From Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

INTERFERING WITH INTERFERONS: INTERPLAY BETWEEN SARS-COV-2 AND INTERFERON RESPONSE

Xi Chen

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Interfering with interferons: Interplay between SARS-CoV-2 and Interferon response

Thesis for Doctoral Degree (Ph.D.)

By

Xi Chen

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Principal Supervisor:
Assistant Professor Soham Gupta
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisor(s):
Associate Professor Ujjwal Neogi
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Docent Ákos Végvári
Karolinska Institutet
Department of Medical biochemistry and biophysics
Division of Chemistry I

Opponent:
Professor Francis Impens
University of Ghent
Department of Biomolecular Medicine

Examination Board:
Docent Johan Lennerstrand
Uppsala University
Department of Medical Sciences

Docent Koustav Ganguly
Karolinska Institutet
Department of Environmental Medicine
Integrative toxicology

Docent Harold Marcotte
Karolinska Institutet
Department of Biosciences and Nutrition

There is only one heroism in the world: to see the world as it is, and to love it.

--Romain Rolland

ABSTRACT

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) caused the coronavirus disease 2019 (COVID-19) pandemic, which has been an ongoing global health crisis. At the beginning of the pandemic, all the efforts were directed toward understanding the pathogenesis of the virus. An early interferon (IFN) response is crucial in initiating and boosting the antiviral response. It was identified that the IFN response is dim and delayed in COVID-19 patients, accompanied by pro-inflammatory cytokine production. Circumvention and dysregulation of interferon (IFN) response were found to be characteristic of the SARS-CoV-2 infection, leading to its pathogenicity and severity in a group of COVID-19 patients. Thus, a better understanding of the pathogenic mechanisms of SARS-CoV-2 infection is crucial for a better therapeutic strategy against the disease. The thesis aimed to characterize the interplay between SARS-CoV-2 and host IFN response.

In **Paper I**, we assessed the susceptibility and cytotoxicity of the first Swedish isolate of SARS-CoV-2 in six cell lines of human origin in comparison to other globally isolated strains. Furthermore, we determined the proteomic landscape during SARS-CoV-2 infection in the susceptible cell lines, using LC-MS/MS-based tandem mass tags (TMT) labeling quantitative proteomics technology. The studies provided an overview of the signaling pathways altered by the SARS-CoV-2, elucidating IFN-signaling pathways.

In **Paper II**, we identified and characterized the expression of antiviral ISGs during SARS-CoV-1, MERS-CoV, and SARS-CoV-2 infection of the Huh7 cell line using TMT-labeled LC-MS/MS. Transcriptomic ISG signatures were identified for SARS-CoV-2 in a time-dependent manner. Furthermore, we identified that SARS-CoV-2 inhibited IFN-β production and showed a muted and delayed activation of ISGs in Huh7 cells. IFN treatment was found to be effective in controlling the virus prior to the establishment of the infection, and IFN treatment post-infection had no effect on the virus. We also showed increased virus production in a senescent Huh cell model.

Paper III explored how the virus infection impacts the IFN signaling pathways (IFN-I/ IFN-III) and interferon-stimulated gene (ISG) expression in COVID-19 patients. Irrespective of the disease status, heterogeneity was observed in the expression of ISGs. We categorized the patients based on type-I, type-II, and antiviral-response-related ISG scores obtained from whole-blood transcriptomics data. We investigated factors like immune cell proportions, neutrophil extracellular traps (NETs), inflammatory factors, metabolic status, and autoimmunity against IFNs, to try to find any association with the ISG score status of the patients. Autoimmune antibodies against IFNs were more prominent in patients with low ISG scores. Furthermore, the expression of ISGs was associated with a perturbation in amino acid and lipid metabolism.

In **Paper IV**, we investigated a potential innate immune evasion mechanism by SARS-CoV-2. We studied the role of a crucial virus protease: papain-like protease (PLpro), which has a potent deubiquitinating and deISGylating activity in inhibiting type-I IFN response. Using immunoprecipitation, we have identified that SARS-CoV-2 interacts with RIG-I signalosome components TRIM25 and RIG-I. Catalytically active PLpro could deubiquitinate the constitutively active 2CARD domain, which leads to the inhibition of interferon response. The

SARS-CoV-2 homologs in other coronaviruses also interacted with TRIM25 and RIG-I and inhibited IFN production. These findings show another innate immune regulatory mechanism by Ub/UbL deconjugated activity of coronavirus PLpro.

In summary, the research covered in this thesis deciphers the significance of interferon response during SARS-CoV-2 infection.

LIST OF SCIENTIFIC PAPERS

I. Elisa Saccon*, **Xi Chen*,** Flora Mikaeloff*, Jimmy Esneider Rodriguez, Laszlo Szekely, Beatriz Sá Vinhas, Shuba Krishnan, Siddappa N Byrareddy, Teresa Frisan, Ákos Végvári, Ali Mirazimi, Ujjwal Neogi, Soham Gupta. Cell-type-resolved quantitative proteomics map of interferon response against SARS-CoV-2. iScience, 2021 May 21;24(5):102420

II. **Xi Chen*,** Elisa Saccon*, K Sofia Appelberg, Flora Mikaeloff, Jimmy Esneider Rodriguez, Beatriz Sá Vinhas, Teresa Frisan, Ákos Végvári, Ali Mirazimi, Ujjwal Neogi, Soham Gupta. Type-I interferon signatures in SARS-CoV-2 infected Huh7 cells. Cell Death Discov. 2021 May 18;7(1):114.

III. Flora Mikaeloff*, Sara Svensson Akusjärvi*, Axel Cederholm*, Ronaldo Lira-Junior, **Xi Chen,** Maike Sperk, Sefanit Rezene, Anoop T Ambikan, Anders Sonnerborg, Carl Johan Treutiger, Nils Landegren, Ujjwal Neogi, Soham Gupta. Expression of interferon stimulated genes and possible regulatory mechanisms in COVID-19. Manuscript format.

IV. **Xi Chen,** Sefanit Rezene, Jácome Toste, Soham Gupta. Papain-like protease of SARS-CoV-2 inhibits type-I IFN by interfering with RIG-I signalosome activation. Manuscript format.

*Equal Contribution

CONTENTS

LIST OF ABBREEVIATION

SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
SARS-CoV-1	Severe Acute Respiratory Syndrome Coronavirus
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
COVID-19	Coronavirus Disease 2019
HCoVs	Human Coronaviruses
CoVs	Coronaviruses
ORFs	Open reading frames
ACE2	Angiotensin-converting enzyme 2
pp	Polyproteins
NSPs	Non-structure proteins
ER	Endoplasmic reticulum
PRRs	Pattern recognition receptors
TLR3	Toll-like receptors 3
RIG-I	Retinoic acid-inducible gene I
MDA5	Melanoma differentiation-associated gene 5
IFN	Interferon
MAVS	Mitochondrial antiviral signaling protein
TNFR	Tumor necrosis factor receptor
IRF	Interferon regulatory factor
IFN-I	Type I interferons
IFNAR	Interferon receptors
JAK1	Janus kinase 1
Tyk2	tyrosine kinase 2
ISREs	interferon-stimulated response elements
ISGs	Interferon stimulatory genes
ARDS	acute respiratory distress syndrome
sHLH	haemophagocytic lymphohistiocytosis
CRS	cytokine release syndrome
TNF	tumor necrosis factor
APS-I	autoimmune polyendocrine syndrome type I

NETs	Neutrophil extracellular traps
FA	Fatty acid synthesis
PLpro	Papain-like protease
3Cpro/Mpro	3-chymotrypsin-like cysteine protease
Ub	Ubiquitin
DUBs	Deubiquitinases
deISGs	DeISGylases
FDR	False discovery rate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay

1 INTRODUCTION

1.1 The Human Coronaviruses (HCoVs) and SARS-CoV-2

Coronaviruses (CoVs) are enveloped single-stranded positive-sense RNA viruses with the biggest genome size (about 30 kb) among the RNA viruses (2). The highly pathogenic human coronaviruses (HCoVs) belong to the family *Coronaviridae* and subfamily *Coronavirinae*. They are divided into four genera named as alpha-coronavirus, beta-coronavirus, gamma-coronavirus, and delta-coronavirus (3). Several epidemics have been caused by the *Coronaviridae* family, but mostly with mild cold symptoms: Human coronavirus HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1 until the outbreak of the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) in Asia in 2002-2003, then the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Saudi Arabia in 2012 (4), and finally, the pandemic coronavirus disease 2019 (COVID-19) caused by the current Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (5).

