2 dataset ($p < 0.05$) that were found in the Coronavirus disease pathway from KEGG 2021 (Pathway: hsa05171, [122]), were retrieved from STRING, and represented in a network created with Cytoscape (Figure 9). This protein-protein interaction network showed several modules of connected proteins, from left to right: Module-1 involving proteins of the complement system [123], Module-2 of proteins associated with the coagulation cascade [124, 125], Module-3 with proteins from antiviral signalling pathways [126] and finally Module-4 consisting of Ribosome associated proteins [127]. Particularly, Module-3 comprises several proteins involved in the IFN signalling pathway and, most interestingly, ISG15 occupies a central position in the network displaying a high number of interactions with several other proteins. Since SARS-CoV-2 PLpro has been shown to interact with ISG15 [87], this result highlights both the importance of ISG15 in coronavirus disease and the possible role of SARS-CoV-2 PLpro in modulation of the cellular response against viral infection.

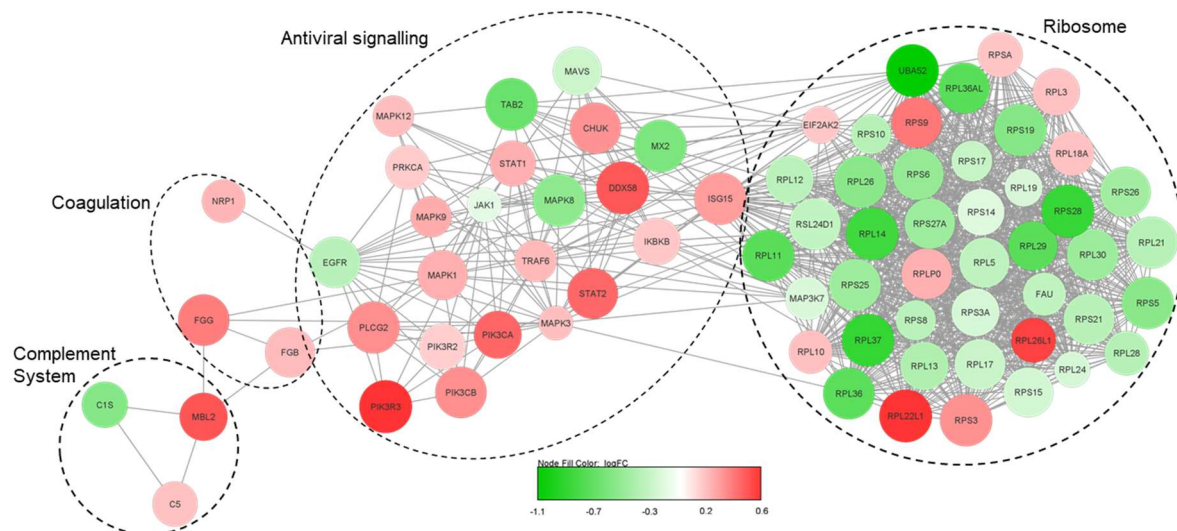


Figure 9. Protein-protein interaction Cytoscape network of the differentially abundant proteins ($p < 0.05$) from the SARS-CoV-2 dataset that were found in the KEGG 2021 Coronavirus disease pathway (Pathway: hsa05171). Nodes represent proteins and edges show protein-protein interactions. Nodes were colour coded according to fold change and the size of the circle is proportional to significance (small = less significant, higher p-value to large = more significant, lower p-value).

The same analysis for the SARS-CoV-1 dataset (Figure 10), shows a smaller number of proteins with significantly altered expression belonging to this pathway, in general, but a similar pattern of expression within each module of connected proteins when compared to SARS-CoV-2. A major difference is the absence of significant changes in the coagulation cascade and in the differential abundance of ISG15.

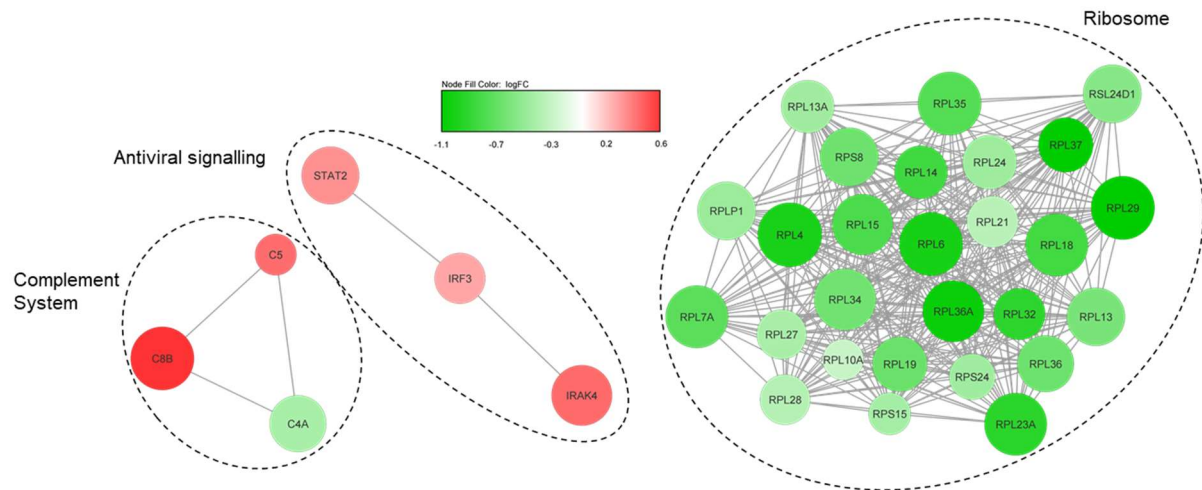


Figure 10. Protein-protein interaction Cytoscape network of the differentially abundant proteins ($p < 0.05$) from the SARS-CoV-1 dataset that were found in the KEGG 2021 Coronavirus disease pathway (Pathway: hsa05171). Nodes represent proteins and edges show protein-protein interactions. Nodes were colour coded according to fold change and the size of the circle is proportional to significance (small = less significant, higher p-value to large = more significant, lower p-value).

The MERS-CoV dataset (Figure 11) shows a distinctive pattern of expression in the sense that the ribosome module is upregulated, the complement and coagulation modules are downregulated, and the antiviral signalling module contains a smaller number of differentially regulated proteins than when compared to SARS-CoV-2. On the other hand, similar to what was observed for the SARS-CoV-2 dataset, ISG15 was upregulated and establishing a link between the antiviral signalling and the ribosome modules.

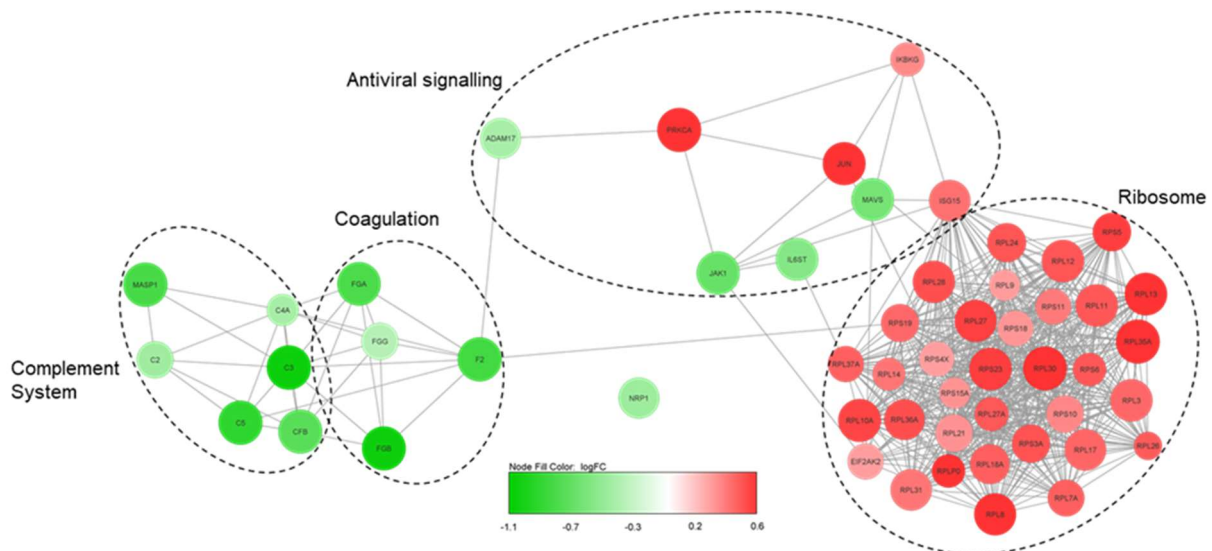


Figure 11. Protein-protein interaction Cytoscape network of the differentially abundant proteins ($p < 0.05$) from the MERS-CoV dataset that were found in the KEGG 2021 Coronavirus disease pathway (Pathway: hsa05171). Nodes represent proteins and edges show protein-protein interactions. Nodes were colour coded according to fold change and the size of the circle is proportional to significance (small = less significant, higher p-value to large = more significant, lower p-value).

2. Role of viral Papain-like protease (PLpro) in modulation of the immune response against SARS-CoV-2 infection

2.1. FLAG-tagged PLpro plasmid design and validation of FLAG-PLpro expression

An *in vitro* model to compare the role of PLpro in immune modulation during SARS-CoV-2, SARS-CoV-1 and MERS-CoV infection was sought. Established cell lines transfected with plasmids encoding the different viral PLpros allow for the expression of the viral protease in a controlled manner, which is necessary to study the viral-host protein interactions. In order to create the plasmid constructs, the SARS-CoV-2, SARS-CoV-1 and MERS-CoV encoded PLpro aminoacidic sequence obtained from the Protein Data Bank (PDB ID 6W9C, 5E6J and 5V6A, respectively) were first aligned using ClustalW, as shown in Figure 12.A.

