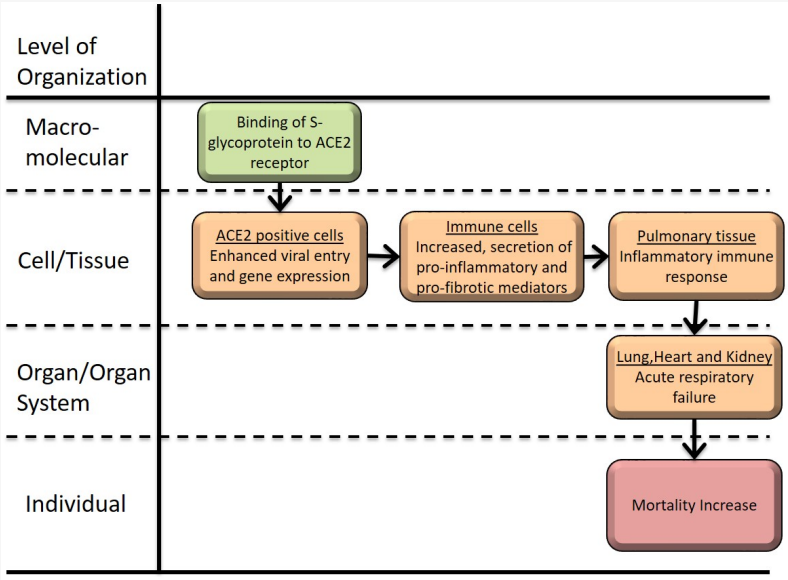
AOP ID and Title:

AOP 320: Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality
Short Title: SARS-CoV-2 leads to acute respiratory distress

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for comment. Do not cite	Under Development	1.96	Included in OECD Work Plan

Abstract

Inhalation of substances, including viral particles, the RNA virus capsid (S) glycoprotein binds the cellular receptor angiotensin-converting enzyme 2 (ACE2) and mediates fusion of the viral and cellular membranes through a pre- to postfusion conformation transition. The S protein is cleaved into S1 and S2 units by a human cell-derived protease (proteolytic enzyme) that is assumed to be Furin.S1 units then bind to its receptor, ACE2. The other fragment, S2, is cleaved by TMPRSS2, a human cell surface serine protease, resulting in cell membrane fusion. The S protein binds the catalytic domain of ACE2 with high affinities likewise, COVID-19 shares 79.6% homology of SARS-CoV and 96% identical at the whole-genome level to a bat coronavirus. The binding of the coronavirus S protein to ACE2 triggers a conformational change in the S protein of the coronavirus, allowing for proteolytic digestion by host cell proteases called TMPRSS2. The AOP reports the S glycoprotein of viral capsid in complex with its host cell receptor ACE2 resulted in acute respiratory distress associated with mortality by cytokine storms and enhanced inflammation in pulmonary tissue. S-glycoprotein of the virus uses ACE2 to get into cells that are found on the surface of epithelial cells in Kidney, Heart, Liver and Lung. However, there is an unexplored relationship for ACE2 levels between fibrotic hypersensitivity and Renin-Angiotensin Pathway which caused acute respiratory distress associated with mortality.

Background

The *ACE2* gene encodes the angiotensin-converting enzyme-2, which has been proved to be the receptor for both the SARS-coronavirus (SARS-CoV) and the human respiratory coronavirus. ACE2 is a key component of blood pressure regulation in the renin-angiotensin system. Angiotensin (Ang) converting enzyme 2 (ACE2) is a homolog of ACE. ACE2 negatively regulates the renin-angiotensin system (RAS) by converting Ang II to Ang-(1-7) and AngI to Ang(1-9). The higher levels of receptor expression achieved by the expression of recombinant ACE2 could be relevant for cell-cell fusion. The underlying mechanisms remain to be elucidated and could play a role in the entry of the cell-free virus into cells and finally increase the acute respiratory distress associated with mortality.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1739	Binding to ACE2	Binding to ACE2

Sequence	Type	Event ID	Title	Short name
2	KE	1738	SARS-CoV-2 cell entry	SARS-CoV-2 cell entry
3	KE	1901	Interferon-I antiviral response, antagonized by SARS-CoV-2	IFN-I response, antagonized
4	KE	1847	Increased SARS-CoV-2 production	SARS-CoV-2 production
5	KE	1848	Toll Like Receptor (TLR) Dysregulation	TLR Activation/Dysregulation
6	KE	1496	Increased, secretion of proinflammatory mediators	Increased proinflammatory mediators
7	KE	1750	Increased inflammatory immune responses	Increased inflammatory immune responses
8	KE	1748	Increase, the risk of acute respiratory failure	Increase, the risk of acute respiratory failure
9	AO	351	Increased Mortality	Increased Mortality

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to ACE2	adjacent	SARS-CoV-2 cell entry	High	High
Increased SARS-CoV-2 production	adjacent	Toll Like Receptor (TLR) Dysregulation	Moderate	Not Specified
Toll Like Receptor (TLR) Dysregulation	adjacent	Increased, secretion of proinflammatory mediators	High	Not Specified
Increased, secretion of proinflammatory mediators	adjacent	Increased inflammatory immune responses	High	Low
Increased inflammatory immune responses	adjacent	Increase, the risk of acute respiratory failure	Moderate	Low
Increase, the risk of acute respiratory failure	adjacent	Increased Mortality	Moderate	Not Specified
SARS-CoV-2 cell entry	adjacent	Interferon-I antiviral response, antagonized by SARS-CoV-2	High	
Interferon-I antiviral response, antagonized by SARS-CoV-2	adjacent	Increased SARS-CoV-2 production	High	

Stressors

Name	Evidence
SARS-CoV	High
HCoV-NL63	Moderate
Sars-CoV-2	High

SARS-CoV

Cryo-EM structure of the SARS coronavirus spike glycoprotein in complex with its host cell receptor ACE2https://doi.org/10.1371/journal.ppat.1007236

*Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, Huan Y, Yang P, Zhang Y, Deng W, Bao L, Zhang B, Liu G, Wang Z, Chappell M, Liu Y, Zheng D, Leibbrandt A, Wada T, Slutsky AS, Liu D, Qin C, Jiang C, Penninger JM (Aug 2005). "A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury". Nature Medicine. **11** (8): 875–9. doi:10.1038/nm1267. PMID 16007097.*

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Conception to < Fetal	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Sex Applicability

Sex	Evidence
Mixed	High

Considerations for Potential Applications of the AOP (optional)

This AOP not only contributes new tools to study entry of the viral particles or Inhalation of stressors into cells and localize its receptor-binding domain of ACE2 but also could serve in the development of novel vaccine immunogens against TMPRSS2 proteases which may inhibit cell entry of COVID-19.