SARS-CoV-2 is a novel beta-coronavirus with 79% genome sequence similarity to SARS-CoV and 50% similarity to MERS-CoV (6). Its genome structure is similar to that of other beta-coronaviruses. The six functional open reading frames (ORFs) are arranged in order from 5′ to 3′: two non-structural proteins ORF1a (replicas) and ORF1b (protease), and four structural proteins S (spike), E (envelope), M (membrane), and N (nucleocapsid). The viral capsid is shaped by the N protein, and the viral genome is encased in an envelope composed of the three structural proteins: S, M, and E. There are also six putative ORFs encoding accessory proteins between the structural genes. Furthermore, the overlapping viral polyproteins 1a and 1ab are further cleaved by the viral proteases to form 16 non-structural proteins with varied functions that aid the virus in establishing infection (7) (Figure 1).

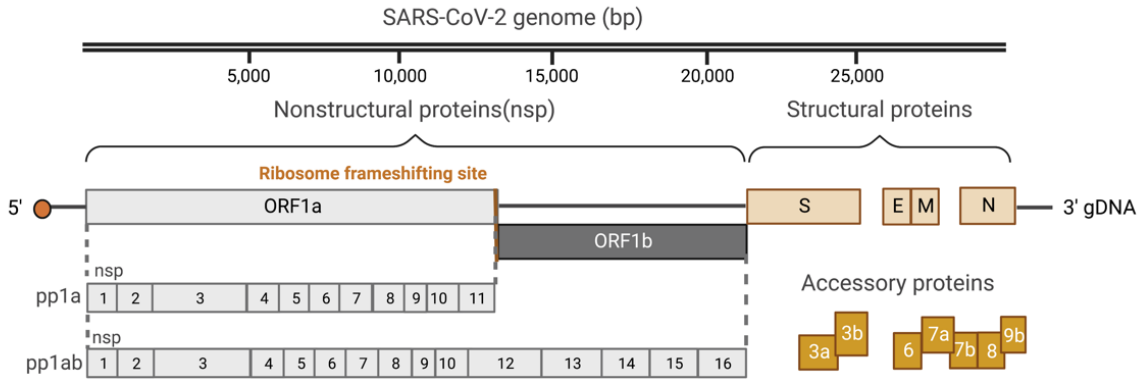

Figure 1: Schematic representation of the structure of the SARS-CoV-2 genome. ORF1a/b is cleaved proteolytically into 16 non-structural proteins (NSPs). Spike (S), Membrane (M), envelop (E), and nucleoprotein (N) are the structural proteins. ORF 3a, 3b, 6, 7a, 7b, 8, 9b. Accessory proteins. Adapted from (1). Created using Biorender.com.

The virus intracellular life cycle can be divided into receptor binding, entry, fusion and uncoating, initial translation, polyprotein processing, translation, viral RNA synthesis, accessory proteins-host interactions, assembly, exocytosis and virus release (8). Coronavirus infection begins with the coronavirus spike (S) protein binding mainly to the known cellular entry receptors angiotensin-converting enzyme 2 (ACE2) (9). Following entry, uncoated genomic RNA is ready for instant translation of the open reading frames ORF1a and ORF1b. Then polyproteins(pp) 1a and 1b are co- and post-translated into the individual Non-structure proteins (NSPs) that comprise the viral replication transcription complex. Translated structural protein then translocate to endoplasmic reticulum (ER) membranes and are presented on their membrane as a preparation for the assembly into virus particles. The nucleocapsid binds viral RNA and assembles into viral particles while budding mediated by secretory vesicular compartments. Finally, the virions are secreted by exocytosis from the infected cells (2). Recent studies have also shown the ability of the beta-coronaviruses to use lysosomal trafficking to egress out of cells (10).

1.2 SARS-CoV-2 and innate immune response

The innate immune response is the first line of protection against viral infections, and it is critical in combating the SARS-CoV-2 (11-13) and thereafter activates the adaptive immune system (14).

Coronaviruses share similarities in their cell entry mechanism. After binding to the receptors, the virus is internalized by endocytosis, followed by viral RNA's release in the cytoplasm. The viral NSPs and host factors initiate the viral replication/transcription complexes and synthesis of viral proteins (15). The pattern recognition receptors (PRRs), possibly the endosomal Toll-like receptors 3 (TLR3) and TLR7/8 (16) or cytosolic sensors retinoic acid-inducible gene I (RIG-I) and RIG-I like receptor melanoma differentiation-associated gene 5 (MDA5) recognize the viral RNA or gene products (Figure 2) that lead to the activation of a signaling cascade resulting in elicitation of type-I and type-III interferon (IFN) response (17). Following recognition of viral RNA by the RIG-I or MDA-5, the conformation of these receptors changes that leads to interaction with the E3-ubiquitin ligase TRIM25, followed by K63-linked ubiquitination of RIG-I/MDA-5 leading to their oligomerization and translocation to the mitochondria. After translocation to the mitochondria, the RIG-I signalosome complex interacts with and activates the mitochondrial adaptor, mitochondrial antiviral signaling protein (MAVS). Through ubiquitination mediated mechanism, the MAVS further activates tumor necrosis factor receptor-associated factor 6 (TRAF6), which through a series of phosphorylation events, results in phosphorylation and dimerization of Interferon regulatory factor (IRF) 3 or 7. Upon activation, the IRF3/7 homo-dimer translocates to the nucleus and binds to IFN-β and IFN-α promoters that lead to their transcriptional activation (18). Not only Type-I interferons (IFN-I), but a variety of ISGs expression is triggered during this step (19). Likewise, TLR3 and TLR7 also follow similar signaling activation cascades.

The released IFN-I can further be sensed in cis- or in trans- by the interferon receptors (IFNAR), which again leads to a signaling cascade that is initiated by the activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2). In a phosphorylation-dependent manner, the activated JAK1/TyK2 promotes the formation of STAT1, STAT2, and IRF9 complexes, translocation of the complex to the nucleus, and binding to interferon-stimulated response

elements (ISREs) of several anti-viral Interferon stimulatory genes (ISG's) like MX1, MX2, IFITs, OASs, ISG15 etc. (20). The diverse antiviral functional ISGs that are triggered can further result in additional amplification loops.

Notably, the IFN-I response may be quite different depending on the state of the responding cell (e.g., metabolic or general activation state) (21), stochastic (22, 23), differs through cell types and microenvironments, and exhibits inter-individual variability in terms of magnitude and kinetics (22-24).

1.3 IFN response and COVID-19 severity

Whether the cellular signaling system can fine-tune an interferon response is pretty important for the individual to control the disease since both excessive and insufficient IFN activation is detrimental to the host (25).