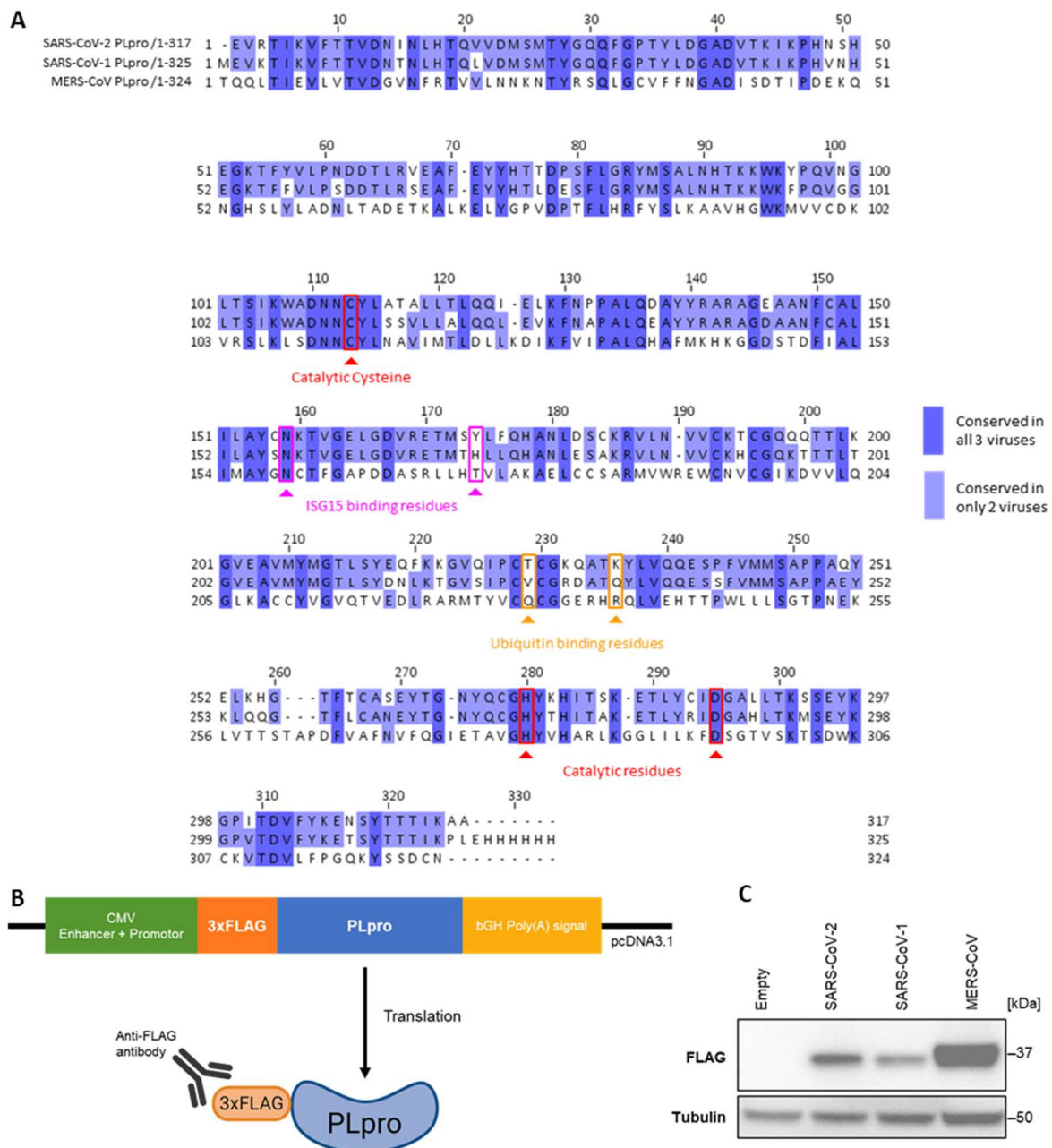


Figure 12. Plasmid design and validation. A. SARS-CoV-2, SARS-CoV-1 and MERS-CoV encoded PLpro aminoacidic sequence alignment (PDB ID 6W9C, 5E6J and 5V6A, respectively) with highlighted Catalytic, ISG15 and Ubiquitin binding residues [114]. B. Illustration of the FLAG-tagged PLpro plasmid maps, translation product and antibody detection for immune assays. C. Immunoblot showing plasmid expression in transfected HeLa cells.

SARS-CoV-2 and SARS-CoV-1 PLpro showed high degree of homology, while MERS-CoV PLpro differed significantly from the other two. Importantly, the 3 core catalytic domains [128], including the catalytically active cysteine, are conserved in the 3 viral strains, however, there are significant differences in the ubiquitin and ISG15 binding domains.

Each sequence was then codon optimized for human expression and inserted into a pcDNA3.1 plasmid vector with a 3xFLAG N-terminal tag for easy protein detection in immune assays, and a bGH Poly(A) signal termination sequence (Figure 12.B). Because they express a wide range of proteins [129], which is ideal to study protein-protein interactions, HeLa cells were selected to be transfected with the plasmids and protein expression was validated by Western Blot (Figure 12.C).

2.2. DUB probing indicates diminished deubiquitinating activity of SARS-CoV-2 PLpro

After plasmid expression was confirmed in transfected HeLa cells, the phenotype of the fusion 3xFLAG-PLpro proteins was assessed. PLpro has been shown to be a Deubiquitinating enzyme in SARS-CoV-1 and MERS-CoV [130, 131], therefore, to evaluate the comparative DUB activity of SARS-CoV-2 PLpro, a DUB probing was performed (Figure 13). The hemagglutinin-tagged ubiquitin-vinyl methyl ester (HA-Ub-VME) probe used is an activity-based probe that mimics the substrate and covalently binds to the DUB active-site by forming a thioether bond with the catalytic cysteine [82, 132]. DUB probing is, thus, a functional assay that reports on deubiquitinase activity. The protein complex comprising the DUB attached to the probe has a higher molecular weight than the DUB alone and can, as such, be easily identified through Western Blot. In accordance to what has been reported by others [87], SARS-CoV-2 PLpro has a diminished deubiquitinating activity when compared to SARS-CoV-1 and MERS-CoV (Figure 13.A). This finding suggests that the role of SARS-CoV-2 PLpro on immune modulation may be more strongly connected to its interaction with ISG15.

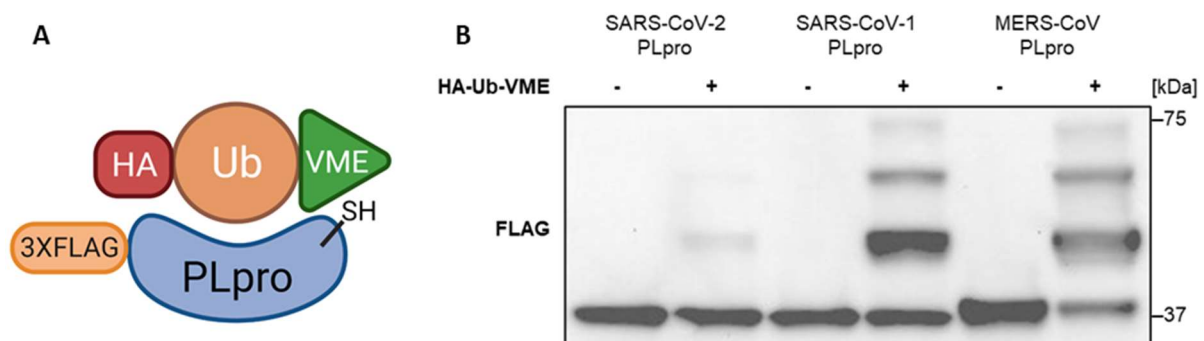


Figure 13. FLAG-tagged PLpro DUB probing. A. Schematic representation of the DUB probing reaction. B. Immunoblot showing the different PLpro constructs and the complexes formed with the HA-Ub-VME probe.

2.3. Interferon treatment highlights deISGylating activity of SARS-CoV-2 PLpro

To assess the comparative deISGylating activity of the different PLpros, an IFN treatment of transfected cells was performed to induce endogenous ISG15 production and conjugation, therefore making deISGylation more evident. Mono-ISG15 has a molecular weight of 15 kDa while ISG15 conjugation with several proteins is visible in a western blot as a smear of bands of varied molecular weight. Firstly, IFN- α and IFN- β treatment at a concentration of 1000 U/ml were compared to determine which type of IFN-I causes the highest level of induction of ISGylation. IFN- β treatment resulted in a stronger induction of ISG15 expression (data not shown) and was, therefore selected for the following IFN treatment experiments. Notably, there was no difference in ubiquitination between the treatments and there was also no variation both in mono-ubiquitin or ubiquitin conjugate levels

between SARS-CoV-2 PLpro and the negative control (data not shown), which further confirms its diminished deubiquitinating activity.

In PLpro transfected cells treated with IFN- β , the levels of mono-ISG15 and Conjugated ISG15 observed for each of the PLpro constructs were measured (Figure 14). SARS-CoV-2 PLpro showed the most prominent inhibition of ISGylation, which suggests that it has a higher specificity to ISG15 than when compared to the PLpro from the other coronaviruses, in accordance to what was reported by others [87]. Interestingly, the decrease in conjugated ISG15 levels is accompanied by a decrease in mono-ISG15 indicating that PLpro may inhibit cellular production of ISG15.

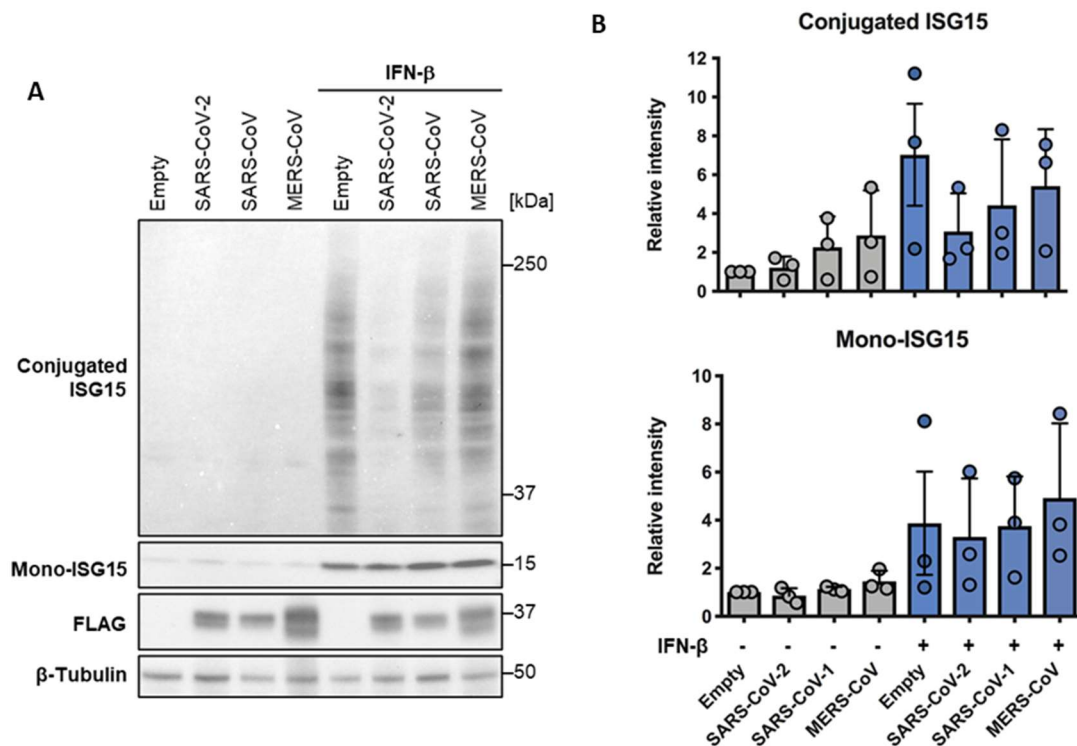


Figure 14. IFN- β treatment to compare the deISGylation activity of the different viral PLpro constructs. A. Western blots of one representative experiment. B. Conjugated and Mono-ISG15 quantification relative to the non-treated control transfected with the Empty plasmid and normalised with the β -tubulin loading control.

2.4. LMW Poly(I:C) treatment reveals no inhibition of IFN production by FLAG-tagged SARS-CoV-2 PLpro

To test if PLpro inhibits IFN production upon activation of RIG-I signalling, HeLa cells were transfected with a plasmid construct and, after 24h, treated with a synthetic dsRNA polymer – Polyinosinic:polycytidylic acid (Poly(I:C)). The Poly(I:C) used here was coupled with a transfection reagent (LyoVec) which ensures its delivery into the cytoplasm of the cells, mimicking viral infection [133], and, since this is a short Low Molecular Weight (LMW) dsRNA polymer, it is preferentially detected by RIG-I [134, 135]. After a screening of several cell lines treated with 2 μ g/ml of LMW Poly(I:C) for 6 or 16 h (Figure 15.A), HeLa cell lines and a 6h-long treatment were selected since a significant increase in IFN- β levels (12.5 fold increase) was observed. Plasmid expression was validated by Western Blot analysis (Figure 15.B). For these conditions, there was an observable decrease in IFN- β production in cells transfected with SARS-CoV-1 and MERS-CoV PLpro. However, in the presence of SARS-CoV-2 PLpro, no inhibition of IFN- β production was noted and, conversely, there was a visible increase (Figure 15.C).

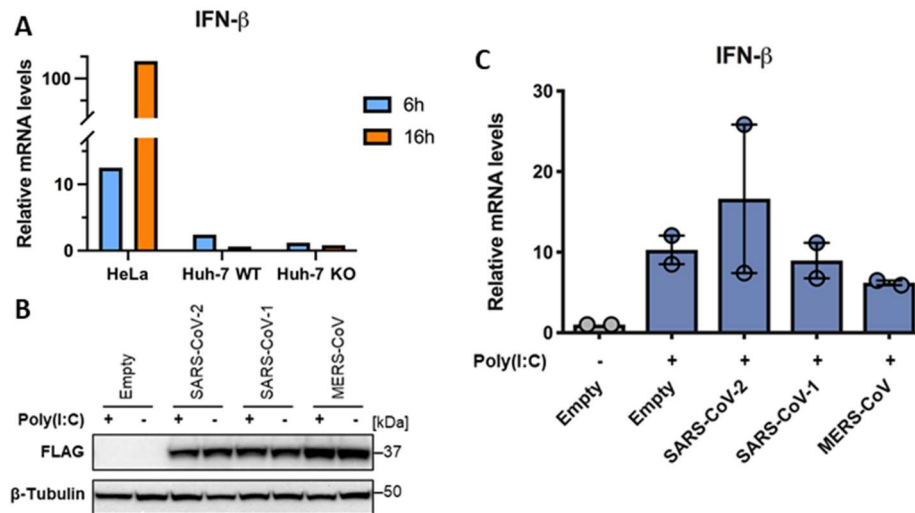


Figure 15. A. Comparison of HeLa, Huh-7 and Huh-7 ISG15 KO cells treated with LMW Poly(I:C) at 2 μ g/ml for 6 or 16 h. B. Immunoblot validating plasmid expression in transfected HeLa cells treated with 2 μ g/ml of LMW Poly(I:C) for 6 h. C. IFN- β production in transfected HeLa cells treated with 2 μ g/ml of LMW Poly(I:C) for 6 h.