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Appendix 1

List of MIEs in this AOP

Event: 1739: Binding to ACE2

Short Name: Binding to ACE2

Key Event Component

Process	Object	Action
receptor binding	angiotensin-converting enzyme 2	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	MolecularInitiatingEvent
Aop:374 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on brain cells (neuronal and non-neuronal) leads to neuroinflammation resulting in encephalitis	MolecularInitiatingEvent
Aop:381 - Binding of viral S-glycoprotein to ACE2 receptor leading to dysgeusia	MolecularInitiatingEvent
Aop:385 - Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	MolecularInitiatingEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	MolecularInitiatingEvent
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	MolecularInitiatingEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	MolecularInitiatingEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	MolecularInitiatingEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	MolecularInitiatingEvent
Aop:427 - ACE2 downregulation following SARS-CoV-2 infection triggers dysregulation of RAAS and can lead to heart failure.	MolecularInitiatingEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	MolecularInitiatingEvent
Aop:428 - Binding of S-protein to ACE2 in enterocytes induces ACE2 dysregulation leading to gut dysbiosis	MolecularInitiatingEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	MolecularInitiatingEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	MolecularInitiatingEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	MolecularInitiatingEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
Mustela lutreola	Mustela lutreola	High	NCBI
Felis catus	Felis catus	Moderate	NCBI
Panthera tigris	Panthera tigris	Moderate	NCBI
Canis familiaris	Canis lupus familiaris	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

The KE is applicable to broad species/life stage/sex. The binding of ACE2 occure in the cells which express ACE2.

Key Event Description

Angiotensin-converting enzyme 2 (ACE2) is an enzyme that can be found either attached to the membrane of the cells (mACE2) in many tissues and in a soluble form form (sACE2).

A table on ACE2 expression levels according to tissues*(Kim et al.)*

	Sample size	ACE2 mean expression	Standard deviation of expression
Intestine	51	9.50	1.183
Kidney	129	9.20	2.410
Stomach	35	8.25	3.715
Bile duct	9	7.23	1.163
Liver	50	6.86	1.351
Oral cavity	32	6.23	1.271
Lung	110	5.83	0.710
Thyroid	59	5.65	0.646
Esophagus	11	5.31	1.552
Bladder	19	5.10	1.809
Breast	113	4.61	0.961
Uterus	25	4.37	1.125
Protaste	52	4.35	1.905

ACE2 receptors in the brain (endothelial, neuronal and glial cells):

The highest ACE2 expression level in the brain was found in the pons and medulla oblongata in the human brainstem, containing the medullary respiratory centers (Lukiw et al., 2020). High ACE2 receptor expression was also found in the amygdala, cerebral cortex and in the regions involved in cardiovascular function and central regulation of blood pressure including the sub-fornical organ, nucleus of the tractus solitarius, paraventricular nucleus, and rostral ventrolateral medulla (Gowrisankar and Clark 2016; Xia and Lazartigues 2010). The neurons and glial cells, like astrocytes and microglia also express ACE-2.

In the brain, ACE2 is expressed in endothelium and vascular smooth muscle cells (Hamming et al., 2004), as well as in neurons and glia (Gallagher et al., 2006; Matsushita et al., 2010; Gowrisankar and Clark, 2016; Xu et al., 2017; de Morais et al., 2018) (from Murta et al., 2020). Astrocytes are the main source of angiotensinogen and express ATR1 and MasR; neurons express ATR1, ACE2, and MasR, and microglia respond to ATR1 activation (Shi et al., 2014; de Morais et al., 2018).

ACE2 receptors in the intestines

The highest levels of ACE2 are found at the luminal surface of the enterocytes, the differentiated epithelial cells in the small intestine, lower levels in the crypt cells and in the colon (Liang et al, 2020; Hashimoto et al., 2012, Fairweather et al. 2012; Kowalczuk et al. 2008).

How it is Measured or Detected

***In vitro* methods supporting interaction between ACE2 and SARS-CoV-2 spike protein**

Several reports using surface plasmon resonance (SPR) or biolayer interferometry binding (BLI) approaches. to study the interaction between recombinant ACE2 and S proteins have determined a dissociation constant (Kd) for SARS-CoV S and SARS-CoV-2 S as follow,

Reference	ACE2 protein	SARS-CoV S	SARS-CoV2 S	Method	Measured Kd
doi:10.1126/science.abb2507	1–615 aa	306–577 aa		SPR	325.8 nM
			1–1208 aa		14.7 nM
doi:10.1001/jama.2020.3786	19–615 aa	306–527 aa		SPR	408.7 nM
			319–541 aa		133.3 nM
Lan et al., 2020	19–615 aa	306–527 aa		SPR	31.6 nM
			319–541 aa		4.7 nM
doi:10.1016/j.cell.2020.02.058	1–614 aa	306–575 aa		BLI	1.2 nM
			328–533 aa		5 nM
doi:10.1126/science.abb2507	1–615 aa	306–577 aa		BLI	13.7 nM
			319–591 aa		34.6 nM

Pseudo typed vesicular stomatitis virus expressing SARS-CoV-2 S (VSV-SARS-S2) expression system can be used efficiently infects cell lines, with Calu-3 human lung adenocarcinoma epithelial cell line, CaCo-2 human colorectal adenocarcinoma colon epithelial cell line and Vero African grey monkey kidney epithelial cell line being the most permissive (Hoffmann et al., 2020; Ou et al., 2020). It can be measured using a wide variety of assays targeting different biological phases of infection and altered cell membrane permeability and cell organelle signaling pathway. Other assay measured alteration in the levels of permissive cell lines all express ACE2 or hACE2-expressing 293T cell (e.g. pNUO1-hACE2, pFUSE-hIgG1-Fc2), as previously demonstrated by indirect immunofluorescence (IF) or by immunoblotting are associated with ELISA(W Tai et al., nature 2020). To prioritize the identified potential KEs for selection and to select a KE to serve as a case study, further in-silico data that ACE2 binds to SARS-CoV-2 S is necessary for virus entry. The above analysis outlined can be used evidence-based assessment of molecular evidence as a MIE.