COVID-19 is a type of respiratory disease that is mild but often causes severe disease in some infected individuals. Severe patients have acute respiratory distress syndrome (ARDS) or multi-organ injuries caused by secondary haemophagocytic lymphohistiocytosis (sHLH). Either ARDS or sHLH is characterized by excessive cytokine release and inflammation, which leads to cytokine release syndrome (CRS) (26). The SARS-CoV-2-induced CRS is also reported in other highly pathogenic respiratory viruses, like SARS-CoV and MERS-CoV. Significantly increased pro-inflammatory plasma cytokines are detected in COVID-19 patients, including IL-6, tumor necrosis factor (TNF), and several chemokines.

The interferon (IFN) family of cytokines play key roles in shaping the immune response against viruses. In the context of COVID-19, IFNs are key for restraining SARS-CoV-2 infection but on the other hand have also been described as drivers of severe symptoms (27).

IFN-I deficiency has been linked to the SARS-CoV-2 pathogenesis and disease severity (28). It has been shown that the residual interferon response by an individual can dictate the disease progression. While a robust IFN response early during the virus infection can control the virus infection resulting in a positive disease outcome, a low-level primary interferon response showed a severe disease outcome, often with delayed IFN-I signaling that leads to the hyper-inflammation and "cytokine storm" (29).

The direct result of hyperinflammation is the recruitment of inflammatory cells in the infection sites, mostly lung tissues. The excessive cytokines and chemokines secretion lead to organ damage and dysregulation of the innate immune system (30). In the clinic, acute respiratory distress syndrome (ARDS) is commonly found in COVID-19 patients (29). The function of these fine-tuning mechanisms in COVID-19 has yet to be extensively discussed.

Due to the fact that SARS-CoV-2 inhibits the IFN-I system, medications that increase this cellular defense mechanism may increase early innate immune responses. IFN-I treatment may be useful in individuals with hereditary deficiencies in the interferon system (31), but not in those with autoimmune phenocopies of these deficiencies (32-34). Over twenty clinical studies are now evaluating the effectiveness of IFN-I therapy (34-36), the optimal time window for interferon therapy, and the advantages against the dangers of interferon therapy. Alternatives such as interferon (type-III IFN) that specifically targets receptors on epithelial cells without the wider effects of IFN-I are also being evaluated in clinical trials (37).

1.4 Regulators of IFN response during COVID-19

1.4.1 Genetic factors regulating IFN response

IFN is key to determining COVID-19 pathogenesis. An efficient IFN stimulation at the early stage prior to severe COVID-19 can create an antiviral environment that is restrictive for the virus and thus can prevent COVID-19 progression. Inborn genetic errors and autoreactive antibodies that interferes IFN response have been linked to life-threatening COVID-19 pneumonia. The presence of neutralizing antibodies against type-I and type-III IFNs can block their interactions with the IFNAR, thus interfering with downstream signaling to elicit an antiviral response. This could enable the virus to propagate without antiviral effectors during the early stages of infection. Predisposition to severe COVID has been observed in patients with autoimmune polyendocrine syndrome type I (APS-I), which causes increased IFN-autoantibody production (38). More than 10% of the life-threatening COVID-19 patients harbored antibodies against type-I IFNs with neutralization ability (31). However, it remains debatable whether the IFN autoantibodies decide the fate of the immune response or whether there are produced by the host in response to pathophysiological changes due to the severe infection that remains to be decided. The importance of the presence of type-I IFN response is further exemplified in studies detecting inborn genetic defects in the receptors, transducers, and the effectors of the type-I IFN signaling in 3.5% of the severe COVID-19 cases (31).

1.4.2 Neutrophil extracellular traps (NETs) regulating the inflammatory response

Genetic factors and cellular activation in response to the disease can also influence the IFN response. The inflammatory complications associated with severe COVID-19 has been linked to the formation of neutrophil extracellular traps (NETs) upon recruitment and infiltration of neutrophil in various organs (39). NETs are released by the activated neutrophils in the form of an extracellular web of chromatin, enzymes, and proteins, having the potential to cause inflammation and coagulation in the COVID-19 infected lungs (40). SARS-CoV-2 infection has been shown to directly activate neutrophils to release NET's and even NET formation was observed to be induced in healthy neutrophils by COVID-19 patient sera (41). NETs have been associated with inflammation in several respiratory diseases and based on them, several mechanisms have been hypothesized for lung damage due to SARS-CoV-2 infection. NET-mediated inflammatory reaction could be due to the NET-IL1β loop, mediated via the inflammasome activation (42). NETs can also be recognized by the DNA receptor cGAS, which can lead to the induction of IFN-signaling pathways causing immune activation (43). NET-bound enzymes can modify immunological factors to regulate immune response against viruses (44).

1.4.1 Virus reprograms the host cell metabolism

The primary function of metabolism is the conversion of nutrients to energy for the maintenance of all cellular functions and the supply of building blocks for protein, lipid, nucleic acid, and certain carbohydrates biosynthesis. Viruses are unable to metabolize on their own and therefore depend entirely on the host metabolism. Their life cycle necessitates the production of large amounts of proteins, glycoproteins, nucleic acids, and sometimes lipids (45). As a result, viral replication, metabolism, and host protection are all interdependent.

In general, infecting viral pathogens capture and redirect host cell metabolic behavior for viral reproduction, disrupting the "normal" homeostasis of cellular metabolism. This disruption of host metabolism results in changes to intracellular metabolites and dysregulation of metabolic enzymes, which may directly or indirectly affect cellular immune responses. DNA viruses have been shown to regulate key metabolic pathways at the transcriptional stage (46), and RNA viruses appear to shape host-cell metabolism through post-translational modifications (47) that are time-dependent in relation to the replication cycles of the respective viruses. Studies on many respiratory viruses, including Influenza A virus and coronaviruses, show that the viruses alter three core metabolic pathways: Glycolysis, fatty acid (FA) synthesis, and glutaminolysis for virus propagation (48).

1.4.5 IFN and Cellular metabolism

While the viruses are known to hijack the cellular metabolism shifting towards aerobic glycolysis for its efficient replication, innate immune responses are also dependent on the cellular metabolism, as the enzymatic activities of intermediate kinases, as well as transcription and translation to biosynthesize proteins require metabolic resources. However, there are complex and reciprocal feedbacks between the IFN-I signaling and the metabolic intermediates.

It has been shown that while on one side, activation of RIG-I signaling decreases the level of glycolytic intermediates downstream of HK-2, on the other hand, low-glucose and blockade of HK-2 causes induction of IFN-β (49). During viral infection, lactate produced under anaerobic glycolysis can suppress IFN-β (49). AMPK/mTOR signaling and Autophagy are known to regulate both these signaling pathways to maintain homeostasis (50). The underlying comorbidities leading to severe COVID-19 are hyperglycemia, altered metabolic profile, and chronic subclinical inflammation. When acutely infected with respiratory viruses, they present with an enhanced hyper-inflammatory IFN-I response suggesting a link between metabolic and immune signaling pathways. IFNs are well described in the inhibition of FA synthesis and cholesterol synthesis (51, 52). However, the mechanism of how immune-metabolism is regulated during severe or fatal COVID-19 needs to be better studied.

1.5 Evasion of innate immune responses

SARS-CoV-2 applies a multidisciplinary approach to evade the IFN response, as in early COVID-19 trials, researchers recognize an insufficient type-I IFN reaction in patients. So far, a variety of SARS-CoV-2 proteins have been reported to play a role in resistance to IFN's antiviral impact. Notably, protein interaction and biochemical studies have shown that non-structural and accessory proteins can interact with host-cellular components and dampen their function. For example, non-structural protein NSP1 binds to 18S ribosomal RNA and reduces global mRNA translation (53), NSP13 interacts with RIG-I signaling component TBK1 (54), NSP15 interacts with NRDP1, an E3-ubiquitin ligase that regulates the production of pro-inflammatory cytokine and type-I IFN, ORF6 interacts with nuclear porin complexes NUP98-RAE1 and KPNA2 and ORF9B interacts with a key outer mitochondrial membrane receptor TOMM70 (55). All of these interactions regulate the signaling components and the transcription factors of IFN signaling pathway. Additionally, NSP16 was shown to inhibit

global mRNA splicing and viral protein expression (56) and NSP8 and NSP9 disturbed the membrane protein trafficking (56), all of these could potentially lead to the decrease of type-I IFN secretion. Several studies have functionally identified the inhibitory action of the SARS-CoV-2 structural, non-structural, and accessory proteins that are detailed in Table 1.