To check the effect of a different concentration of LMW Poly(I:C) and also compare HeLa to more biologically relevant cell lines for COVID-19, the LMW Poly(I:C) concentration was adjusted to 1 μ g/ml and Calu-3, a human lung cell line, Caco2, a human colon epithelial cell line, and HeLa were treated. Calu-3 did not show PLpro expression after transfection (data not shown). For HeLa cells, the results were similar to what was observed before in the sense that SARS-CoV-2 PLpro caused no inhibition of IFN- β production while there was an observable decrease in the presence of SARS-CoV-1 PLpro. The PLpro of MERS-CoV, on the other hand, did not cause inhibition of IFN- β production in this experiment. Caco-2 (Figure 16.B) showed inducibility of RIG-I signalling and the effect of the PLpros on IFN- β production was observed to be similar to that seen in HeLa cells (Figure 16.A).

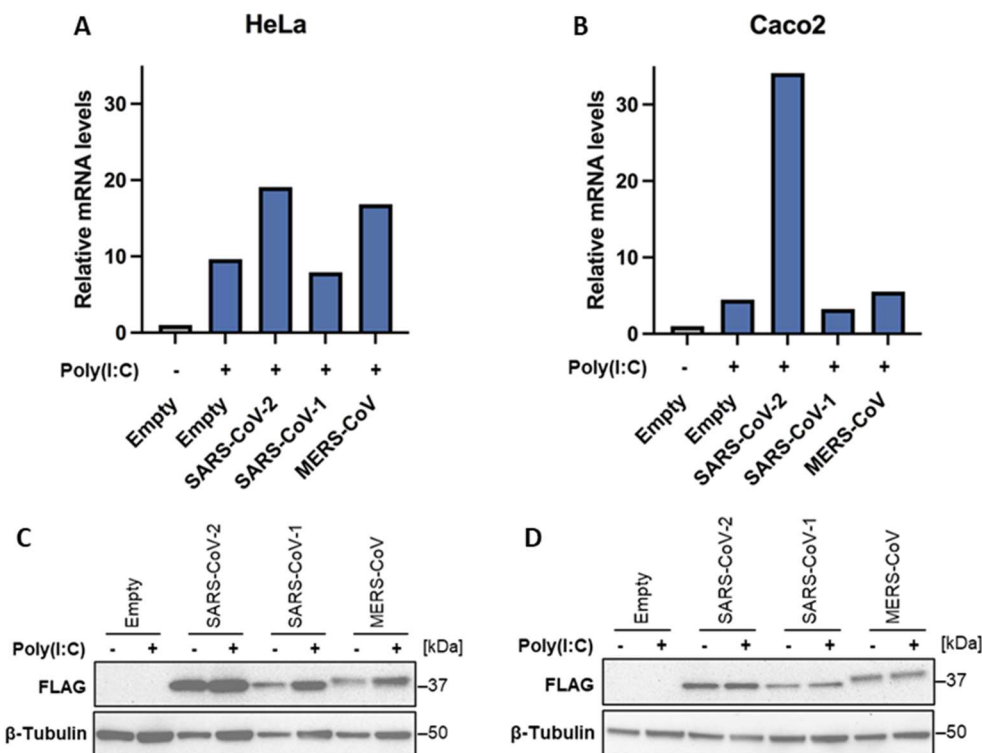


Figure 16. Comparison between IFN- β production in transfected HeLa (A) and Caco2 (B) cells treated with 1 μ g/ml of LMW Poly(I:C) for 6 h. Protein expression in HeLa (C) and Caco2 (D) was verified through western blot.

Since this observation directly contradicts findings reported by others [87], we hypothesized that the reason why no inhibition of IFN- β production was observed for SARS-CoV-2 PLpro may lie with the N-terminal tag interfering with the folding and interaction of PLpro with its substrates, or that this viral protease is very specific for MDA5 and the LMW Poly(I:C) used is not stimulating this signalling pathway adequately.

2.5. LMW Poly(I:C) treatment shows no inhibition of IFN- β production by V5-tagged SARS-CoV-2 PLpro

To test the hypothesis of the N-terminal FLAG tag interfering with the folding of PLpro, the experiment was now repeated using similar plasmids encoding the viral PLpros with a C-terminal V5-tag (Figure 17.A), which have been validated in a previous study [84]. After confirmation of Plasmid expression in HeLa cells by Western Blot analysis (Figure 17.B), the relative mRNA levels of IFN- β were measured (Figure 17.C). Once more, an increase in IFN- β production in the presence of SARS-CoV-2 was observed. This finding indicates that the N-terminal position of the FLAG tag used in the previous PLpro plasmid constructs is not the cause of the absence of inhibition of IFN- β production by SARS-CoV-2 3xFLAG-PLpro.

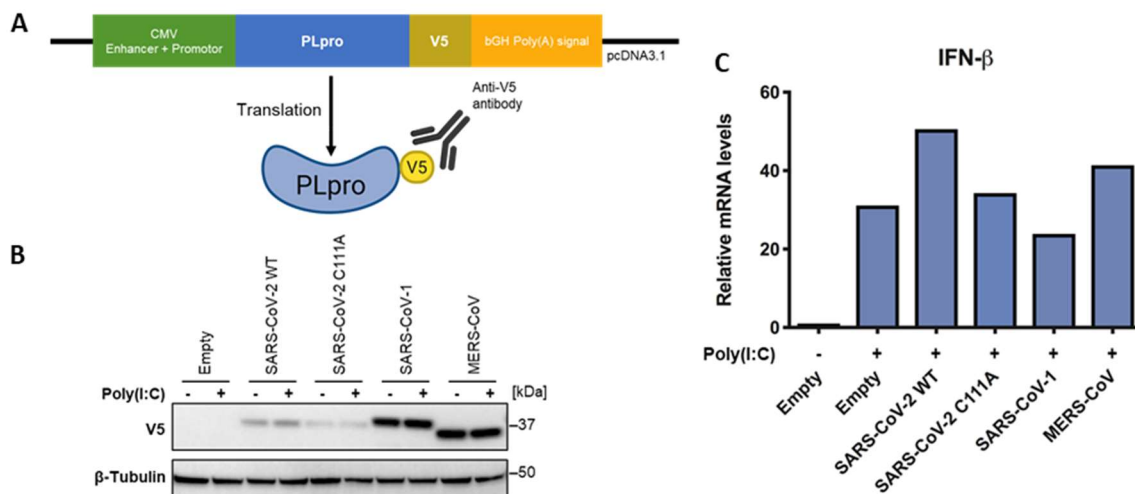


Figure 17. A. Illustration of the V5-tagged PLpro Plasmid maps, translation product and antibody detection for immune assays. B. Western Blot validation of V5-tagged plasmid expression in transfected HeLa cells treated with 1 μ g/ml of LMW Poly(I:C) for 6 h. C. IFN- β production in transfected HeLa cells treated with 1 μ g/ml of LMW Poly(I:C) for 6 h.

2.6. HMW Poly(I:C) treatment indicates inhibition of MDA5 signalling by FLAG-tagged SARS-CoV-2 PLpro

To test if PLpro inhibits IFN production upon activation of MDA5 signalling, HeLa cells were transfected with FLAG-tagged plasmids and now treated with High Molecular Weight (HMW) Poly(I:C), which preferentially induces this signalling pathway [134, 135] (Figure 18). Western Blot analysis revealed poor expression of SARS-CoV-1 PLpro which explains the lack of induction of IFN- β observed in the qPCR results. In the presence of SARS-CoV-2 and MERS-CoV PLpro, there was now a visible inhibition of IFN- β production. This suggests that SARS-CoV-2 PLpro has a specific inhibitory action on MDA5 signalling, but not RIG-I.

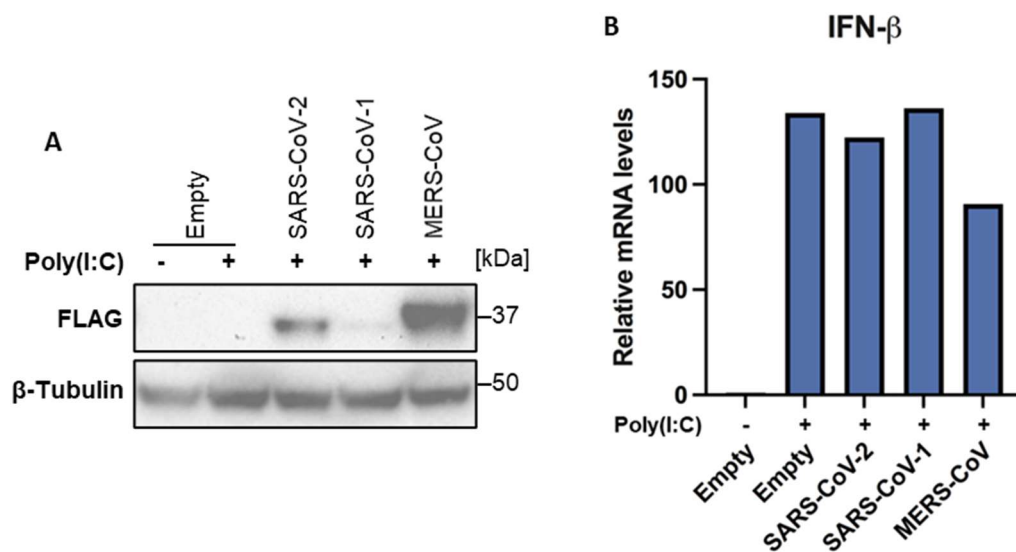


Figure 18. HMW Poly(I:C) treatment of HeLa Cells at 1 μ g/ml for 6 h. A. Western Blot validation of FLAG-tagged plasmid expression in transfected cells. B. IFN- β production in transfected cells.

Discussion

Despite the unprecedented worldwide effort employed to study COVID-19, there are many aspects of SARS-CoV-2 infection that remain unknown. To study viral-host interactions, an appropriate cell model that recapitulates SARS-CoV-2 infection *in vitro* is essential. Even though the African green monkey Vero-E6 cell line is commonly used in coronavirus research, its lack of genes encoding IFN-I makes this model not suitable for the study of the immune response to hCoV infection [94]. As such, cell lines of human origin are more physiologically relevant *in vitro* models.

Several cell lines were tested in the search for a model to compare the effects of SARS-CoV-2, SARS-CoV-1 and MERS-CoV infection on the host cell (Figure 5). Importantly, membrane receptors ACE2 and TMPRSS2 have been identified as mediators of SARS-CoV-2 cell entry and infection [6]. Vero-E6, Calu-3 and Caco2, which co-expressed ACE2 and TMPRSS2, showed the most susceptibility to SARS-CoV-2 infection. Despite lacking ACE2 expression, the Huh-7 and 293FT cell lines showed strong TMPRSS2 expression and were also susceptible to infection. These findings indicate that the presence of ACE2 and co-expression of ACE2 and TMPRSS2 strongly correlate with cell susceptibility to viral infection, however TMPRSS2 alone can mediate viral cell entry, even if to a lesser extent. In fact, the presence of these membrane receptors is not exclusively required to mediate viral cell entry. SARS-CoV-2 can also take advantage of different cell receptors, such as NRP1 and CD147 [23, 24], and has been shown to enter target cells via endocytosis, even if in a delayed manner [28]. This allows for a broader spectrum of target cells and tissues, which manifests *in vivo* by COVID-19 side effects and disease-associated pathogenic manifestations in several host organs. Indeed, COVID-19 is primarily associated with lung injury, but loss of taste and smell is also widely reported and patients with severe forms of the disease also suffer liver damage, cardiovascular complications, coagulation disorders, etc. [28, 32-34, 41].