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List of Key Events in the AOP

Event: 1738: SARS-CoV-2 cell entry

Short Name: SARS-CoV-2 cell entry

Key Event Component

Process	Object	Action
membrane fusion	transmembrane protease serine 2	occurrence
endocytosis involved in viral entry into host cell	cathepsin L1 (human)	occurrence
viral entry into host cell	viral genome	occurrence
viral entry into host cell	viral protein	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	KeyEvent
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	KeyEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Manis javanica	Manis javanica	Low	NCBI
Canis familiaris	Canis lupus familiaris	Moderate	NCBI
Macaca fascicularis	Macaca fascicularis	Not Specified	NCBI
Mesocricetus auratus	Mesocricetus auratus	Not Specified	NCBI
Mustela putorius furo	Mustela putorius furo	Not Specified	NCBI
Felis catus	Felis catus	Moderate	NCBI
Mustela lutreola	Mustela lutreola	High	NCBI
Neovison vison	Neovison vison	High	NCBI
Panthera tigris	Panthera tigris	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

TMPRSS2 vertebrates (Lam et al., 2020)

NRP1 in human & rodents (but also present in monkey and other vertebrates (Lu and Meng, 2015)

The ability for SARS-CoV-2 to use multiple host pathways for viral entry, means that it is critical to map which viral entry pathway is prevalent in specific cell types. This is key for understanding coronavirus biology, but also use informed decisions to select cells for cell-based genetic and small-molecule screens and to interpret data. In fact, a combination of protease inhibitors that block both TRMPSS2 and cathepsin L is the most efficient combination to block coronavirus infection (Yamamoto et al., 2020, Shang et al., 2020, Shirato et al., 2018). In accordance, SARS-CoV-2 entry processes are highly dependent on endocytosis and endocytic maturation in cells that do not express TMPRSS2, such as VeroE6 or 293T cells (Murgolo et al., 2021, Kang et al., 2020, Mirabelli et al., 2020, Riva et al., 2020). However, even in these cells, heterologous expression of TMPRSS2 abrogates the pharmacological blockade of cathepsin inhibitors (Kawase et al., 2012, Hoffmann et al., 2020a). Treatment of SARS-CoV-2 with trypsin enables viral cell surface entry, even when TMPRSS2 is absent. Moreover, TMPRSS2 is more efficient to promote viral entry than cathepsins (Lamers et al., 2020), as when both factors are present,d cathepsin inhibitors are less effective than TMPRSS2 inhibitors (Hoffmann et al., 2020b). Therefore it is critical to map which cells contain the different types of proteases.

In summary, TMPRSS2 appears to be expressed in a wide range of healthy adult organs, but in restricted cell types, including:

- AT2 and clara cells of lungs
- sinusoidal endothelium, and hepatocyte of the liver,
- endocrine cells of the prostate,
- goblet cells , and enterocytes of the small intestine,
- intercalated cells, and the proximal tubular of the kidney.
- Ciliated, secretory and suprabasal of nasal
- spermatogonial stem cells of testes
- cyto tropoblast and peri vascular cells of placenta
- The nasal epithelium expresses various combinations of factors that, in principle, could facilitate SARS-CoV-2 infection, but it also expresses robust basal levels of RFs, which may act as a strong protective barrier in this tissue.

There is a shift in TMPRSS2 regulation during nasal epithelium differentiation in young individuals that is not occurring in old individuals (Lin et al., 1999, Lucas et al., 2008, Singh et al., 2020).

Only a small minority of human respiratory and intestinal cells have genes that express both ACE2 and TMPRSS2. Amongst the ones that do, three main cell types were identified: A) lung cells called type II pneumocytes (which help maintain air sacs, known as alveoli); B) intestinal cells called enterocytes, which help the body absorb nutrients; and C) goblet cells in the nasal passage, which secrete mucus (Ziegler et al., 2020).

The clinical manifestations of COVID-19 include not only complications from acute myocardial injury, elevated liver enzymes, and acute kidney injury in patients presenting to hospitals, but also gastrointestinal symptoms in community patients experiencing milder forms of the disease (Madjid et al., 2020, Pan et al., 2020).

NRP-1:

All life stages

The expression of isoforms 1 (NRP1) and 2 (NRP2) does not seem to overlap. Isoform 1 is expressed by the blood vessels of different tissues. In the developing embryo it is found predominantly in the nervous system. In adult tissues, it is highly expressed in heart and placenta; moderately in lung, liver, skeletal muscle, kidney and pancreas; and low in adult brain. Isoform 2 is found in liver hepatocytes, kidney distal and proximal tubules. Expressed in colon and 234 other tissues with Low tissue specificity (UniProtKB).

The expression of NRP1 protein in gastric cancer was not related to gender or age (Cao et al., 2020).

Sex Applicability:

TMPRSS2:

Androgen receptors (ARs) play a key role in the transcription of TMPRSS2 (Fig. 1). This may explain the predominance of males to COVID-19 infection, fatality, and severity because males tend to have a higher expression and activation of ARs than females, which is due to the presence of dihydrotestosterone (DHT).

Regulation of COVID-19 severity and fatality by sex hormones. Females have aromatase, the enzyme that converts androgen substrates into estrogen. On the other hand, males have steroid 5α reductase, the enzyme that is responsible for the conversion of testosterone into dihydrotestosterone (DHT). In case of males, DHT activates androgen receptor (AR) that binds to the androgen response element (ARE) present in the promoter of TMPRSS2 gene, leading to its transcription. This ultimately results into enhanced processing of viral spike protein for greater entry and spread of SARS-CoV-2 into host cells. On the other hand,in females, estrogen activates estrogen receptor (ER), which binds to the estrogen response element (ERE) present in the promoter of eNOS gene to drive its transcription and catalyze the formation of nitric oxide (NO) from L-arginine. This NO is involved in vasodilation as well as inhibition of viral replication.

NRP-1:

For more information difference of NRP1 expression between male and female see https://www.proteinatlas.org/ENSG00000099250-NRP1/tissue.

The expression of NRP1 protein in gastric cancer was not related to gender, age. The expression of NRP1 protein in gastric cancer is closely correlated to clinical stage, tumor size, TNM stage, differentiation, and lymph node metastasis (Cao et al., 2020).