Table 1: Evasion mechanisms of SARS-CoV-2 proteins

Viral Protein	Affected Step	Antagonism Mechanism	References
NSP3, NSP4, NSP6	Formation of DMVs	Not known	(57, 58)
NSP10, NSP12, NSP13, NSP14, NSP16	Capping	Not known	(58-60)
N	Blockage of RIG-I RNA recognition	Interation with the DExD/H helicase domain	(61)
NSP3	Cleavage of ISG15	Antagonism of ISG15-dependent MDA-5 activation	(62, 63)
NSP5	Cleavage of RIG-I at the last 10 N-terminal amino acids	Blockage of its ability to signal through MAVS Promotion of the ubiquitination and proteasome-mediated degradation of MAVS	(64)
N	Inhbition of RIG-I CARD domain activation	Interaction with TRIM25	(65-67)
NSP1, NSP3, NSP5, NSP12, NSP13, NSP14, M, N, ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b	Reduction of MAVS mediated IFN-B promoter activities	Not known	(68-70)
NSP1, NSP5, NSP6, NSP12, NSP13, NSP14, NSP15, ORF6, ORF3b	Inhibition of IRF3 phosphorylation/nuclear translocation	Cofactor of Nsp16, required for RNA cap methylation	(68-76)
NSP3, NSP5	Cleavage of IRF3	Interacts with TBK1	(75, 76)
NSP13, M, ORF7a, ORF9b	Inhibition of MAVS signaling complex	Interaction with TBK1, interaction with TOM70	(54, 55, 70, 77-80)
NSP5, NSP9, ORF9b	Inhibition of NF-kB pathway	Interaction with Nup69 to block p65 translocation, blockage of Nemo K69-linked polyubiquitination Cleavage of TAB1	(76, 81, 82)
NSP1, NSP6, NSP13, M, N, ORF3a, ORF6, ORF7a, ORF7b	Inhibition of STAT1/STAT2 phosphorylation	Not known	(70, 83, 84)
ORF6	Blockage of STAT1/STAT2 nuclear translocation	Interaction with the nucleopore Nup98	(85-87)
NSP14	IFNAR1 lysosomal degradation	Not known	(62)
NSP3, NSP5	Inhibition of ISGs	Not known	(53, 62, 69)
NSP16	Inhibition of pre-mRNA splicing	Binding to U1 and U2	(56)
NSP8, NSP9	Disruption of protein trafficcking	Binding to SRP complex leading to the inhibition of signal peptide recognition	(56)
NSP1	Blockage of mRNAs export	Binding to the mRNA entry channel overlapping mRNA path. Interaction with export receptor heterodimer NXF1-NXT	(56, 88-91)

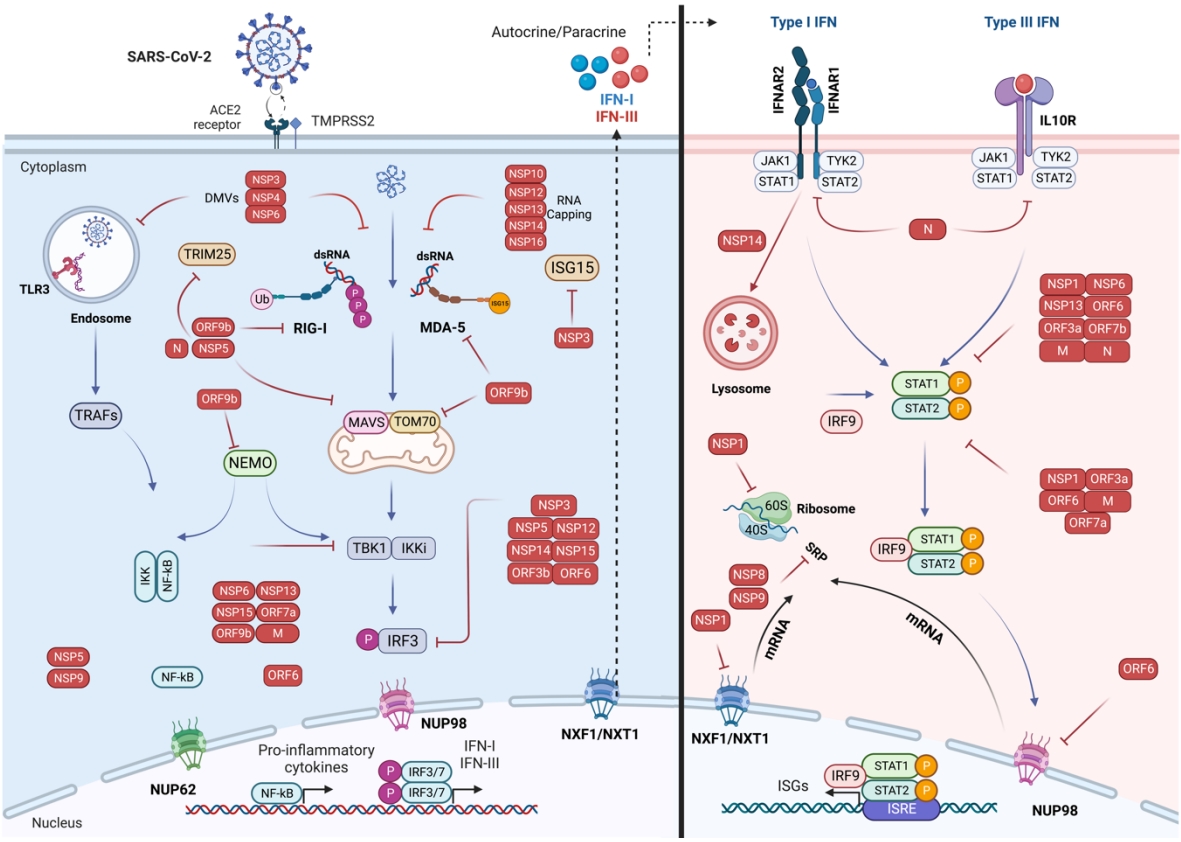

Figure 2: Antagonization of IFN signaling pathways by SARS-CoV-2. Adapted from (1). Created with BioRender.com

1.6 Role of ubiquitin and ubiquitin-like deconjugases in the regulation of innate responses

SARS-CoV-2 Proteases: SARS-CoV-2 encodes two functional proteases, the papain-like protease (PLpro) and the 3-chymotrypsin-like cysteine protease (Mpro or 3CLpro). As a cysteine protease, PLpro is important for viral protein maturation through digesting the viral proteins, thus dysregulating host inflammatory responses and affecting host type-I IFN antiviral immune responses (92).