From the cell lines considered, Huh-7, a human hepatocyte-derived cellular carcinoma cell line, has been previously observed to sustain SARS-CoV-1 and MERS-CoV replication [120, 121], and now also SARS-CoV-2 (Figure 5). This cell line was thus selected to perform a proteomics study aiming at comparing the viral induced cellular translational changes upon infection of each of the three hCoVs. Proteomics datasets obtained from earlier studies [94, 111] were now reanalysed to characterize the global host cell response to viral infection. The most significant changes in protein abundance in mock-infected versus virus-infected Huh-7 cells at a MOI of 1 were previously noted at 48 hpi for SARS-CoV-2 and SARS-CoV-1 and, since MERS-CoV was observed to be highly cytopathic and by 48 hpi all the cells died, the 24 hpi timepoint for this virus was considered. There were large protein clusters with significant increase and decrease in abundance for each dataset (Figure 6).

Pathway enrichment analysis of the significantly differentially abundant proteins revealed several key cellular pathways which are altered during viral infection. Particularly, among the most significant changes inflicted by SARS-CoV-2 infection, this analysis identified pathways related to endocytosis and lysosomes, ribosome and protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, and carbon metabolism (Figure 7.A). These can be correlated to various steps of viral infection such as viral cell entry, hijacking of the host translation machinery, and interfering with signalling and metabolism. Several other global proteomics studies on SARS-CoV-2 infection have been performed in different cell lines such as Caco-2 [96], Vero-6 [136, 137] and ACE2-expressing A549 cells [95]. Regardless of the cell model used, and in line with our findings, the differentially regulated

pathways found were commonly associated with splicing and translation [95, 96, 110, 137], protein homeostasis [96] and central carbon metabolism [95, 96, 136, 137].

Comparing SARS-CoV-2, SARS-CoV-1 and MERS-CoV infection, an overlap of 3 common significantly altered pathways was detected in the virus datasets (Figure 7). Interestingly, one of these pathways is identified in the KEGG 2021 database as Coronavirus disease – COVID-19 (Pathway: hsa05171). This pathway compiles information from numerous sources regarding the mechanisms of coronavirus infection, with particular focus on SARS-CoV-2, and consists of proteins related to endocytosis, IFN and RLR signalling, translation, complement and coagulation cascades, among others, and all known interactions between SARS-CoV-2 and human cellular proteins [122]. When comparing the SARS-CoV-2, SARS-CoV-1 and MERS-CoV datasets, variations in differential protein abundance within this pathway were noted (Figure 8). This finding shows that the viruses interact with the host cell machinery in different ways and elicit distinct host responses to infection.

To further investigate the modulation of the host cellular environment by SARS-CoV-2, SARS-CoV-1 and MERS-CoV, a protein-protein interaction analysis of the differentially abundant proteins belonging to the Coronavirus disease pathway was performed for each of the datasets.

The SARS-CoV-2 protein-protein interaction network showed several modules of connected proteins related to the complement system, coagulation cascade, antiviral signalling pathways and a module consisting of Ribosome associated proteins (Figure 9). Particularly, the antiviral signalling module comprises several proteins of the IFN pathway being that the majority of them are upregulated. This indicates that the host cell mounts a strong innate immune response to SARS-CoV-2 at 48 hpi. Since coronaviruses employ several mechanisms of viral evasion of innate immunity, the host IFN response is often delayed and diminished during the early phases of infection, allowing the virus to propagate uncontrollably. Facing a high viral load, the host later enters a state of hyperinflammation where a cytokine storm is established, aggravating tissue damage. A late production IFN, as is observed here, exacerbates the acute inflammatory response and constitutes a driving force behind COVID-19 pathogenesis [42]. The balance between the ability of SARS-CoV-2 to modulate the cellular environment upon infection and the efficacy of the host immune response determines disease outcome [43]. Following this line of thought, most interestingly, ISG15 occupies a central position in the network displaying a high number of interactions with several other proteins. Since SARS-CoV-2 PLpro has been shown to interact with ISG15 [87], this result highlights both the importance of ISG15 in coronavirus disease and the possible role of SARS-CoV-2 PLpro in modulation of the cellular response against viral infection. The Ribosome module, on the other hand, includes mostly downregulated proteins. This suggests that SARS-CoV-2 infection leads to host shut-off which is consistent with findings from several studies that identified a number of viral proteins that block global mRNA translation [76-78]. The presence of a module containing upregulated proteins belonging to the coagulation cascade strengthens the association between SARS-CoV-2 infection and RAS dysregulation, as well as the consequent cardiovascular complications caused by COVID-19 [32]. Also, complement activation has been reported in severe cases of COVID-19 [45], which is consistent with the identification of a module with differentially regulated proteins of the complement system. Furthermore, other proteomics studies of patient samples have also identified prominent changes in the coagulation and complement system pathways [100, 138].

For SARS-CoV-1, the network obtained shows a smaller number of proteins with significantly altered abundance belonging to the Coronavirus disease pathway, the coagulation module is missing, but

there is a similar pattern of protein abundance within the remaining modules (Figure 10). SARS-CoV-1 also utilizes the ACE2 receptor and dysregulation of the coagulation cascade has been observed in SARS patients [32]. Indeed, a transcriptomics analysis by Tang et al. has showed upregulation of the expression of genes associated with the coagulation pathway in Huh-7 cells at 2 and 4 hpi [139]. This upregulation might be restricted to an early stage of infection, which could explain why it was not observed here, at 48 hpi. Complement overactivation has also been reported in SARS [46], which is consistent with the complement module found here. The impact of SARS-CoV-1 on the IFN pathway is visible within the antiviral signalling module. A major difference in this network when compared to the previous is the absence of differential regulation of ISG15. This indicates that SARS-CoV-1 interference with the IFN pathway may be less dependent on ISG15 than for SARS-CoV-2.

In the MERS-CoV network, the 4 protein modules observed in the SARS-CoV-2 network are also easily identified, however, this dataset shows a distinctive pattern of protein abundance (Figure 11). The antiviral signalling module shows a smaller number of altered proteins, indicating that MERS-CoV has a distinct impact on the host immune response to viral infection. Conversely, the ribosome module is upregulated which suggests that MERS-CoV does not achieve host shut-off at 24 hpi. The effect of MERS-CoV infection in the coagulation cascade was noticed here, which is consistent with the coagulation disorders reported in patients with MERS [41]. Since there is no evidence of MERS-CoV interacting with ACE2 or of the existence of MERS-associated endotheliitis, the mechanism through which this virus affects coagulation remains unknown. Complement overactivation has previously been associated to immunopathogenesis in MERS [47], and indeed, this network displays a larger complement module with downregulated proteins. This suggests that MERS-CoV can affect the coagulation system at several levels.

As ISG15 appears to have a prominent role in the immune response to SARS-CoV-2, we set out to study its encoded Papain-like protease (PLpro) which has been shown to have deISGylating and deubiquitinating (DUB) activities in previous hCoVs [18]. Importantly, SARS-CoV-2 and SARS-CoV-1 PLpro showed high degree of homology, while MERS-CoV PLpro differed significantly from the other two. The core catalytic domains [128], including the catalytically active cysteine, are conserved among the three viral strains, however, there are significant differences in the ubiquitin and ISG15 binding domains.

Plasmids encoding SARS-CoV-2, SARS-CoV-1 and MERS-CoV PLpro with an N-terminal 3xFLAG-tag were here built as a tool to compare the role of PLpro in immune modulation during infection of the three hCoVs (Figure 12). Due to the fact that HeLa cells express a wide range of proteins [129], which is ideal to study viral-host protein-protein interactions, this cell line was selected to be transfected with the plasmids, and protein expression was validated through Western Blot.

The phenotype of the fusion 3xFLAG-PLpro proteins was then assessed. Since PLpro has shown a strong DUB activity in SARS-CoV-1 and MERS-CoV infection [130, 131], a DUB probing assay was performed to compare the DUB activity of SARS-CoV-2 PLpro with the previous hCoVs (Figure 13). The HA-Ub-VME probe used mimics the ubiquitin substrate and covalently binds to the active site of the DUB enzyme, therefore acting as an activity-based probe [82, 132]. SARS-CoV-2 PLpro showed a diminished deubiquitinating activity when compared to SARS-CoV-1 and MERS-CoV. This is in accordance with a recent study by Shin et al., where it was observed that the PLpro of SARS-CoV-2 cleaved K48-linked di-ubiquitin less efficiently than SARS-CoV-1 [87]. This finding suggests that the role of SARS-CoV-2 PLpro on immune modulation may centre around its interaction with ISG15.

IFN treatment induces endogenous ISG15 production and conjugation, therefore allowing for a clearer observation of deISGylation activity. In transfected cells treated with IFN- β , SARS-CoV-2 PLpro showed the most prominent inhibition of ISGylation, which suggests a higher specificity binding to ISG15 than when compared to PLpros from SARS-CoV-1 and MERS-CoV-1 (Figure 14), similarly to what was also reported by Shin et al. [87]. Notably, the decrease in conjugated ISG15 levels was accompanied by a decrease in mono-ISG15, indicating that PLpro may inhibit cellular production of ISG15.

Having assessed the DUB and deISGylating activity of the different PLpros, their degree of IFN antagonism was then analysed. For this, a synthetic dsRNA polymer – Poly(I:C) – was used in combination with a transfection reagent which ensures its delivery into the cytoplasm of the cells, mimicking viral infection and stimulating IFN signalling [133]. Importantly, host recognition of dsRNA is length-dependent, in the sense that RIG-I recognises short dsRNAs whereas MDA5 selectively recognizes long dsRNAs [134].

To measure PLpro's inhibition of the IFN pathway downstream of RIG-I signalling, first, a short LMW Poly(I:C) was used. Several cell lines, concentrations of Poly(I:C) and durations of the treatment were tested. Across all experiments, a common trend of a decrease of IFN- β production was observed in the presence of SARS-CoV-1 PLpro, but, in the case of SARS-CoV-2 no inhibition of IFN- β was noted and, conversely, there was a visible increase (Figure 15 and Figure 16). This observation contradicts findings from a similar study, where the authors reported a 5-fold inhibition of IFN- β production in A549 cells transfected with SARS-CoV-2 PLpro and treated with 500 ng poly(I:C) for 18 h [87]. However, the type of Poly(I:C) used in this study is not specified and there are no mentions of it being coupled with a transfection reagent. Naked Poly(I:C), as opposed to transfected, can also be recognized by TLR3 on top of activating RIG-I/MDA5 signalling, leading to a broader induction of IFN production [133, 140]. Thus, this study does not inform on the specific signalling pathway with which SARS-CoV-2 interferes.

As such, we hypothesized that the reason why we observed no inhibition of IFN- β production in the presence of SARS-CoV-2 PLpro may lie with the N-terminal FLAG tag interfering with the folding and interaction of the protease with its substrates, or that PLpro is very specific for MDA5 signalling which is not adequately stimulated by LMW Poly(I:C).

To investigate the hypothesis of the N-terminal FLAG-tag interfering with the folding of the PLpro fusion protein, similar plasmids encoding the viral PLpros with a C-terminal V5-tag, which have been validated in a recent study [84], were then tested, using the previous experimental setup (Figure 17). Again, an increase in IFN- β production in the presence of SARS-CoV-2 was observed, which ruled out this hypothesis. Next, longer High Molecular Weight (HMW) Poly(I:C), which preferentially binds to MDA5 [134, 135], was used to test if PLpro inhibits IFN production upon activation of MDA5 signalling (Figure 18). In the presence of SARS-CoV-2, there was now an observable inhibition of IFN- β production. Together, these results suggest that SARS-CoV-2 PLpro has a specific inhibitory action on MDA5 signalling, but not RIG-I. As SARS-CoV-2 PLpro shows a strong deISGylating activity, this inhibition is likely ISG15-dependent. This is in line with another study by Liu et al. that has recently identified a mechanism through which SARS-CoV-2 PLpro inhibits MDA5 signalling. The authors reported that ISGylation of the caspase activation and recruitment domains activates MDA5, triggering IFN production and the mounting of the innate immune response against viral infection. SARS-CoV-2 PLpro directly deISGylates MDA5 therefore suppressing this signalling pathway [84].