SARS-CoV-2 Spike protein co-opts VEGF-A/Neuropilin-1 receptor signalling to induce analgesia had same results on both male and female rodents(Moutal et al., 2020).

Key Event Description

Coronavirus is recognized by the binding of S protein on the viral surface and angiotensin-converting enzyme 2 (ACE2) receptor on the cellular membrane, followed by viral entry via processing of S protein by transmembrane serine protease 2 (TMPRSS2) (Hoffmann et al., 2020b). ACE2 is expressed on epithelial cells of the lung and intestine, and also can be found in the heart, kidney, adipose, and male and female reproductive tissues (Lukassen et al., 2020, Lamers et al., 2020, Chen et al., 2020, Jing et al., 2020, Subramanian et al., 2020).

SARS-CoV-2 is an enveloped virus characterized by displaying spike proteins at the viral surface (Juraszek et al., 2021). Spike is critical for viral entry (Hoffmann et al., 2020b) and is the primary target of vaccines and therapeutic strategies, as this protein is the immunodominant target for antibodies (Yuan et al., 2020, Ju et al., 2020, Robbiani et al., 2020, Premkumar et al., 2020, Liu et al., 2020). Spike is composed of S1 and S2 subdomains. S1 contains the N-terminal (NTD) and receptor-binding (RBD) domains, and the S2 contains the fusion peptide (FP), heptad repeat 1 (HR1) and HR2, the transmembrane (TM) and cytoplasmic domains (CD) (Lan et al., 2020). S1 leads to the recognition of the angiotensin-converting enzyme 2 (ACE2) receptor and S2 is involved in membrane fusion (Hoffmann et al., 2020b, Letko et al., 2020, Shang et al., 2020).

Upon binding to ACE2, the spike protein needs to be activated (or primed) through proteolytic cleavage (by a host protease) to allow membrane fusion. Fusion is a key step in viral entry as it is the way to release SARS-CoV-2 genetic material inside the cell. Cleavage happens between its spike's S1 and S2 domains, liberating S2 that inserts its N-terminal domain into a host cell membrane and mediates membrane fusion (Millet and Whittaker, 2018). Many proteases were identified to activate coronaviruses including furin, cathepsin L, trypsin-like serine proteases TMPRSS2, TMPRSS4, TMPRSS11, and human airway trypsin-like protease (HATs). These may operate at four different stages of the virus infection cycle: (a) pro-protein convertases (e.g., furin) during virus packaging in virus-producing cells, (b) extracellular proteases (e.g., elastase) after virus release into extracellular space, (c) cell surface proteases [e.g., type II transmembrane serine protease (TMPRSS2)] after virus attachment to virus-targeting cells, and (d) lysosomal proteases (e.g., cathepsin L) after virus endocytosis in virus-targeting cells (Li, 2016). SARS-CoV-2 lipidic envelope may fuse with two distinct membrane types, depending on the host protease(s) responsible for cleaving the spike protein: (i) cell surface following activation by serine proteases such as TMPRSS2 and furin (Hoffmann et al., 2020b); or (ii) endocytic pathway within the endosomal–lysosomal compartments including processing by lysosomal cathepsin L (Yang and Shen, 2020). These flexibility for host cell factors mediating viral entry, highlights that the

availability of factors existing in a cell type dictates the mechanism of viral entry (Kawase et al., 2012). When TMPRSS2 (or other serine proteases such as TMPRSS4 (Zang et al., 2020) or human airway trypsin-like protease [HAT] (Bestle et al., 2020a)) is expressed, fusion of the virus with the cell surface membrane is preferred (Shirato et al., 2018), while in their absence, the virus can penetrate the cell by endocytosis(Kawase et al., 2012). A third factor has also been shown to facilitate SARS-CoV-2 entry in cells that have ACE2 and even promote, although to very low levels, SARS-CoV-2 entry in cells that lack ACE2 and TMPRSS2 which is the neuropilin-1 (NRP-1) (Cantuti-Castelvetri et al., 2020). This key event deals with SARS-CoV-2 entry in host cells and is divided in three categories: TMPRSS2, capthesin L and NRP-1.

TMPRSS2 Spike cleavage:

TMPRSS2 (transmembrane serine protease 2, (https://www.ncbi.nlm.nih.gov/gene/7113) is a cell-surface protease (Hartenian et al., 2020) that facilitates entry of viruses into host cells by proteolytically cleaving and activating viral envelope glycoproteins. Viruses found to use this protein for cell entry include Influenza virus and the human coronaviruses HCoV-229E, MERS-CoV, SARS-CoV and SARS-CoV-2 (COVID-19 virus).

TMPRSS2 is a membrane bound serine protease also known as epitheliasin. TMPRSS2 belongs to the S1A class of serine proteases alongside proteins such as factor Xa and trypsin. Whilst there is evidence that TMPRSS2 autoclaves to generate a secreted protease, its physiological function has not been clearly identified. However, it is known to play a crucial role in facilitating entry of coronavirus particles into cells by cleaving the spike protein (Huggins, 2020).

After ACE2 receptor binding, SARS-CoV-2 S proteins can be subsequently cleaved and activated by host cell-surface protease at the S1/S2 and S2’ sites, generating the subunits S1 and S2 that remain non-covalently linked. Cleavage leads to activation of the S2 domain that drives fusion of the viral and host membranes (Hartenian et al., 2020, Walls et al., 2016). For other coronaviruses, processing of spike was proposed to be sequential with S1/S2 cleavage preceding that of S2. Cleavage at S1/S2 may be crucial for inducing conformational changes required for receptor binding or exposure of the S2 site to host proteases.

The S1/S2 site of SARS-CoV-2 S protein contains an insertion of four amino acids providing a minimal furin cleavage site (RRAR685↓) (that is absent in SARS-CoV). Interestingly, the furin cleavage site has been implicated in increased viral pathogensis (Bestle et al., 2020b, Huggins, 2020). Processing of the spike protein by furin at the S1/S2 cleavage site is thought to occur following viral replication in the endoplasmic reticulum Golgi intermediate compartment (ERGIC) (Hasan et al., 2020). The spike S2’ cleavage site of SARS-CoV-2 possesses a paired dibasic motif with a single KR segment (KR815↓) (as SARS-CoV) that is recognized by trypsin-like serine proteases such as TMPRSS2. **The current data support a model for SARS-CoV-2 entry in which furin-mediated cleavage at the S1/S2 site pre-primes spike during biogenesis, facilitating the activation for membrane fusion by a second cleavage event at S2’ by TMPRSS2 following ACE2 binding** (Bestle et al., 2020b, Johnson et al., 2020).