ISG15 is one of the early induced IFN-stimulated genes (ISGs), and it is part of a class of proteins sharing structural homology to ubiquitin (Ub), termed as ubiquitin-like protein (UbL), which belongs to the member of the ubiquitin system (93). The ubiquitin system is composed of enzymes that catalyze ubiquitination and deubiquitination, as well as ubiquitin receptors that recognize and read the ubiquitin code. These components work in concert to control almost all host cellular activities, including host-pathogen interactions (94). By contrast, monoubiquitination does not result in protein degradation but may be used to alter protein localization and function (95). ISGylation is an IFN-stimulated and controlled process that appears to mirror ubiquitination activities associated with enzymatic activity regulation rather than protein degradation. ISGylation, like ubiquitination, is a cascade of biochemical processes involving the E1, E2, and E3 enzymes. To summarize, ISG15 is generated as a precursor protein (165 amino acids) similar to ubiquitin that is then cleaved to expose di-glycine residues in the form of an LRLRGG motif at its C-terminus. This GG motif is adenylated in the presence

of ATP, and ISG15 is then transported successively from E1 to E3 enzymes to a lysine residue inside the target substrate (96). Due to the numerous parallels between ubiquitination and ISGylation processes, it is not surprising that they overlap and compete. While the E1 enzymes are specialized for ISGylation and ubiquitination, several of the ubiquitination enzymes (for example, the E2 enzyme UbcH8) have a comparable function in the ISGylation pathway (93). Both ubiquitination and ISGylation are reversible, which is a characteristic shared by both mechanisms. De-ubiquitinating and de-ISGylation enzymes (for example, UBP43) function to de-ubiquitinate and/or de-ISGylate conjugated proteins (97).

Coronavirus PLpro can act as deubiquitinases (DUBs) and deISGylases (deISGs) by removing ubiquitin (Ub) or ISG15 conjugated to lysine residues on proteins and can antagonize the innate immune response (97). Furthermore, since several metabolic pathways are also regulated by ISGylation, these pathways can also be targeted by the viral deISGylases.

2 RESEARCH AIMS

The overall aim of the project was to understand how SARS-CoV-2 remodels the host interferon signaling pathways. The specific aim for each paper was:

Paper I: To create a cell type resolve proteomics map of interferon response against SARS-CoV-2.

Paper II: To compare the dynamics of ISG's during SARS-CoV, MERS-CoV, and SARS-CoV-2 infection and their regulation by SARS-CoV-2.

Paper III: To reveal the expression of interferon stimulated genes and possible regulatory mechanisms in COVID-19.

Paper IV: To determine the role of coronavirus proteases in the regulation of host ubiquitylation/ ISGylation.

3 MATERIALS AND METHODS

The thesis contains both published and prospective papers where all the experimental methods are described in detail. This section presents the specific methodologies used in the studies.

3.1 Ethical Considerations

In **Paper III**, samples originating from COVID-19 patient cohort were included. The patient's whole blood samples were collected from SARS-CoV-2 infected patients (April-July 2020) attending the South Hospital, Stockholm. All aspects of the study were performed according to the Helsinki declaration from handling of sensitive information, respect for autonomy, medical, psychological, sociological, and social welfare of the participants. Informed consent was obtained to participate in the study. All the samples were anonymized and coded by the clinician before we could gain access to them. Ethical approval was provided by the Regional Ethics Review Board of Stockholm.

3.2 Sample Collection

In **Paper III**, samples were included from the COVID-19 cohort, whole blood was collected in Tempus^{TM} blood RNA tubes and EDTA tubes at the South Hospital, Stockholm. RNA was extracted from the Tempus^{TM} blood RNA tubes for whole blood transcriptomics and Plasma was separated from the EDTA tubes were stored at -80ºC to be used for plasma proteomics, metabolomics, and other immunological assays.

3.3 Cell models

Paper I and II, studied the behavior of the virus in different cell-line infection models. In **paper I**, Vero E6 cell line that is derived from kidney epithelial cells of African Green monkey was used to propagate and titrate the virus. In **paper I**, to determine the susceptibility of the virus in different human cell lines the following cell lines were used: Caco2 cells (derived from human colon adenocarcinoma), Calu-3 cells (derived from non-small cell lung cancer), 293FT cells (derived from human embryonic kidney cells), Huh7 cells (derived from hepatocyte cellular carcinoma cells), A549 cells (derived from human lung adenocarcinoma tissue), 16HBE (derived from human bronchial epithelial cells). In **paper II**, Huh7 cell line was used to paper the viral modulation of the IFN-signaling. Huh7 cells and Caco2 cells were used to study the senescent model of infection. In **paper IV**, to study the interaction of catalytic domain of SARS-CoV-2 PLpro and its effect on IFN-I signaling HeLa cells (derived from human cervical epithelial adenocarcinoma) was used.

3.4 Viruses and Virus infection

In **Paper I and II**, we used the first isolated Swedish strain of SARS-CoV-2 (Genbank accession number MT093571). In **Paper II**, the SARS-CoV-1 and MERS-CoV strains were obtained from Swedish Public Health Agency. The TCID50 virus titers were determined using Vero E6 cells. Cell lines were infected with a determined infective dose of the virus for 1hr in DMEM containing 5% FBS. Following infection, the cell lines were washed with PBS and further incubated in DMEM containing 5%FBS for the indicated time points. All the virus infections were performed under BSL-3 conditions.

3.5 Plasmids and Transfection

In **Paper IV**, plasmids encoding the catalytic domain of the SARS-CoV-2 PLpro WT (NSP3 746 – 1070 amino acids) and C111A catalytic mutant was synthesized in pcDNA3.1 vector with C-terminal 3xFlag-tag. Its corresponding C-terminal V5-tag and GST-RIG-I-2CARD domain and plasmids encoding GST-tagged TRIM25 fragments of RING, RING-BB, BB, CCD and SPRY were a kind gift from Michaela Gack (Cleveland Clinic, Florida). C-terminal V5-tagged PLpro from SARS-CoV-1, MERS-CoV, NL63-CoV was provided by Susan C Baker (Loyola University, Chicago). The plasmids alongside their corresponding empty vector were used to transfect HeLa cells using either Lipofectamine 3000 (Life technologies) or jetPEI (Polypus transfection) according to manufacturer's guidelines. After 2 days cells were harvested for either protein expression or for immunoprecipitation.

3.6 Analytical methods

3.6.1 Western blot

The protein expression levels of the ISGs in the SARS-CoV-2 infected cell lines were determined using western blot analysis (**paper I and II**). Western blot was also used in **paper IV** to determine the expression of plasmids and determining co-immunoprecipitated proteins.

3.6.2 Immunoprecipitation

Immunoprecipitation (IP) is a tool to identify interacting partners of a specific protein using a target protein-specific antibody in conjunction with affinity beads. In **paper IV**, we used immunoprecipitation by using Flag-tagged and V5-tagged Agarose beads to explore the potential substrates of the Papain-like protease of coronaviruses. Glutathione Sepharose 4B beads (Cytiva) were used to pull-down GST-tagged to RIG-I-2CARD to identify its ubiquitination status under harsh lysis condition (1%SDS containing buffer).

3.6.3 Microscopy

Localization of intracellular proteins can be determined using Immunofluorescence staining (IF) and confocal microscopy. In order to understand the polarization of Calu-3 cells in **Paper I**, this method was applied to investigate the localization of β-catenin, β-actin and nuclei. β-catenin and β-actin are essential for the organization of polarized epithelium.

As the size of viruses are generally small, to further understand the virus production and morphology, in **paper I**, scanning electron microscopy (SEM) was used to detect the SARS-CoV-2 particles on the surface of the infected cell lines.

3.6.4 Polymerase chain reaction (PCR)

Taqman probe based Quantitative polymerase chain reaction (qPCR) was used to determine the virus production in the cell culture supernatant. The viral RNA was determined by measuring the N-gene or the E-gene and relative quantification was done using serial dilution of virus stock with known TCID50 virus titer value as a standard. SYBR green based qPCR was performed targeting IFN-β, ISGs (ISG15, IFIT1, RIG-I, MX2) and p21 in cDNA converted from extracted RNA of mock or virus-infected cell lines (**paper I and II**) and targeting IFN-β in **paper IV** from PLpro transfected cells. The values were normalized to the

human GAPDH as housekeeping gene. Relative fold change was calculated by using Δ(ΔCT) method as per Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. All qPCRs were performed in ABI7500 Fast instrument.