It is also interesting to note that, in a similar way as MDA5 ISGylation, ubiquitination is responsible for stabilizing RIG-I upon sensing of viral RNA, triggering IFN signalling [83]. Even if at a basal level, ISGylation of MDA5 may be sufficient to activate this PRR, RIG-I-mediated IFN production leads to an increase in ISG15 expression, which will further induce MDA5 signalling. As ISG15 negatively regulates RIG-I, after the initial stages of infection, a 'sensor switch' might occur where MDA5 signalling predominates over RIG-I activity [84]. Since SARS-CoV-1 and MERS-CoV PLpro show a strong deubiquitinating activity, their IFN antagonism may be more connected to suppression of RIG-I signalling, early in coronavirus infection. Indeed, SARS-CoV-1 PLpro has been previously found to deubiquitinate RIG-I leading to suppression of IFN production [86]. In contrast, SARS-CoV-2 PLpro, which shows greater deISGylating activity, may inhibit IFN production through blocking of MDA5 signalling, at a later stage of infection. Nevertheless, this mechanism of IFN antagonization through MDA5 deISGylation is likely conserved among coronaviruses, rendering PLpro a potential therapeutic target against the already known hCoVs, but also other novel emergent strains that may arise in the future [84, 87].

Importantly, some limitations are acknowledged for this study. Foremost, the analysis is restricted to cell lines, which might not constitute physiologically representative models to study the mechanisms of infection *in vivo*. More complex *in vitro* models such as three-dimensional (3D) organotypic cultures can better mimic human tissue and allow the study of coronavirus infection in a more relevant context [141, 142]. However, handling of 3D organoids is technically challenging, time-consuming, and expensive, and cell lines can still provide an overview of the complexity of the interactions between SARS-CoV-2 and host cellular targets. Furthermore, the experiments are restricted to discrete timepoints and more detailed temporal analysis might better elucidate on the dynamics of viral infection. Also, due to time constraints, it was only possible to perform one experimental replicate for some of the experiments. As such, only observable changes were reported, without statistical validation. Nonetheless, the results obtained are consistent to what has been reported by others, as explained above. Also, this work will be continued by other members of the lab to fully validate the plasmid constructs before moving on to the next experiments. Finally, previous *in vivo* and *in vitro* studies on SARS-CoV-1 PLpro have reported conflicting results [18]. Therefore, further analysis is warranted to clarify the substrate specificity of PLpro and discover what factors influence its activity during viral infection.

Conclusions and future perspectives

Overall, this study highlights the differences and similarities in the modulation of cellular environment upon SARS-CoV-2, SARS-CoV-1 and MERS-CoV infection. Even though assumptions cannot be made about the mechanism of infection and immune regulation of novel strains based on findings from previous ones, many common molecular therapeutic targets can be found amongst different hCoVs. A deeper understanding of the interplay between the host immune response to coronavirus infection and the mechanisms of viral escape may be a useful tool to fight viral diseases and prevent future outbreaks.

As previously discussed, dysregulation of IFN signalling has been widely reported during coronavirus infection. IFNs, therefore, have great therapeutic potential. In fact, administration of IFN- β 1a, in combination with other antiviral drugs, was shown effective at reducing mortality of hospitalized patients and has been approved in different therapies against COVID-19 in Iran [143, 144]. Even though a robust early IFN response is essential to control viral infection, at a later stage of disease, IFN supplementation can drive hyperinflammation and a cytokine storm, aggravating tissue and systemic damage. Thus, there are still many questions surrounding the timing, type of interferon, and route of administration and IFN therapies for COVID-19 are not yet widely approved [145].

Importantly, this and other proteomics analysis identified different ISG signatures in SARS-CoV-2, SARS-CoV-1 and MERS-CoV infection. A follow-up mechanistic study including an siRNA screening could be performed to selectively knock-out gene expression of each ISG and identify which ones are essential for an effective innate immune response against viral infection [146]. A common set of ISGs could potentially be identified and targeted to develop more universal therapies for coronavirus-related diseases.

Here, an *in vitro* model to study the interactions between the viral PLpro and host cellular proteins was established. After complete validation of the phenotype of the fusion 3xFLAG-PLpros produced from our plasmid constructs, co-immunoprecipitation (CO-IP) followed by mass spectrometry (MS) and bioinformatic analysis of the viral-host protein complexes will be carried out to identify the cellular targets of the different viral PLpros. A functional *in vitro* immunofluorescence assay will also be performed to evaluate the co-localization of the viral and target proteins in the cellular compartments.

Since deISGylation was identified as a key function of SARS-CoV-2 PLpro, infection of the ISG15 KO Huh-7 cells can be carried out to evaluate differences in infectivity. This would help determine if ISG15 can be specifically targeted to control viral infection and reveal its impact on immunometabolism during coronavirus infection.

Lastly, the World Health Organization has currently identified 4 new variants of concern – Alpha, Beta, Gamma and, the most recent, Delta SARS-CoV-2 – which show increased transmissibility, virulence or resistance to vaccines and other therapies [147]. An analysis similar to what was performed here to compare the changes in the host cellular environment and even the role of the PLpro of the different emerging strains of SARS-CoV-2, could show how mechanisms of infection and viral immune escape evolve, paving the way to more effective antiviral therapies.

Supplementary data

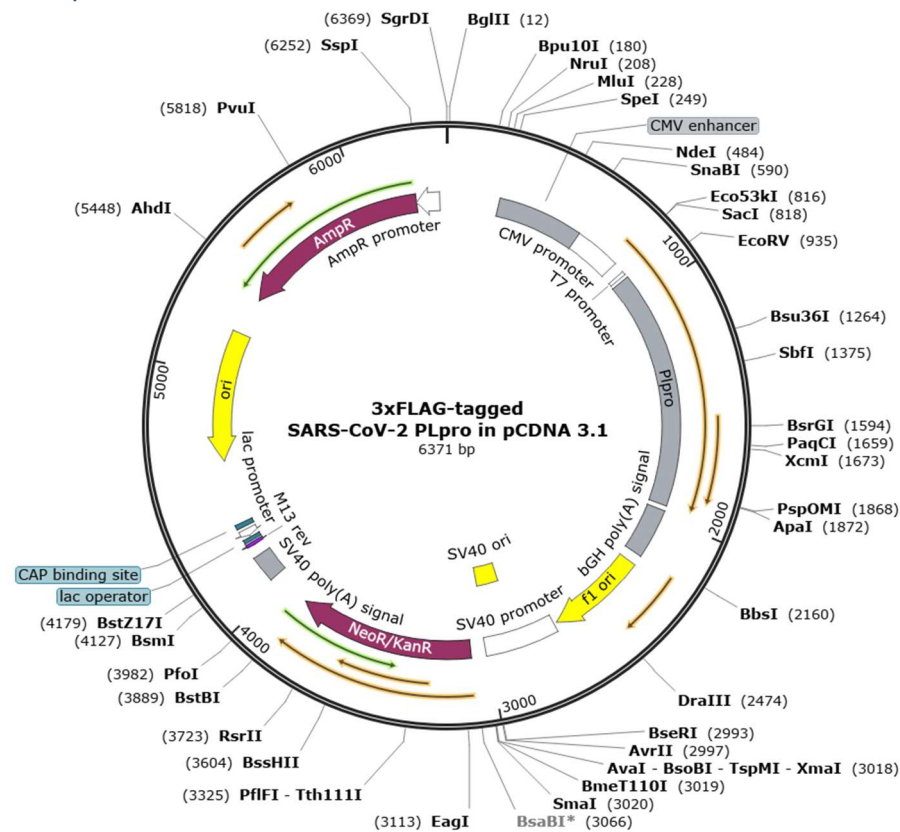


Figure 19. Plasmid map of 3xFLAG-tagged SARS-CoV-2 PLpro with relevant components and restriction enzyme recognition sites.

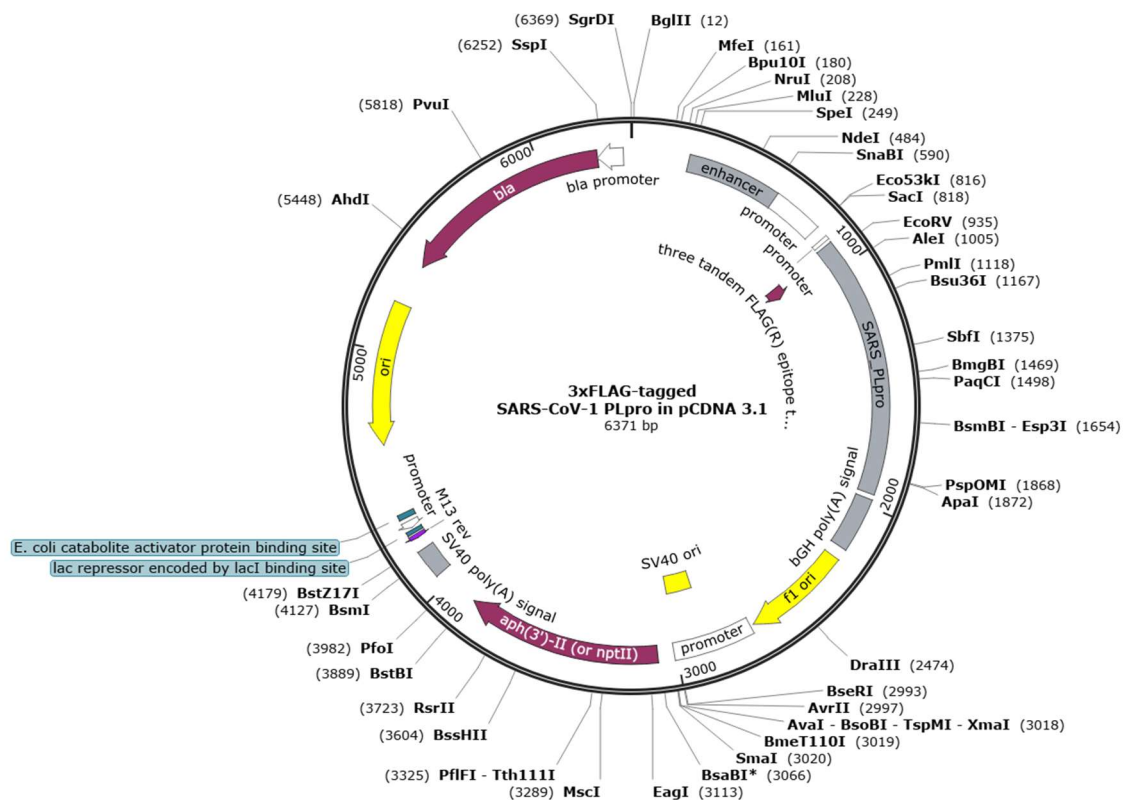
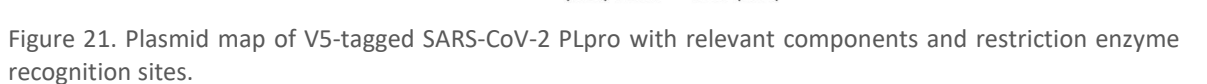
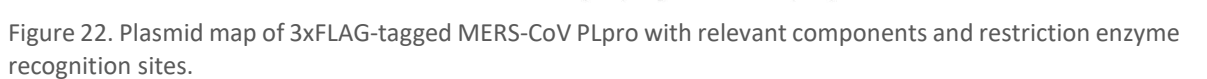


Figure 20. Plasmid map of 3xFLAG-tagged SARS-CoV-1 PLpro with relevant components and restriction enzyme recognition sites.