Virus	S1/S2 site	S2’ site
SARS-CoV-2	TNSP**RRAR**|SVA	PSKPS**KR**|SFIEDL
SARS-CoV	S----LLR|STS	PLKPT**KR**|SFIEDL

Camostat mesylate, an inhibitor of TMPRSS2, blocks SARS-CoV-2 infection of lung cells like Calu-3 cells but not Huh7.5 and Vero E6 cells. Cell entry was assessed using a viral isolate and viral pseudotypes (artificial viruses) expressing the COVID-19 spike (S) protein. The ability of the viral pseudotypes (expressing S protein from SARS-CoV and SARS-CoV-2) to enter human and animal cell lines was demonstrated, showing that SARS-CoV-2 can enter similar cell lines as SARS-CoV. Amino acid analysis and cell culture experiments showed that, like SARS-CoV, SARS-CoV-2 spike protein binds to human and bat angiotensin-converting enzyme 2 (ACE2) and uses a cellular protease TMPRSS2 for priming. Priming activates the spike protein to facilitate viral fusion and entry into cells. Cell culture experiments were performed using immortalized cell lines and primary human lung cells (Hoffmann et al., 2020b, Rahman et al., 2020).

Spike binding to neuropilin-1:

Neuropilin-1 (NRP1) is a transmembrane glycoprotein that serves as a cell surface receptor for semaphorins and various ligands involved in angiogenesis in vertebrates. NRP1 is expressed in neurons, blood vessels (endothelial cells), immune cells and many other cell types in the mammalian body (maternal fetal interface) and binds a range of structurally and functionally diverse extracellular ligands to modulate organ development and function (Raimondi et al., 2016). NRP1 is well described as a co-receptor for members of the class 3 semaphorins (SEMA3) or vascular endothelial growth factors (VEGFs) (Gelfand et al., 2014). Structurally, NRP1 comprises seven sub-domains, of which the first five are extracellular; two CUB domains (a1 and a2), two coagulation factor V/VIII domains (FV/VIII; b1 and b2) and a meprin, A5 μ-phosphatase domain (MAM; c). NRP1 contains only a short cytosolic tail with a PDZ-binding domain lacking internal signaling activity. The different ligand families bind to different sites of NRP1; SEMA3A binding requires the first three sub-domains of NRP1 (a1, a2, and b1), whereas binding of VEGF-A requires the b1 and b2 domains (Muhl et al., 2017). Additional studies conducted by means of in silico computational technology to identify and validate inhibitors of the interaction between NRP1 and SARS-CoV-2 Spike protein are reported in (Perez-Miller et al., 2020). Represents a schematic picture of VEGF-A triggered phosphorylation of VEGF-R2. Screening of NRP-1/VEGF-A165 inhibitors by in-cell Western (Perez-Miller et al., 2020).v NRP1 acts as a co-receptor for SARS-CoV-2.

NRP1 is a receptor for furin-cleaved SARS-CoV-2 spike peptide (Cantuti-Castelvetri et al., 2020, Daly et al., 2020, Johnson et al., 2020). Blockade of NRP1 reduces infectivity and entry, and alteration of the furin site leads to loss of NRP1 dependence, reduced replication in Calu3, augmented replication in Vero E6, and attenuated disease in a hamster pathogenesis disease model (Johnson et al., 2020). In fact, a small sequence of amino acids was found that appeared to mimic a protein sequence found in human proteins that interact with NRP1. The spike protein of SARS-CoV-2 binding with NRP1 aids viral infection of human cells. This was confirmed by applying a range of structural and biochemical approaches to establish that the spike protein of SARS-CoV-2 does indeed bind to NRP1. The host protease furin cleaves the full-length precursor S glycoprotein into two associated polypeptides: S1 and S2. Cleavage of S generates a polybasic RRAR C-terminal sequence on S1, which conforms to a C-end rule (CendR) motif that binds to cell surface neuropilin-1 (NRP1) and neuropilin-2 (NRP2) receptors. It was reported that the S1 CendR motif directly bound NRP1 by X-ray crystallography and biochemical approaches. Blocking this interaction using RNAi or selective inhibitors reduced SARS-CoV-2 entry and infectivity in cell culture (Daly et al., 2020).

NRP1, known to bind furin-cleaved substrates, significantly potentiates SARS-CoV-2 infectivity, which was revealed by a monoclonal blocking antibody against NRP1. It was found that a SARS-CoV-2 mutant with an altered furin cleavage site did not depend on NRP1 for infectivity. Pathological analysis of olfactory epithelium obtained from human COVID-19 autopsies revealed that SARS-CoV-2 infected NRP1-positive cells faced the nasal cavity (Cantuti-Castelvetri et al., 2020). Furthermore, it has been found that NRP1 is a new potential SARS-CoV-2 infection mediator implicated in the neurologic features and central nervous system involvement of COVID-19. Preclinical studies have suggested that NRP1, a transmembrane receptor that lacks a cytosolic protein kinase domain and exhibits high expression in the respiratory and olfactory epithelium, may also be implicated in COVID-19 by enhancing the entry of SARS-CoV-2 into the brain through the olfactory epithelium. NRP1 is also expressed in the CNS, including olfactory-related regions such as the olfactory tubercles and paraolfactory gyri. Supporting the potential role of NRP1 as an additional SARS-CoV-2 infection mediator implicated in the neurologic manifestations of COVID-19. Accordingly, the neurotropism of SARS-CoV-2 via NRP1-expressing cells in the CNS merits further investigation (Davies et al., 2020).

Up-regulation of NRP1 protein in diabetic kidney cells hints at its importance in a population at risk of severe COVID-19. Involvement of NRP-1 in immune function is compelling, given the role of an exaggerated immune response in disease severity and deaths due to COVID-19. NRP-1 has been suggested to be an immune checkpoint of T cell memory. It is unknown whether involvement and up-regulation of NRP-1 in COVID-19 may translate into disease outcome and long-term consequences, including possible immune dysfunction (Mayi et al., 2021).