3.6.5 Immunological Assays

To determine the levels of plasma soluble inflammatory factors and autoantibodies several immunogiocal assays were employed in **paper III**. Flowcytometry was used to determine the levels of IFNs in patient plasma using a bead based LEGENDplex assay (Biolegend). The assay was run on Fortessa (BD Bioscience) and analysed using FlowJo 10.8.1 (Treestar Inc) software.

Enzyme-linked immunosorbent assay (ELISA) was used to quantify the plasma levels of Neopterin (Tecan) and S100A8/A9 (R&D systems). All the above-mentioned assays were performed as per manufacturers guidelines. A modified version of ELISA was used to measure the circulating NET marker MPO-DNA. The plates were coated with anti-MPO antibody (Bio-Rad Laboratories) for capture and detection was performed using anti-dsDNA antibody from the Cell Death Detection ELISA kit (Roche Diagnostic). The optical density was determined using microplate reader. IFN-autoantibodies were measured in a bead based inhouse assay of collaborator. Recombinant protein coated magnetic beads having fluorescent markers (Magplex) were incubated with inactivated and diluted plasma samples, following which the antibody binding was detected by labeled anti-human IgG and FlexMap 3D (Luminex) instrument.

3.7 Experimental Assays

3.7.1 Drug treatments

To study any inhibitor effect of SARS-CoV-2 in RIG-I signaling, RIG-I agonists LMW polyI:C/lyovec and acitretin were used to stimulate the activation of RIG-I signaling after virus infection in **paper II**. In **paper IV**, both the LMW polyI:C and HMW polyI:C was used to activate the RIG-I signaling following transfection with PLpro constructs to determine their inhibitory effect on IFN-I production. In **paper II**, IFN-β and IFN-α 2a were used to treat the cells both pre- and post-infection to see the effect of interferon on the establishment of virus infection and activation of ISGs. Again, in **paper II**, low-dose etoposide treatment of Huh7 cells for 6 days was used to induce cellular senescence. All the apoptotic cells were discarded, and only attached cells with a changed morphology and expressing increased p21 was considered to be senescent and were used for virus infection.

3.8 High-throughput Data

3.8.1 Quantitative cellular proteomics

In **Paper I and II,** quantitative proteomics were carried out to understand the global proteomic dynamics during Coronavirus infection in different cell lines as described previously (98). Briefly, the peptides obtained from the digestion of precipitated proteins extracted from the lysis buffer with SDS, were labeled with isobaric TMTpro^{TM} reagents. The fractionated labeled peptides were analyzed on Ultimate 3000 UHPLC (ThermoFisher Scientific, USA). The proteomics data were acquired on an Orbitrap Fusion Lumos™ tribrid mass spectrometer (ThermoFisher Scientific, USA). In **Paper II**, to characterize the IFN response following

SARS-CoV-2 infection in Huh7 cells, the quantitative proteomics dataset of 48h SARS-CoV-2 infected Huh7 cells were extracted from a previous study from the lab (99).

3.8.2 Transcriptomics, plasma proteomics and plasma metabolomics

The transcriptomics data, from the SARS-CoV-2 infected Huh7 cells (**paper II**) and the whole-blood of the COVID-19 cohort (**paper III**), were extracted from earlier studies (98, 100). The RNA sequencing data was generated using Illumina NovaSeq6000 in S4 mode. The Olink plasma proteomics data and the metabolomics data of the cohort were also obtained from another study (101), where Olink proteomics was performed using Olink Immuno-Oncology Panel (Olink, Sweden) and plasma metabolomics was performed using the Metabolon HD4 (Metabolon Inc, NC, US) (101).

3.9 Bioinformatic analysis

3.9.1 Quantitative proteomics analysis

In **Paper I and II**, the quantitative proteomics data was analyzed for both the host-cellular proteins and the virus proteins based on SwissProt human and SARS-CoV-1/SARS-CoV-2 databases respectively using the search engine Mascot Server v2.5.1 (MatrixScience Ltd., UK) in Proteome Discoverer v2.4 (ThermoFisher Scientific, USA) software.

3.9.2 Transcriptomics, plasma proteomics and plasma metabolomics analysis

In **Paper III**, transcriptomics, Olink proteomics data and metabolomics data were retrieved and re-analyzed (100). Transcriptomics data were filtered for low variance (variance < 0.2) and transformed using variance-stabilizing transformation (VST) from R package Deseq2 (102). The transcriptomic analysis was performed using protein coding genes belonging to three Reactome (https://reactome.org) pathways: ‘antiviral mechanisms’, ‘interferon alpha/beta’ and ‘interferon gamma’. Normalized values of the plasma soluble factors from Olink proteomics data and log2 transformed metabolomics data were used for further categorical analysis. In **paper II**, the transcriptomics data analysis was performed as described previously (98).

3.10 Statistical Analysis

Statistical analyses for proteomics and transcriptomics were performed in R package LIMMA (**Paper I and II**). In **paper III**, the differential gene expression analysis of the whole-blood transcriptomics data was performed using R package DESeq2 (102) and the differential abundance analysis of the plasma metabolomics and proteomics data was performed using R package LIMMA (103). All statistics was performed using GraphPad Prism (Version 8.0.0) or R. The normality distribution of the dataset determined the choice of statistical methods used. For normally distributed data parametric tests were applied, unpaired T test is selected. Means of continuous variables were compared using Mann-Whitney U test. False discovery rate (FDR) was applied to correct multiple comparisons too decrease risk of false positive.

4 RESULTS AND DISCUSSION

This chapter briefly outlines the main result of each study and discusses their relevance.

4.1 Cell-type-resolved Interferon response against SARS-CoV-2

SARS-CoV-2 is a highly pathogenic coronavirus that caused the coronavirus disease 2019 (COVID-2019) pandemic. The virus mainly infects the lung but also causes pathological changes in multiple organs like the heart, gastrointestinal tract, liver, kidney, brain etc. (104, 105). While 3D organoids can mimic different organs, the generation of the organoids can be challenging, and thus a simple and fast established cell model to study the virus pathogenesis can be a good choice.

In **Paper I**, we tested SARS-CoV-2 virus infectivity in different cell lines: Vero-E6, Calu3, A549, Caco2, Huh7, 293FT, and 16HBE by performing infection with moi 0.1 and moi 1 over a period of 120h. Among the six different human cell lines of varied origin that were tested, Caco2 (Colon) and Calu3 (lung) showed the highest virus production. Especially, potentially polarized Calu3 showed significantly higher infectivity compared to the non-polarized cells. 293FT (kidney) and Huh7 (liver) showed moderate virus production. Lung cell lines 16HBE, and A549 cells showed very low virus production. In the cytotoxicity detection assay, the virus-specific cytotoxicity was observed only in Calu3 cells. While virus-like particles were found to be attached to the cell surface and cellular projections in Calu3 and Caco2 cells using scanning electron microscopy, no cytopathogenicity was observed in Caco2 cells. Among the infected cell lines, high virus production correlated with the high expression of ACE2 receptors in Vero-E6, Calu3, and Caco2 cells. Conversely, Huh7 and 293FT cells strongly expressed TMPRSS2 but lacked ACE2 expression. The changes in the global proteomic landscape upon SARS-CoV-2 infection of Calu3, Caco2, Huh7, and 293FT cells at 24hpi was investigated by performing TMT-labeling quantitative proteomics. Calu3 showed major changes in protein abundance upon infection, with 6462 proteins differentially expressed in infected cells compared to the mock. The Caco2 cells showed a significant difference in the abundance of 177 proteins. No change in the protein abundance was observed in Huh7 and 293FT cells. We found 132 commonly dysregulated proteins in both Calu3 and Caco2 cells. Among these proteins, 44 were significantly upregulated, and 44 were downregulated. Reactome pathway analysis on the commonly dysregulated proteins presented a strong enrichment of type-I and type-II interferon signaling pathways and RIG-I/MDA-5 signaling pathways. Expression of Interferon stimulated genes (IFIT1, MX1, MX2, ISG15, and RIG-I) quantified by qPCR observed a significant increase in Caco2 and Calu3 cells, while 293FT and Huh7 cells did not show any significant change. The qPCR results corroborated with the western blot analysis. Combining the Calu3 and Caco2 proteomics data with the 48hpi proteomics data of Huh7 cells suggested interferon signaling to be commonly dysregulated in these three cell lines. However, a distinct pattern of expression of ISGs were observed between these cell lines with only DDX58, STAT1, STAT2, ISG15 and IFIT1 to be commonly upregulated in all the three.