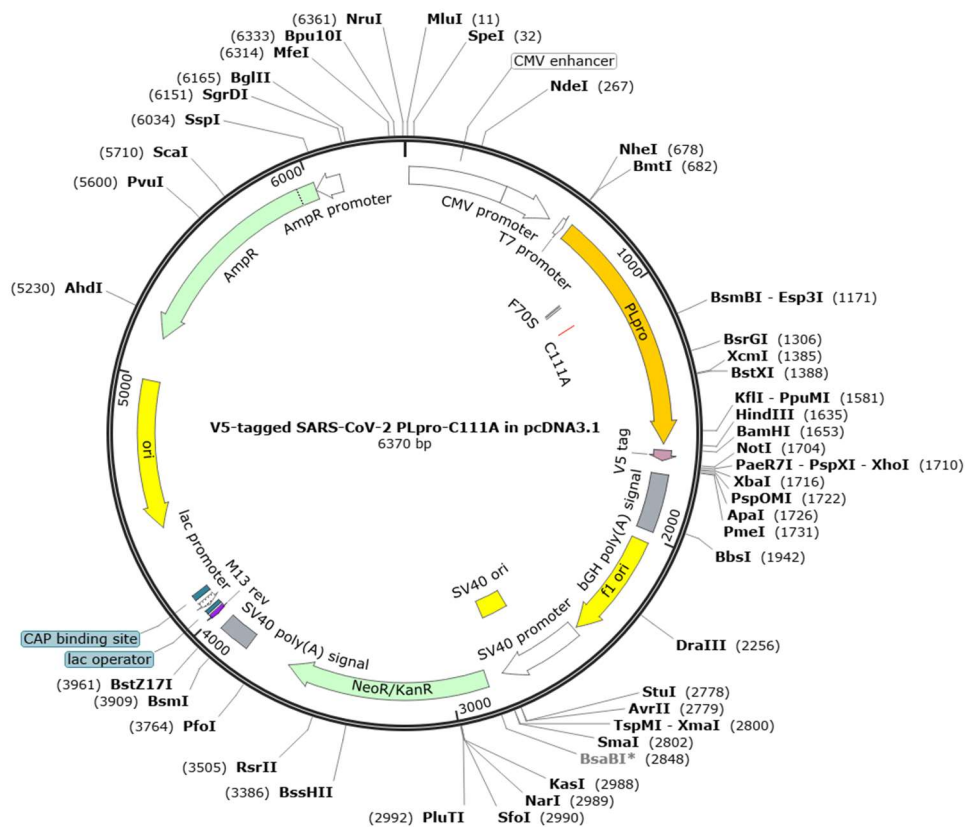


Figure 24. Plasmid map of V5-tagged catalytic mutant (C111A) SARS-CoV-2 PLpro with relevant components and restriction enzyme recognition sites.

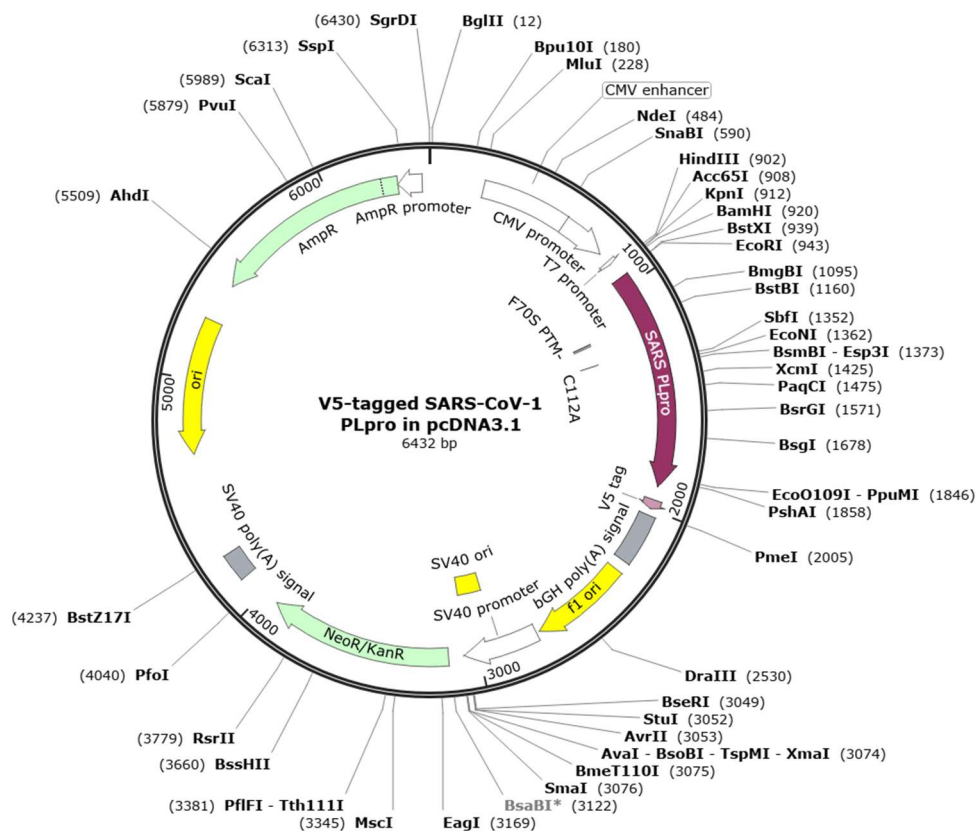


Figure 23. Plasmid map of V5-tagged SARS-CoV-1 PLpro with relevant components and restriction enzyme recognition sites.

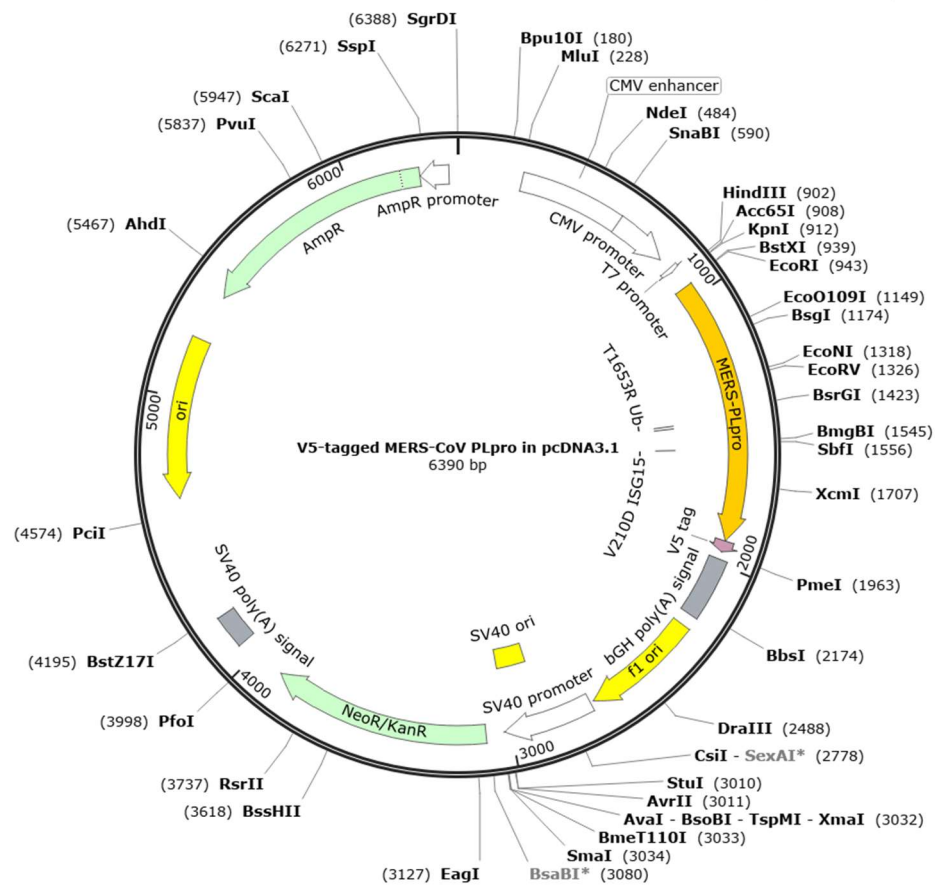


Figure 25. Plasmid map of V5-tagged MERS-CoV PLpro with relevant components and restriction enzyme recognition sites.

References

1. Payne, S., *Family Coronaviridae*. Viruses, 2017: p. 149-158.
2. Organization, W.H. *Severe Acute Respiratory Syndrome (SARS)*. 29.06.2021]; Available from: https://www.who.int/health-topics/severe-acute-respiratory-syndrome#tab=tab_1.
3. WHO. *Middle East respiratory syndrome coronavirus (MERS-CoV)*. 2019 17.06.2021]; Available from: [https://www.who.int/en/news-room/fact-sheets/detail/middle-east-respiratory-syndrome-coronavirus-\(mers-cov\)](https://www.who.int/en/news-room/fact-sheets/detail/middle-east-respiratory-syndrome-coronavirus-(mers-cov)).
4. (ECDC), E.C.f.D.P.a.C. *COVID-19 situation update worldwide, as of week 21, updated 10 June 2021*. 2021 17.06.2021]; Available from: <https://www.ecdc.europa.eu/en/geographical-distribution-2019-ncov-cases>.
5. Prompetchara, E., C. Ketloy, and T. Palaga, *Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic*. Asian Pacific Journal of Allergy and Immunology, 2020. **38**(1): p. 1-9.
6. Hoffmann, M., et al., *SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor*. Cell, 2020. **181**(2).
7. Li, W., et al., *Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus*. Nature, 2003. **426**(6965): p. 450-454.
8. Raj, V.S., et al., *Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC*. Nature, 2013. **495**(7440): p. 251-254.
9. (JHU), C.f.S.S.a.E.C.a.J.H.U. *COVID-19 Dashboard*. 17.06.2021]; Available from: <https://gisanddata.maps.arcgis.com/apps/dashboards/bda7594740fd40299423467b48e9ecf6>.
10. Prevention, U.S.D.o.H.H.S.C.f.D.C.a. *SARS (10 Years After)*. 2016 17.06.2021]; Available from: <https://www.cdc.gov/dotw/sars/index.html>.
11. WHO-China, *Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19)*. 2020.
12. Ke, R., et al., *Estimating the reproductive number R_0 of SARS-CoV-2 in the United States and eight European countries and implications for vaccination*. Journal of Theoretical Biology, 2021. **517**: p. 110621.
13. Petrosillo, N., et al., *COVID-19, SARS and MERS: are they closely related?* Clinical Microbiology and Infection, 2020. **26**(6): p. 729-734.
14. WHO. *Middle East respiratory syndrome*. 2021 17.06.2021]; Available from: <http://www.emro.who.int/health-topics/mers-cov/mers-outbreaks.html>.
15. Ansariniya, H., et al., *Comparison of Immune Response between SARS, MERS, and COVID-19 Infection, Perspective on Vaccine Design and Development*. BioMed Research International, 2021. **2021**: p. 8870425.
16. Yuki, K., M. Fujiogi, and S. Koutsogiannaki, *COVID-19 pathophysiology: A review*. Clinical immunology (Orlando, Fla.), 2020. **215**: p. 108427-108427.
17. Rana, R., V. Rath, and N.K. Ganguly, *A comprehensive overview of proteomics approach for COVID 19: new perspectives in target therapy strategies*. Journal of Proteins and Proteomics, 2020. **11**(4): p. 223-232.
18. Lei, J., Y. Kusov, and R. Hilgenfeld, *Nsp3 of coronaviruses: Structures and functions of a large multi-domain protein*. Antiviral research, 2018. **149**: p. 58-74.
19. Chen, Y., Q. Liu, and D. Guo, *Emerging coronaviruses: Genome structure, replication, and pathogenesis*. Journal of Medical Virology, 2020. **92**(4): p. 418-423.
20. Báez-Santos, Y.M., S.E. St. John, and A.D. Mesecar, *The SARS-coronavirus papain-like protease: Structure, function and inhibition by designed antiviral compounds*. Antiviral Research, 2015. **115**: p. 21-38.