The main feature of NRP1 co-receptor is to form complexes with multiple other receptors. Hence, there is a competition between receptors to complex with NRP-1, which may determine their abilities both quantitatively and qualitatively to transduce signals. It is tempting to hypothesize that the occupancy of NRP-1 with one receptor may thus decrease its availability for virus entry. Recent proteomics work has shown that NRP-1 can form a complex with the α7 nicotinic receptor in mice. Both receptors are expressed in the human nasal and pulmonary epithelium (Mayi et al., 2021).

NRP1, is highly expressed in the respiratory and olfactory epithelium; it is also expressed in the CNS, including olfactory related regions such as the olfactory tubercles and paraolfactory gyri (Davies et al., 2020).

More information on tissue distribution and protein expression of NRP1 can be found in https://www.proteinatlas.org/ENSG000000992 50-NRP1

Spike entry via lysosomal cathepsins and endocytosis:

Evidence shows the role of TMPRSS2 and other serine proteases in activating the coronavirus spike protein for plasma membrane fusion. However, studies using various cell culture systems showed that SARS-CoV2 could enter cells via an alternative endosomal–lysosomal pathway. Evidence came from studies demonstrating that lysosomotropic agents reduced SARS-CoV replication in cells lacking TMPRSS2 and other studies, using highly potent and specific small-molecule cathepsin inhibitors, to understand the role of cathepsins in processing and activating the spike for membrane fusion, mainly of cathepsin L (one of the 11 cathepsins) (Rossi et al., 2004, Simmons et al., 2005). SARS-CoV-2 and other coronaviruses can establish infection through endosomal entry in commonly used in vitro cell culture systems. Of relevance, inhibitors of the endosomal pathway, as the cathepsin inhibitor Z-FA-FMK and PIKfyve inhibitor apilimod, blocked viral entry in Huh7.5 and Vero E6 cells but not Calu-3 cells.

Viral entry leads to delivery of virion proteins and translation of viral proteins immediately:

Coronavirus is a class of viruses that have single-stranded positive-sense RNA genomes in their envelopes [Kim D, *et al., 2020*]. The virus contains a 29.7 kB positive-sense RNA genome flanked by 5' and 3' untranslated regions of 265 and 342 nucleotides, respectively that contain cis-acting secondary RNA structures essential for RNA synthesis [Huston N. C. *et al., 2021*]. The genome just prior to the 5′ end contains the transcriptional regulatory sequence leader (TRS-L) [Budzilowicx C.J., *et al., 1985*]. The SARS-CoV genome is polycistronic and contains 14 open reading frames (ORFs) that are expressed by poorly understood mechanisms [Snijder E. J., *et al.*, 2003]. Preceding each ORF there are other TRSs called the body TRS (TRS B). The 5′ two-thirds of the genome contains two large, overlapping, nonstructural ORFs and the 3′ third contains the remainder ORFs [Di H., *et al.*, 2018]. Genome expression starts with the translation of two large ORFs of the 5′ two-thirds: ORF1a of 4382 amino acids and ORF1ab of 7073 amino acid that occurs via a programmed (- 1) ribosomal frameshifting [Snijder E. J., *et al.*, 2003], yielding pp1a and pp1ab. These two polyproteins are cleaved into 16 subunits by two viral proteinases encoded by ORF1a, nsp3, and nsp5 that contain a papain-like protease domain and a 3C-like protease domain [Sacco M. D. *et al., 2020*]. The processing products are a group of replicative enzymes, named nsp1-nsp16, that assemble into a viral replication and transcription complex (RTC) associated with membranes of endoplasmic reticulum (ER) with the help of various membrane-associated viral proteins [Klein S., *et al., 2021*, Snijder E. J*., et al., 2020,* V'Kovski P. , *et al., 2021*]. This association leads to replication factories or organelles, that are originate new membranous structures that are observed by electron mciroscopy . They are a feature of all coronaviridae and the site of viral replication and transcription hidden from innate immune molecules.

How it is Measured or Detected

SARS-CoV2 entry can be determined by many different ways:

1) quantitative RT-PCR specific to the subgenomic mRNA of the N transcript, in cells manipulated with host factors that express of not TMPRSS2, cathepsinL, neuropilin-1, hACE2 [Glowacka I, et al. (2011)], or exogenous addition of HAT or furin.

2) using spike-pseudotyped viral particles expressing GFP/luciferase/bgalactosidase and comparing with vesicular stomatitis virus G seudotyped particles expressing the same reporter analysed in manipulated cultured with cell lines, followed by determining fluorescence, biolumincescence, luciferase activity in cell lysates [Hoffmann M, et al. (2020)].

TMPRSS2:

TMPRSS2 gene expression can be measured by RNAseq and microarray (Baughn et al., 2020).

Expression levels of TMPRSS2 can be measured by RNA in situ hybridization (RNA-ISH) (Qiao et al., 2020)

NRP-1:

Several methods have been identified in the literature for measuring and detecting NRP1 receptor binding. Briefly described:

1. X-ray crystallography and biochemical approaches help to show that the S1 CendR motif directly bound NRP1 (1). Binding of the S1 fragment to NRP1 was assessed and ability of SARS-CoV-2 to use NRP1 to infect cells was measured in angiotensin-converting enzyme-2 (ACE-2)-expressing cell lines by knocking out NRP1 expression, blocking NRP1 with 3 different anti-NRP1 monoclonal antibodies, or using NRP1 small molecule antagonists (Centers for Disease Control and Prevention, 2020, Daly et al., 2020).

Key findings (Centers for Disease Control and Prevention, 2020, Daly et al., 2020):

• The S1 fragment of the cleaved SARS-CoV-2 spike protein binds to the cell surface receptor neuropilin-1 (NRP1).

• SARS-CoV-2 utilizes NRP1 for cell entry as evidenced by decreased infectivity of cells in the presence of: NRP1 deletion ($p < 0.01$). Three different anti-NRP1 monoclonal antibodies ($p < 0.001$). Selective NRP1 antagonist, EG00229 ($p < 0.01$).

2. Cell lines were modified to express ACE2 and TMPRSS2, the two known SARS-CoV-2 host factors, and NRP1 to assess the contribution of NRP1 to infection. Autopsy specimens from multiple airway sites were stained with antibodies against SARS-CoV-2 proteins, ACE2, and NRP1, to visualize co-localization of proteins (6, 15).