4.2 SARS-CoV-2 regulation of Interferon response

The severity of COVID has been associated with dysregulation of type-I interferon (IFN-I) response. A characterization of SARS-CoV-2 mediated regulation of IFN response is required to develop novel and rational therapeutic approaches for SARS-CoV-2. Huh7 is an established cell line for SARS-CoV-1 and MERS-CoV infection, and we have shown in the paper I that Huh7 is moderately infected with SARS-CoV-2.

In **Paper II,** using the Huh7 infection model, we compared the dynamics of ISG's in SARS-CoV-2 compared to SARS-CoV-1 and MERS-CoV and their regulation by SARS-CoV-2. We employed a TMT-labeling quantitative proteomics strategy in SARS-CoV-1 and MERS-CoV infected Huh7 cell lines to characterize the differences in global proteomic response against human coronaviruses. We reused our previous study's quantitative proteomics datasets of SARS-CoV-2 infected Huh7 cells 48hpi. The commonly regulated pathways by all three viruses belonged to RNA processing, RNA translation, and infectious diseases. Analysis of IFN-related proteins showed very little overlap between the three. No overlap was observed between SARS-CoV-1 and MERS-CoV, while SARS-CoV-2 and MERS-CoV had 13 commonly dysregulated IFN signaling proteins, including ISG15 and IFIT1. Only two IFN-regulated proteins, STAT1 and EIF4A2 were commonly upregulated between SARS-CoV and SARS-CoV-2. Overall, the network analysis showed a distinct pattern of the IFN signaling response in the three coronaviruses.

In **Paper II,** we further analyzed our previous study's proteomics and transcriptomics datasets. The protein-protein interaction network of the dysregulated genes showed two distinct clusters, the proteins of cluster-1 were related to the RIG-I/DDX58 and IFN-I signaling, while the proteins of cluster-2 were associated with the components of nucleoporin complex and karyopherin family belonging to transporter proteins. We observed a delayed activation of RIG-I and dysregulation of ISGs based on proteomics data, while no significant changes were found in the transcription level of IFN-β either at 24hpi or 48hpi in the SARS-CoV-2 infected Huh7 cells. In contrast to Caco2 and Calu-3 cells (**Paper I**), no enhanced expression of RIG-I and MDA5 was noted at 24 hpi in Huh7 cells. Aligning with the proteomics data, western blot analysis showed a non-significant increase in the expression of ISGs like RIG-I, MDA5 and ISG15 levels at 48hpi. As an antiviral cytokine, ISG15 can conjugate to varied viral and cellular proteins and regulate their functions. While the infection of SARS-CoV-2 did not change the mRNA levels of ISG15, we observed a notable decrease in the conjugated form at 24hpi and an observable increase at 48 hpi in a dose-dependent manner, indicating towards possibility of modulation of cellular protein ISGylation and as well IFN-response by the virus which can influence the cellular environment. Indeed, established SARS-CoV-2 infection in Huh7 cells could inhibit IFN-β production upon RIG-I/MDA5 activation by RIG-I agonists poly (I:C) and Acitretin. Furthermore, the virus production was not affected by treatment with either IFN-α2a or IFN-β post-infection. On the other hand, Huh7 cells pre-treated with IFN-I, either IFN-α2a or IFN-β, showed reduced virus production compared to the non-treated cells suggesting that the pre-existence of IFN response can control the virus replication while once the infection was established in the cells, the virus could escape or counteract the cellular immune response. As aged population show more vulnerabilities to SARS-CoV-2 infection, the cellular senescence can be a key modulating factor regarding to the virus infection. To address to this notion, we induced senescence in Huh7 cells by using low-dose etoposide treatment for 6 days. The

senescence was evaluated by p21 mRNA levels. Virus production in senescent Huh7 cells significantly boosted compared to the non-senescent cells. The qPCR data of the ISGs expression showed that the senescent cells have higher IFN-response with increased levels of IFN-β and other ISGs tested. SARS-CoV-2 infection decreased the IFIT1 expression but not other genes. Since earlier we had observed differences in global proteome response in different cell lines upon SARS-CoV-2 infection, we replicated the same treatment in Caco2 cells. Caco2 cells showed increased resistance to the etoposide. A very low level of senescence could be achieved as determined by p21 levels and no major observable change in cell morphology or growth. In contrast to Huh7, etoposide-treated Caco2 cells could sufficiently reduce the cell susceptibility to SARS-CoV-2 infection. This also correlated with an increase in IFIT1 mRNA level that was inhibited by SARS-CoV-2 infection in senescent Huh7 cells. These results suggested a regulation of SARS-CoV-2 based on cell type and IFIT could be an important anti-virus ISG in controlling SARS-CoV-2 infection.

4.3 ISGs expression and possible regulatory mechanism in COVID-19 patients

IFN signaling plays an important role in anti-SARS-CoV-2 infection as the first line of defense. The initial interferon response relies on the sensing of the viral RNA by RIG-I and MDA-5 cytoplasmic pattern-recognition receptors that lead to the production of IFN-I and, in turn activates several ISGs with varied antiviral functions. There are a few regulators in the functions of interferons, including the genetic defects associated with interferon deficiency, IFN-autoantibodies, and cellular metabolism. In **Paper I and Paper II**, we have characterized cell type-specific ISG signatures upon SARS-CoV-2 infection and the virus's capability to regulate the IFN response. In **Paper III**, based on a COVID-19 patient cohort, we investigated the ISG landscape based on whole blood-transcriptomics data complementing with immunological assays. The analysis of whole blood transcriptomics showed a significant increase in ISGs, especially the type-I IFN signaling genes in mild and severe COVID-19 patients. Since the ISGs are activated through the JAK-STAT signaling pathway upon cellular recognition of type-I, type-II, and type-III in an autocrine or paracrine fashion, we further evaluated the transcript level of related gene components. While IFN-λ1 and IFN-γ transcript expression significantly decreased in the severe group, no significant changes were detected in the plasma IFN-I/-II and III levels using FACS-based LEGENDplex assay.

A further hierarchical consensus clustering was done based on our transcriptomics data. Irrespective of the disease severity, a clustering pattern (cluster-1, cluster-2, and cluster-3) of the genes associated with IFN-signaling was noted, showing differences in ISG scores (low, high, and moderate, respectively), which links the standardized count of ISGs with type-I, -II and anti-viral signaling pathways. Next, to resolve the heterogeneity of ISGs profile in mild and severe COVID-19 patients. The cluster-1 has a similar ISG expression with the Healthy control. The cluster-2, with the highest ISG scores, showed a significant increase in activated dendritic cells and neutrophils counts assessed by digital cell quantification (DCQ). The cluster-3 had significantly decreased count in monocyte and M2 macrophage.