21. Snijder, E.J., E. Decroly, and J. Ziebuhr, *Chapter Three - The Nonstructural Proteins Directing Coronavirus RNA Synthesis and Processing*, in *Advances in Virus Research*, J. Ziebuhr, Editor. 2016, Academic Press. p. 59-126.
22. Song, Z., et al., *From SARS to MERS, Thrusting Coronaviruses into the Spotlight*. *Viruses*, 2019. **11**(1).
23. Cantuti-Castelvetri, L., et al., *Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity*. *Science*, 2020. **370**(6518): p. 856-860.
24. Wang, K., et al., *CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells*. *Signal Transduction and Targeted Therapy*, 2020. **5**(1): p. 283.
25. Coutard, B., et al., *The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade*. *Antiviral Research*, 2020. **176**: p. 104742.
26. Ziegler, C.G.K., et al., *SARS-CoV-2 receptor ACE2 is an interferon-stimulated gene in human airway epithelial cells and is detected in specific cell subsets across tissues*. *Cell*, 2020.
27. Qing, E., et al., *Evaluating MERS-CoV Entry Pathways*, in *MERS Coronavirus: Methods and Protocols*, R. Vijay, Editor. 2020, Springer US: New York, NY. p. 9-20.
28. Koch, J., et al., *TMPRSS2 expression dictates the entry route used by SARS-CoV-2 to infect host cells*. *The EMBO Journal*. **n/a**(n/a): p. e107821.
29. InvivoGen. *Repurposing approved drugs to target SARS-CoV-2*. 2020 [cited 2020; Available from: <https://www.invivogen.com/spotlight-covid-19-treatment-repurposed-drugs>].
30. Xu, J., et al., *Systematic Comparison of Two Animal-to-Human Transmitted Human Coronaviruses: SARS-CoV-2 and SARS-CoV*. *Viruses*, 2020. **12**(2): p. 244.
31. Wrapp, D., et al., *Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation*. *Science*, 2020. **367**(6483): p. 1260.
32. Varga, Z., et al., *Endothelial cell infection and endotheliitis in COVID-19*. *The Lancet*, 2020. **395**(10234): p. 1417-1418.
33. Ding, Y., et al., *Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways*. *The Journal of pathology*, 2004. **203**(2): p. 622-630.
34. Xu, L., et al., *Liver injury during highly pathogenic human coronavirus infections*. *Liver International*, 2020. **40**(5): p. 998-1004.
35. Hou, Y.J., et al., *SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract*. *Cell*, 2020.
36. Kuba, K., et al., *A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury*. *Nature Medicine*, 2005. **11**(8): p. 875-879.
37. Gurwitz, D., *Angiotensin receptor blockers as tentative SARS-CoV-2 therapeutics*. *Drug Development Research*, 2020.
38. Imai, Y., et al., *Angiotensin-converting enzyme 2 protects from severe acute lung failure*. *Nature*, 2005. **436**(7047): p. 112-116.
39. Yau, J.W., H. Teoh, and S. Verma, *Endothelial cell control of thrombosis*. *BMC cardiovascular disorders*, 2015. **15**: p. 130-130.
40. Pearson, J.D., *Endothelial cell function and thrombosis*. *Best Practice & Research Clinical Haematology*, 1999. **12**(3): p. 329-341.
41. Giannis, D., I.A. Ziogas, and P. Gianni, *Coagulation disorders in coronavirus infected patients: COVID-19, SARS-CoV-1, MERS-CoV and lessons from the past*. *Journal of Clinical Virology*, 2020. **127**: p. 104362.
42. Portela Sousa, C. and C. Brites, *Immune response in SARS-CoV-2 infection: the role of interferons type I and type III*. *The Brazilian Journal of Infectious Diseases*, 2020. **24**(5): p. 428-433.
43. Kikkert, M., *Innate Immune Evasion by Human Respiratory RNA Viruses*. *Journal of Innate Immunity*, 2020. **12**(1): p. 4-20.

44. Yoshikawa, T., et al., *Severe Acute Respiratory Syndrome (SARS) Coronavirus-Induced Lung Epithelial Cytokines Exacerbate SARS Pathogenesis by Modulating Intrinsic Functions of Monocyte-Derived Macrophages and Dendritic Cells*. Journal of Virology, 2009. **83**(7): p. 3039.
45. Cao, X., *COVID-19: immunopathology and its implications for therapy*. Nature Reviews Immunology, 2020.
46. Gralinski, L.E., et al., *Complement Activation Contributes to Severe Acute Respiratory Syndrome Coronavirus Pathogenesis*. mBio, 2018. **9**(5): p. e01753-18.
47. Jiang, Y., et al., *Complement Receptor C5aR1 Inhibition Reduces Pyroptosis in hDPP4-Transgenic Mice Infected with MERS-CoV*. Viruses, 2019. **11**(1): p. 39.
48. Park, A. and A. Iwasaki, *Type I and Type III Interferons - Induction, Signaling, Evasion, and Application to Combat COVID-19*. Cell Host & Microbe, 2020.
49. Yin, X., et al., *MDA5 Governs the Innate Immune Response to SARS-CoV-2 in Lung Epithelial Cells*. Cell Reports, 2021. **34**(2).
50. Thompson, M.R., et al., *Pattern Recognition Receptors and the Innate Immune Response to Viral Infection*. Viruses, 2011. **3**(6).
51. Stanifer, M.L., K. Pervolaraki, and S. Boulant, *Differential Regulation of Type I and Type III Interferon Signaling*. International Journal of Molecular Sciences, 2019. **20**(6): p. 1445.
52. Platanitis, E., et al., *A molecular switch from STAT2-IRF9 to ISGF3 underlies interferon-induced gene transcription*. Nature Communications, 2019. **10**(1): p. 2921.
53. Sallard, E., et al., *Type 1 interferons as a potential treatment against COVID-19*. Antiviral research, 2020. **178**: p. 104791-104791.
54. Blanco-Melo, D., et al., *Imbalanced host response to SARS-CoV-2 drives development of COVID-19*. 2020: CellPress.
55. Broz, P. and V.M. Dixit, *Inflammasomes: mechanism of assembly, regulation and signalling*. Nature Reviews Immunology, 2016. **16**(7): p. 407-420.
56. Fung, S.-Y., et al., *A tug-of-war between severe acute respiratory syndrome coronavirus 2 and host antiviral defence: lessons from other pathogenic viruses*. Emerging Microbes & Infections, 2020. **9**(1): p. 558-570.
57. Qian, Y., et al., *Direct activation of endothelial cells by SARS-CoV-2 nucleocapsid protein is blocked by Simvastatin*. bioRxiv, 2021: p. 2021.02.14.431174.
58. Perico, L., et al., *Immunity, endothelial injury and complement-induced coagulopathy in COVID-19*. Nature Reviews Nephrology, 2021. **17**(1): p. 46-64.
59. Shi, Y., et al., *COVID-19 infection: the perspectives on immune responses*. Cell Death & Differentiation, 2020. **27**(5): p. 1451-1454.
60. de Wit, E., et al., *SARS and MERS: recent insights into emerging coronaviruses*. Nature Reviews Microbiology, 2016. **14**(8): p. 523-534.
61. Hadjadj, J., et al., *Impaired type I interferon activity and exacerbated inflammatory responses in severe Covid-19 patients*. medRxiv, 2020: p. 2020.04.19.20068015.
62. Kindler, E., V. Thiel, and F. Weber, *Chapter Seven - Interaction of SARS and MERS Coronaviruses with the Antiviral Interferon Response*, in *Advances in Virus Research*, J. Ziebuhr, Editor. 2016, Academic Press. p. 219-243.
63. Rai, K.R., et al., *Acute Infection of Viral Pathogens and Their Innate Immune Escape*. Frontiers in Microbiology, 2021. **12**(1358).
64. Viswanathan, T., et al., *Structural basis of RNA cap modification by SARS-CoV-2*. Nature Communications, 2020. **11**(1): p. 3718.
65. Encinar, J.A. and J.A. Menendez, *Potential Drugs Targeting Early Innate Immune Evasion of SARS-Coronavirus 2 via 2'-O-Methylation of Viral RNA*. Viruses, 2020. **12**(5): p. 525.
66. Hsin, F., et al., *Distinct Inductions of and Responses to Type I and Type III Interferons Promote Infections in Two SARS-CoV-2 Isolates*. bioRxiv, 2020: p. 2020.04.30.071357.

-
67. Jeong Yoon Lee, S.B.a.J.M., *Middle East Respiratory Syndrome Coronavirus-Encoded Accessory Proteins Impair MDA5-and TBK1-Mediated Activation of NF- κ B*. Journal of Microbiology and Biotechnology, 2019. **29**(8): p. 1316-1323.
 68. Siu, K.-L., et al., *Severe Acute Respiratory Syndrome Coronavirus M Protein Inhibits Type I Interferon Production by Impeding the Formation of TRAF3.TANK.TBK1/IKKepsilon complex*. Journal of Biological Chemistry, 2009. **284**(24): p. 16202-16209.
 69. Yang, D., et al., *Attenuated Interferon and Proinflammatory Response in SARS-CoV-2–Infected Human Dendritic Cells Is Associated With Viral Antagonism of STAT1 Phosphorylation*. The Journal of Infectious Diseases, 2020.
 70. Yuen, C.-K., et al., *SARS-CoV-2 nsp13, nsp14, nsp15 and orf6 function as potent interferon antagonists*. Emerging Microbes & Infections, 2020. **9**(1): p. 1418-1428.
 71. Konno, Y., et al., *SARS-CoV-2 ORF3b Is a Potent Interferon Antagonist Whose Activity Is Increased by a Naturally Occurring Elongation Variant*. Cell Reports, 2020. **32**(12).
 72. Miorin, L., et al., *SARS-CoV-2 Orf6 hijacks Nup98 to block STAT nuclear import and antagonize interferon signaling*. Proceedings of the National Academy of Sciences, 2020. **117**(45): p. 28344-28354.
 73. Menachery, V.D., et al., *Pathogenic Influenza Viruses and Coronaviruses Utilize Similar and Contrasting Approaches To Control Interferon-Stimulated Gene Responses*. mBio, 2014. **5**(3): p. e01174-14.
 74. Thornbrough, J.M., et al., *Middle East Respiratory Syndrome Coronavirus NS4b Protein Inhibits Host RNase L Activation*. mBio, 2016. **7**(2): p. e00258-16.
 75. Finkel, Y., et al., *SARS-CoV-2 uses a multipronged strategy to impede host protein synthesis*. Nature, 2021. **594**(7862): p. 240-245.
 76. Banerjee, A.K., et al., *SARS-CoV-2 Disrupts Splicing, Translation, and Protein Trafficking to Suppress Host Defenses*. Cell, 2020. **183**(5): p. 1325-1339.e21.
 77. Schubert, K., et al., *SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation*. Nature Structural & Molecular Biology, 2020. **27**(10): p. 959-966.
 78. Addetia, A., et al., *SARS-CoV-2 ORF6 Disrupts Bidirectional Nucleocytoplasmic Transport through Interactions with Rae1 and Nup98*. mBio, 2021. **12**(2): p. e00065-21.
 79. Nandi, D., et al., *The ubiquitin-proteasome system*. J Biosci, 2006. **31**(1): p. 137-55.
 80. Isaacson, M.K. and H.L. Ploegh, *Ubiquitination, Ubiquitin-like Modifiers, and Deubiquitination in Viral Infection*. Cell Host & Microbe, 2009. **5**(6): p. 559-570.
 81. Villarroya-Beltri, C., S. Guerra, and F. Sánchez-Madrid, *ISGylation – a key to lock the cell gates for preventing the spread of threats*. Journal of Cell Science, 2017. **130**(18): p. 2961-2969.
 82. Hewings, D.S., et al., *Activity-based probes for the ubiquitin conjugation–deconjugation machinery: new chemistries, new tools, and new insights*. The FEBS Journal, 2017. **284**(10): p. 1555-1576.
 83. Rehwinkel, J. and M.U. Gack, *RIG-I-like receptors: their regulation and roles in RNA sensing*. Nature Reviews Immunology, 2020. **20**(9): p. 537-551.
 84. Liu, G., et al., *ISG15-dependent activation of the sensor MDA5 is antagonized by the SARS-CoV-2 papain-like protease to evade host innate immunity*. Nature Microbiology, 2021. **6**(4): p. 467-478.
 85. Li, S.-W., et al., *SARS Coronavirus Papain-Like Protease Inhibits the TLR7 Signaling Pathway through Removing Lys63-Linked Polyubiquitination of TRAF3 and TRAF6*. International Journal of Molecular Sciences, 2016. **17**(5): p. 678.
 86. Chen, X., et al., *SARS coronavirus papain-like protease inhibits the type I interferon signaling pathway through interaction with the STING-TRAF3-TBK1 complex*. Protein & Cell, 2014. **5**(5): p. 369-381.
 87. Shin, D., et al., *Papain-like protease regulates SARS-CoV-2 viral spread and innate immunity*. Nature, 2020. **587**(7835): p. 657-662.
-