Key findings (Cantuti-Castelvetri et al., 2020, Centers for Disease Control and Prevention, 2020):

• Infectivity of cells expressing angiotensin converting enzyme-2 (ACE2, receptor for SARS-CoV-2), transmembrane protease serine-2 (TSS2, primes the Spike [S] protein), and neuropilin-1 (NRP1) with pseudovirus expressing the SARS-CoV-2 S1 protein was approximately 3-fold higher than in cells expressing either ACE2 or TSS2 alone ($p<0.05$).

• Analysis of autopsy tissue from COVID-19 patients showed co-localization of the SARS-CoV-2 spike (S) protein and NRP1 in olfactory and respiratory epithelium.

Virtual screen of nearly 0.5 million compounds against the NRP-1 CendR site, resulting in nearly 1,000 hits. A pharmacophore model was derived from the identified ligands, considering both steric and electronic requirements. Preparation of receptor protein and grid for virtual screening, docking of known NRP-1 targeting compounds, ELISA based NRP1-VEGF-A165 protein binding assay; more details on methodology in the referenced paper (Perez-Miller et al., 2020)

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Event: 1901: Interferon-I antiviral response, antagonized by SARS-CoV-2

Short Name: IFN-I response, antagonized

Key Event Component

Process	Object	Action
type I interferon signaling pathway	interferon alpha	decreased
type I interferon signaling pathway	interferon beta	decreased
cellular response to exogenous dsRNA	RNA viral genome	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	KeyEvent

Stressors

Name
Stressor:624 SARS-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
mink	Mustela lutreola	High	NCBI
cat	Felis catus	High	NCBI
rhesus macaque	Macaca mulatta	High	NCBI
dog	Canis lupus familiaris	Moderate	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Broad mammalian host range based on spike protein tropism for and binding to ACE2 (Conceicao et al. 2020; Wu et al. 2020) and cross-species ACE2 structural analysis (Damas et al. 2020). Some literature found on non-human hosts indicates that NSPs and accessory proteins can interact in a similar manner with bird (chicken) and other mammal proteins in the IFN-I pathway (Moustaqil et al. 2021; Rui et al. 2021).

Key Event Description

SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of ~30 kb, sequence orientation in a 5’ to 3’ direction typical of positive sense and reflective of the resulting mRNA (doi:https://doi.org/10.1016/j.cell.2020.04.01). The SARS-CoV-2 genome contains a 5’-untranslated region (UTR; 265 bp), ORF1ab (21,289 bp) holding two overlapping open reading frames (13,217 bp and 21,289 bp, respectively) that encode two polyproteins (Kim et al. 2020; O’Leary et al. 2020). Viral transcription and replication is explained in depth in KE1847. Briefly, the first event upon cell entry is the primary translation of the ORF1a and ORF1b genomic RNA to produce non-structural proteins (NSPs). The ORF1a produces polypeptide 1a (pp1a, 440–500 kDa) that is cleaved into NSP-1 through NSP-11. A -1-ribosome frameshift occurs immediately upstream of the ORF1a stop codon, to allow translation through ORF1b, yielding 740–810 kDa polypeptide pp1ab, which is cleaved into 15 NSPs (duplications of NSP1-11 and five additional proteins, NSP12-16). Viral proteases NSP3 and NSP5 cleave the polypeptides through domains functioning as a papain-like protease and a 3C-like protease, respectively (doi:https://doi.org/10.1016/j.cell.2020.04.01). The NSPs, structural proteins, and accessory proteins are encoded by 10 ORFs in the SARS-CoV-2 RNA genome. They may have multiple functions during viral replication as well as in evasion of the host innate immune response, thus augmenting viral replication and spread **(Amor et al. 2020).** Extensive protein-protein interaction **(Gordon et al. 2020)** and viral protein-host RNA interaction networks have been demonstrated between the viral NSPs and accessory proteins and host molecules.

This key event is focused on the specific viral:host protein interactions within the infected cell that are involved in theIFN-I antiviral response pathways. IFN-I is the main component of the innate immune system that is suppressed by the SARS-CoV-2 coronavirus early in infection. The primary form of host intracellular virus surveillance detects viral components to induce an immediate systemic type I interferon (IFN) response. Cellular RNA sensors called pattern recognition receptors (PRRs) such as RIG-I, MDA5 and LGP2 detect the presence of viral RNAs and promote nuclear translocation of the transcription factor IRF3, leading to transcription, translation, and secretion of IFN-α and IFN-β. This in turn leads to interaction with the IFN receptor (IFNAR), phosphorylation of STAT1 and 2, and transcription and translation of hundreds of antiviral genes **(Quarleri and Delpino, 2021).**

Interactions between SARS-CoV-2 proteins and human RNAs thwart the IFN response upon infection: NSP1 binds to 40S ribosomal RNA in the mRNA entry channel of the ribosome to inhibit host mRNA translation; NSP8 and NSP9 displace signal recognition particle proteins (SRP54, 27 and 19) to bind to the 7SL RNA and block protein trafficking to the cell membrane (Banerjee et al. 2020; Gordon et al. 2020). Xia et al. (2020) found that NSP6 and NSP13 antagonize IFN-I production via distinct mechanisms: NSP6 binds TANK binding kinase 1 (TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation, and NSP13 binds and blocks TBK1 phosphorylation. NSP14 induces lysosomal degradation of type 1 IFNAR to prevent STAT activation (Hayn et al. 2021). ORF6 hijacks KPNA2 to block IRF3, and Nup98/RAE1 to block STAT nuclear import, to silence IFN-I gene expression (Xia and Shi, 2020). ORF7a suppresses STAT2 phosphorylation and ORF7b suppresses STAT1 and STAT2 phosphorylation to block ISGF3 complex formation with IRF9 (Xia and Shi, 2020). ORF8 interacts and downregulates MHC-I (Zhang et al 2020), and has been reported to block INFβ expression, but the mechanism has not been identified (Rashid et al. 2021; Li et al. 2020). ORF9b antagonizes Type I Interferons by targeting multiple components of RIG-I/MDA-5-MAVS, TOMM70, NEMO and cGAS-STING signalling (Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al 2020).

Following is a table of the current state of knowledge of SARS-CoV-2 protein putative functions in relation to IFN-I antiviral response antagonism.