As there was increased neutrophils (DCQ) and immature neutrophils (FACS) counts, we measured the plasma Alarmin (S100A8/A9), a marker of neutrophil activation and MPO: DNA, a marker for neutrophil extracellular traps (NETs). NETs can be released by activated neutrophils and have been shown to induce type-I IFN in pDCs through cGAS-STING

signaling. Alarmin levels were significantly higher both in cluster-2 and cluster-3 compared to cluster-1. However, cluster-2 did not show any significant increase in MPO:DNA levels, while MPO:DNA complexes were significantly higher in cluster 3 compared to cluster-1 and -2. The evaluation of Neopterin, a marker of activated macrophages, had a similar trend to Alarmin. Autoantibodies against type-I and type-III IFN is associated with disease severity and mortality. We have performed an auto-antibody array against IFNs. The observed IFN-autoantibodies did not show any association with disease severity. An interesting observation was that cluster-2 showed suppression in antibodies against the SARS-CoV-2 spike and RBD-domain but not to nucleocapsid protein. Inflammatory cytokines measured by Olink onco-immunology panel linked IL10, CXCL10, MCP-2, MCP-3, and IFN-γ levels to the trend in ISG scores observed between the clusters, with the highest levels in cluster-2. Accumulated evidence shows crosstalk between cellular metabolism and IFN signaling during viral infection. We analyzed the ISGs cluster-specific changes in the global plasma metabolomics data. The correlation between the ISG scores and specific metabolites shows that type-I and type-II ISG scores were negatively associated with amino acid and lipid metabolism, which indicates that the hyperinflammatory state may lead to a disorder in lipid and amino acid metabolism.

4.4 SARS-CoV-2 Papain-like protease regulates Interferon response by interfering with RIG-I signalosome activation.

In **Paper IV**, we aimed to determine the role of coronavirus papain-like protease (PLpro) as a viral factor in regulating host IFN response. SARS-CoV-2 is an enveloped single-stranded positive-sense RNA virus. The immune response initiates with the recognition of the viral RNA. The 3' end of the positive sense RNA and the replication product negative sense RNA are recognized by RIG-I and MDA-5, respectively (18, 106). The activation of RIG-I is dependent on K63-linked ubiquitination that is orchestrated by the E3-ligase activity of TRIM25 (107). As one of the two coronavirus proteases, PLpro is responsible for the cleavage and maturation of viral polyproteins and replicase-transcriptase assembling. It has also been reported that PLpro has both deISGylase and deubiquitinase activities (99), and thus it can interfere with host cellular signaling pathways. In **Paper IV**, using co-immunoprecipitation assays, we found that PLpro interacts with TRIM25, an E3-ligase essential for activating RIG-I signalosome. Using different TRIM25 domains, we further determined that B-Box and SPRY domains strongly interacted with PLpro and CCD domain showed very weak binding. PLpro did not show any interaction with the RING domain of TRIM25. Since TRIM25 is a known regulatory partner of RIG-I activation, next, we checked its interaction of PLpro with the two RNA sensors: RIG-I and MDA-5. The result shows that the interaction with TRIM25, RIG-I or MDA-5 is catalytic domain independent. The PLpro WT had more impacts on the inhibition of IFN-β induction than PLpro C111A mutant. Catalytically active PLpro caused the deconjugation of endogenous ubiquitin bound to the constitutively active 2CARD domain of RIG-I, suggesting an interference with RIG-I signalosome activation that could have led to inhibition of IFN-I. Finally, we reported that the homologs of SARS-CoV-2 PLpro in other human coronaviruses share the similar property of interaction with RIG-I signalosome and hampered the IFN production in poly (I:C) treated HeLa cells. However, SARS-CoV-2 and its homologs interacted with TRIM25 and RIG-I in different affinities.

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

This work was initiated at the beginning of the SARS-CoV-2 pandemic. Little information was available on the pathogenesis of the virus, and that required a suitable cell model to study. During the course of the thesis, we have learned a lot about SARS-CoV-2 pathophysiology due to the global research effort. Through our research, we identified suitable laboratory cell models for the *in vitro* virus infection and how the expression of the antiviral ISGs can differ based on the cell lines originating from different organs, indicating that the virus pathogenesis may have organ-specific variations. The antiviral response against SARS-CoV-2 was also observed to differ from other pathogenic coronaviruses, and SARS-CoV-2 could inhibit and delay the interferon response. Thus, any therapeutic intervention based on information from the previous epidemics of SARS-CoV-1 and MERS-CoV should be used with caution. That also holds true for interferon-based treatment strategies. Our *in vitro* results as well as other *in vitro* and clinical results indicate that the early interferon response before establishment of the virus infection is effective. While in the later stages of the disease it is ineffective and may further add to the dysregulated interferon response coupled with hyperinflammation. However, we have observed a heterogeneity in the ISG response irrespective of the disease status that was linked to lipid, amino acid and energy metabolic alterations. The viral proteins play a major role in the dysregulation of immune-metabolic responses, of which the SARS-CoV-2 encoded ubiquitin and ISG15 deconjugase PLpro evades innate immune responses by interfering with different steps of the cellular signaling. Increasing knowledge on the virus pathogenesis has also created several unanswered questions that still remains open.

- In **Paper I**, we have observed cell line specific differences in global proteomic changes upon SARS-CoV-2 infection. However, these are modified cell lines and may not represent actual physiological condition. Thus, to understand these differences further proteomic based studies are required in organ specific organoid models. That could also be extended to autopsy tissue materials where the viral RNA is detected. Another curious observation in the **paper I** was that while the Caco2 infection model showed high level of virus production, but no cytopathogenicity in the cell line was noted. Even scanning electron microscopy showed virus particles on the surface of the cell. Thus, it will be interesting to study any alternate or novel ways the virus employs to be released out of the cell without causing any apoptosis.

- In **Paper II**, we made an observation on late enhanced ISGylation in Huh7 cells, though not significant. ISGylation is a post-translational modification that regulates several immune-metabolic pathways. Identification of ISGylome and Ubiquitylome upon SARS-CoV-2 infection can highlight how cellular signaling pathways are regulated by the virus. Another interesting observation in paper II was an observable link between suppression of IFIT1 and enhanced virus production in senescent Huh7 cells. This indicates IFIT1 may play a role in controlling the virus infection that should be explored further.

- **In Paper III**, using patient derived materials and multi-omics approaches we have observed high ISG expression was associated with perturbation in lipid and amino-aid metabolisms. The role of the identified lipids and the amino acids in the regulation of the ISGs and their mechanisms should be studied in detail. Presently knowledge on the links between the immune and metabolic pathways are limited and investigation of this can lead to novel therapeutic alternatives not only for viral infections but also for other inflammatory conditions. In paper III we have also observed presence of autoantibodies against different IFN subtypes. But the subtype specific functional roles of the autoantibodies in attenuation of IFN-signaling should be ascertained.

- In **Paper IV,** we have studied the interaction of PLpro of coronaviruses with the RIG-I signalosome components and the subsequent effect on inhibition of IFN-I. PLpro was observed to be interacting with E3-ligase TRIM25 that is involved in several ubiquitination/ISGylation dependent signaling processes. The viral DUBs usually interact with E3-ligases and acts upon their substrates. Thus, efforts should be made to identify the interaction of PLpro with TRIM25 better using crystallographic studies and inhibitor designing, that could inhibit the interaction of PLpro with TRIM25 and thus inhibiting its effect on different signaling pathways. Furthermore, other cellular substrates of PLpro should be identified by co-immunoprecipitation and mass-spectrometry to ascertain its direct protein interactions and its consequence.

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