88. McClain, C.B. and N. Vabret, *SARS-CoV-2: the many pros of targeting PLpro*. Signal Transduction and Targeted Therapy, 2020. **5**(1): p. 223.
89. Sperk, M., et al., *Utility of Proteomics in Emerging and Re-Emerging Infectious Diseases Caused by RNA Viruses*. Journal of Proteome Research, 2020. **19**(11): p. 4259-4274.
90. Gerold, G., J. Bruening, and T. Pietschmann, *Decoding protein networks during virus entry by quantitative proteomics*. Virus Research, 2016. **218**: p. 25-39.
91. Zhou, S., et al., *Viral proteomics: The emerging cutting-edge of virus research*. Science China Life Sciences, 2011. **54**(6): p. 502-512.
92. Whetton, A.D., et al., *Proteomics and Informatics for Understanding Phases and Identifying Biomarkers in COVID-19 Disease*. Journal of Proteome Research, 2020. **19**(11): p. 4219-4232.
93. Zhou, P., et al., *A pneumonia outbreak associated with a new coronavirus of probable bat origin*. Nature, 2020. **579**(7798): p. 270-273.
94. Saccon, E., et al., *Cell-type-resolved quantitative proteomics map of interferon response against SARS-CoV-2*. iScience, 2021. **24**(5): p. 102420.
95. Stukalov, A., et al., *Multilevel proteomics reveals host perturbations by SARS-CoV-2 and SARS-CoV*. Nature, 2021. **594**(7862): p. 246-252.
96. Bojkova, D., et al., *Proteomics of SARS-CoV-2-infected host cells reveals therapy targets*. Nature, 2020. **583**(7816): p. 469-472.
97. Yang, Y., et al., *Plasma IP-10 and MCP-3 levels are highly associated with disease severity and predict the progression of COVID-19*. Journal of Allergy and Clinical Immunology, 2020. **146**(1): p. 119-127.e4.
98. Wei, X., et al., *Elevations of serum cancer biomarkers correlate with severity of COVID-19*. Journal of Medical Virology, 2020. **92**(10): p. 2036-2041.
99. Qiu, M., et al., *Antibody responses to individual proteins of SARS coronavirus and their neutralization activities*. Microbes and Infection, 2005. **7**(5): p. 882-889.
100. Shen, B., et al., *Proteomic and Metabolomic Characterization of COVID-19 Patient Sera*. Cell, 2020. **182**(1): p. 59-72.e15.
101. Renuse, S., et al., *Development of mass spectrometry-based targeted assay for direct detection of novel SARS-CoV-2 coronavirus from clinical specimens*. medRxiv, 2020: p. 2020.08.05.20168948.
102. Pushpakom, S., et al., *Drug repurposing: progress, challenges and recommendations*. Nature Reviews Drug Discovery, 2019. **18**(1): p. 41-58.
103. Mukherjee, A., et al., *Proteomics advances towards developing SARS-CoV-2 therapeutics using in silico drug repurposing approaches*. Drug Discovery Today: Technologies, 2021.
104. Gordon, D.E., et al., *A SARS-CoV-2 protein interaction map reveals targets for drug repurposing*. Nature, 2020. **583**(7816): p. 459-468.
105. ClinicalTrials.gov. *IFN Beta-1b and Ribavirin for Covid-19*. 01.07.2021]; Available from: <https://clinicaltrials.gov/ct2/show/NCT04494399>.
106. India, C.t.r.-. *CTRI/2021/01/030231*. 2021 01.07.2021]; Available from: http://ctri.nic.in/Clinicaltrials/pdf_generate.php?trialid=50985&EncHid=&modid=&compid=%27,%2750985det%27.
107. Institute, M. *COVID-19 Tracker*. 2020 01.07.2021]; Available from: <https://airtable.com/shrSAi6t5WFwqo3GM/tblEzPQS5fnc0FHfYR/viwJJuMA1iolgAcPs?blocks=bipZFzhJ7wHPv7x9z>.
108. Grein, J., et al., *Compassionate Use of Remdesivir for Patients with Severe Covid-19*. New England Journal of Medicine, 2020. **382**(24): p. 2327-2336.
109. Rohde, C., S. Becker, and V. Krahling, *Marburg virus regulates the IRE1/XBP1-dependent unfolded protein response to ensure efficient viral replication*. Emerg Microbes Infect, 2019. **8**(1): p. 1300-1313.

-
110. Appelberg, S., et al., *Dysregulation in Akt/mTOR/HIF-1 signaling identified by proteo-transcriptomics of SARS-CoV-2 infected cells*. *Emerging Microbes & Infections*, 2020. **9**(1): p. 1748-1760.
111. Chen, X., et al., *Type-I interferon signatures in SARS-CoV-2 infected Huh7 cells*. *Cell Death Discovery*, 2021. **7**(1): p. 114.
112. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. *Nucleic Acids Research*, 2015. **43**(7): p. e47-e47.
113. Team, R.C. *A language and environment for statistical computing*. 2020; Available from: <https://www.R-project.org/>.
114. Xie, Z., et al., *Gene Set Knowledge Discovery with Enrichr*. *Current Protocols*, 2021. **1**(3): p. e90.
115. Kanehisa, M., et al., *KEGG: integrating viruses and cellular organisms*. *Nucleic Acids Research*, 2020. **49**(D1): p. D545-D551.
116. Wickham, H., *ggplot2: Elegant Graphics for Data Analysis*. 2016: Springer-Verlag.
117. Chen, H. *VennDiagram: Generate High-Resolution Venn and Euler Plots*. *R package version 1.6.20*. 2018; Available from: <https://CRAN.R-project.org/package=VennDiagram>.
118. Warnes, G.R., et al. *gplots: Various R Programming Tools for Plotting Data*. *R package version 3.1.1*. 2020; Available from: <https://CRAN.R-project.org/package=gplots>.
119. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks*. *Genome Res*, 2003. **13**(11): p. 2498-504.
120. DeDiego, M.L., et al., *A Severe Acute Respiratory Syndrome Coronavirus That Lacks the E Gene Is Attenuated In Vitro and In Vivo*. *Journal of Virology*, 2007. **81**(4): p. 1701-1713.
121. de Wilde, A.H., et al., *MERS-coronavirus replication induces severe in vitro cytopathology and is strongly inhibited by cyclosporin A or interferon- α treatment*. *Journal of General Virology*, 2013. **94**(8): p. 1749-1760.
122. KEGG. *PATHWAY: hsa05171*. 03.07.2021]; Available from: <https://www.genome.jp/entry/pathway+hsa05171>.
123. CA Jr, J., et al., *Immunobiology: The Immune System in Health and Disease*. 5th edition ed. 2001.
124. Battin, C., et al., *Neuropilin-1 Acts as a Receptor for Complement Split Products*. *Frontiers in Immunology*, 2019. **10**(2209).
125. Simurda, T., et al., *Genetic Variants in the FGB and FGG Genes Mapping in the Beta and Gamma Nodules of the Fibrinogen Molecule in Congenital Quantitative Fibrinogen Disorders Associated with a Thrombotic Phenotype*. *International Journal of Molecular Sciences*, 2020. **21**(13): p. 4616.
126. Seth, R.B., L. Sun, and Z.J. Chen, *Antiviral innate immunity pathways*. *Cell Research*, 2006. **16**(2): p. 141-147.
127. Zhou, X., et al., *Ribosomal proteins: functions beyond the ribosome*. *Journal of Molecular Cell Biology*, 2015. **7**(2): p. 92-104.
128. Klemm, T., et al., *Mechanism and inhibition of the papain-like protease, PLpro, of SARS-CoV-2*. *The EMBO Journal*, 2020. **39**(18): p. e106275.
129. Fountoulakis, M., et al., *Protein profile of the HeLa cell line*. *Journal of Chromatography A*, 2004. **1038**(1): p. 247-265.
130. Barretto, N., et al., *The Papain-Like Protease of Severe Acute Respiratory Syndrome Coronavirus Has Deubiquitinating Activity*. *Journal of Virology*, 2005. **79**(24): p. 15189-15198.
131. Mielech, A.M., et al., *MERS-CoV papain-like protease has deISGylating and deubiquitinating activities*. *Virology*, 2014. **450-451**: p. 64-70.
132. Misaghi, S., et al., *Structure of the Ubiquitin Hydrolase UCH-L3 Complexed with a Suicide Substrate*. *Journal of Biological Chemistry*, 2005. **280**(2): p. 1512-1520.
133. InvivoGen. *Poly(I:C) (LMW) / LyoVec™*. 25.06.2021]; Available from: <https://www.invivogen.com/polyic-lmw-lyovec>.
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References

134. Kato , H., et al., *Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5*. Journal of Experimental Medicine, 2008. **205**(7): p. 1601-1610.
135. Reikine, S., J.B. Nguyen, and Y. Modis, *Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5*. Frontiers in Immunology, 2014. **5**(342).
136. Grenga, L., et al., *Shotgun proteomics analysis of SARS-CoV-2-infected cells and how it can optimize whole viral particle antigen production for vaccines*. Emerging Microbes & Infections, 2020. **9**(1): p. 1712-1721.
137. Zecha, J., et al., *Data, Reagents, Assays and Merits of Proteomics for SARS-CoV-2 Research and Testing*. Molecular & Cellular Proteomics, 2020. **19**(9): p. 1503-1522.
138. Li, J., et al., *Virus-Host Interactome and Proteomic Survey Reveal Potential Virulence Factors Influencing SARS-CoV-2 Pathogenesis*. Med, 2021. **2**(1): p. 99-112.e7.
139. Tang, B.S.F., et al., *Comparative Host Gene Transcription by Microarray Analysis Early after Infection of the Huh7 Cell Line by Severe Acute Respiratory Syndrome Coronavirus and Human Coronavirus 229E*. Journal of Virology, 2005. **79**(10): p. 6180-6193.
140. Gitlin, L., et al., *Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus*. Proceedings of the National Academy of Sciences, 2006. **103**(22): p. 8459-8464.
141. Clevers, H., *COVID-19: organoids go viral*. Nature Reviews Molecular Cell Biology, 2020. **21**(7): p. 355-356.
142. Sridhar, A., et al., *A Perspective on Organoids for Virology Research*. Viruses, 2020. **12**(11): p. 1341.
143. ClinicalTrials.gov. *Interferon Beta 1a in Hospitalized COVID-19 Patients (IB1aIC)*. 2020 02.07.2021]; Available from: <https://clinicaltrials.gov/ct2/show/NCT04350671?term=Interferon&cond=Covid19&phase=3&draw=2&rank=1>.
144. ClinicalTrials.gov. *Umifenovir in Hospitalized COVID-19 Patients (UAIIC)*. 02.07.2021]; Available from: <https://clinicaltrials.gov/ct2/show/NCT04350684?term=Interferon&cond=Covid19&phase=3&draw=2&rank=3>.
145. Calabrese, L.H., T. Lenfant, and C. Calabrese, *Interferon therapy for COVID-19 and emerging infections: Prospects and concerns*. Cleveland Clinic Journal of Medicine, 2020.
146. Mohr, S.E. and N. Perrimon, *RNAi screening: new approaches, understandings, and organisms*. WIREs RNA, 2012. **3**(2): p. 145-158.
147. Organization, W.H. *SARS-CoV-2 Variants of Concern and Variants of Interest, updated 15 June 2021*. 2021 01.07.2021]; Available from: <https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/>.