Gene	Protein	Function	Role in early innate immune evasion
ORF1a	NSP1	NSP1 antagonizes interferon induction to suppress host antiviral response.	DNA Polymerase Alpha Complex: Regulates the activation of IFN-I through cytosolic RNA-DNA synthesis (POLA1/2-PRIM1/2) and primes DNA replication in the nucleus (Gordon et al. 2020; Chaudhuri et al. 2020). Can also inhibit host gene expression by binding to ribosomes and modifying host mRNAs (Shi et al. 2020; Schubert et al. 2020; Thoms et al. 2020).
	NSP2	While not essential for viral replication, deletion of NSP2 diminishes viral growth and RNA synthesis	Translation repression through binding GIGYF2and EIF4E2 (4EHP) (Gupta et al. 2021)
	NSP3	Papain-like protease (Plpro); Cleaves the ORF1a and 1ab polypeptides	Suppresses IFN-I: Cleaves IRF3 (Moustaqil et al. 2021); binds/cleaves ISG15 (Rui et al. 2021; Shin et al. 2020; Liu et al. 2021; Klemm et al. 2020)
	NSP5	3C-like protease (3CLpro); Cleaves the ORF1a and 1ab polypeptides	Binds STING (Rui et al. 2021)
	NSP6	Limits autophagosome expansion	Suppresses IFN-I expression: Binds TBK-1 to supress IRF3 phosphorylation (Xia et al. 2020; Quarleri and Delpino, 2021)
	NSP7	In complex with NSP8 forms primase as part of multimeric RNA-dependent RNA replicase (RdRp)	
	NSP8	Replication complex with NSP7, NSP9 and NSP12	Binds SRP72/54/19 (Gordon et al. 2020) and 7SL RNA to block IFN membrane transport (Banerjee et al. 2020)

	NSP9	Replication complex with NSP7, NSP8 and NSP12	Binds SRP and 7SL RNA with NSP8 to block IFN membrane transport (Banerjee et al. 2020)
ORF1b	NSP13	Helicase and triphosphatase that initiates the first step in viral mRNA capping.	Binds TBK1 (Xia et al. 2020)
	NSP14		Induces lysosomal degradation of IFNAR1 (Hayn et al. 2021)
ORF2	Spike (S)	ACE2 interaction, cell entry	
ORF3a	ORF3a	Interacts with M, S, E and 7a; form viroporins; immune evasion	Binds STING (Rui et al 2021)
ORF4	Envelope (E)	Viral assembly and budding	
ORF5	Membrane (M)	Viral assembly	Interacts with RIG-I and MAVS sensors of viral RNA (Fu et al 2020)
ORF6	ORF6	Viral pathogenesis and virulence; interacts with ORF8; promotes RNA polymerase activity	Hijacks the nuclear importin Karyopherin a 2 (KPNA2) to block IRF3 (Xia and Shi, 2020) and Nup98/RAE1 to block STAT nuclear import (Miorin et al. 2020; Kato et al. 2020), leading to the silence of downstream ISGs
ORF7a	ORF7a	Interacts with S, ORF3a; immune evasion	Suppresses STAT2 phosphorylation to block IFN-I response (Xia and Shi, 2020).
ORF7b	ORF7b	Structural component of virion	Suppresses STAT1 and STAT2 phosphorylation to block IFN-I response (Xia and Shi, 2020)
ORF8	ORF8	Immune evasion	Interacts and downregulates MHC-I (Zhang et al. 2020). May inhibit type I interferon (IFN-β) and interferon-stimulated response element (ISRE) (Rashid et al. 2020; Li et al. 2020)
ORF9	Nucleocapsid (N)	Stabilizes viral RNA	Attenuates stress granule formation: G3BP1/2 (Chen et al. 2020; Cascarina et al. 2020); G3BP1 also interacts with RIG-I (Kim et al. 2019) and STAT1/2 (Mu et al. 2020)
ORF9b	ORF9b	Immune evasion	Membrane protein antagonizes Type I Interferons by targeting multiple components of RIG-I/MDA-5-MAVS, TOMM70, NEMO, and cGAS-STING signaling pathways (Fu et al. 2020; Chen et al. 2020; Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al 2020)

How it is Measured or Detected

Detection of IFN-I suppression involves measuring gene promoter/transcription activation (luciferase assays), gene up/down regulation (quantitative PCR), protein-protein interaction (immunoprecipitation, immunoblotting) or in-situ co-location of viral and host proteins (immunofluorescent or confocal microscopy) in cell culture. Examples of methods used include the following:

Interferon I decrease (Xia et al. 2020):

- IFN-I production and signaling luciferase reporter assays
- Co-immunoprecipitation and western blot
- Indirect immunofluorescence assays
- DNA assembly and RNA transcription of a luciferase replicon for SARS-CoV-2
- Replicon RNA electroporation and luciferase reporter assay

SARS-CoV-2 ORF9b inhibits RIG-I-MAVS antiviral signaling (Wu et al. 2021)

- Viral- and host-protein-specific antibodies
- Immunoprecipitation
- Immunofluorescent microscopy
- Dual-luciferase reporter assays
- Fluorescence quantification immunoblotting

SARS-CoV-2-Human Protein-Protein Interaction Map (Gordon et al. 2020)

- Cloning and expression of viral proteins via plasmid transfection into HEK293T cell line
- Protein affinity purification using MagStrep beads with detection by anti-strep western blot of cell lysate
- Global analysis of SARS-CoV-2 host interacting proteins using affinity purification-mass spectrometry

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Event: 1847: Increased SARS-CoV-2 production

Short Name: SARS-CoV-2 production

Key Event Component

Process	Object	Action
viral RNA genome replication	viral RNA-directed RNA polymerase complex	increased
positive stranded viral RNA replication	viral RNA-directed RNA polymerase complex	increased
viral RNA genome packaging	viral assembly compartment	increased
mRNA transcription	ssRNA viral genome	increased
viral translation	ssRNA viral genome	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	Moderate	NCBI
Mustela putorius furo	Mustela putorius furo	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Broad mammalian host range has been demonstrated based on spike protein tropism for and binding to ACE2 [Conceicao *et al.* 2020; Wu *et al.* 2020] and cross-species ACE2 structural analysis [Damas *et al.* 2020]. No literature has been found on primary translation and molecular interactions of nsps in non-human host cells, but it is assumed to occur if the virus replicates in other species.

Very broad mammalian tropism: human, bat, cat, dog, civet, ferret, horse, pig, sheep, goat, water buffalo, cattle, rabbit, hamster, mouse

Key Event Description

This KE1847 "Increase coronavirus production" deals with how the genome of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is translated,

replicated, and transcribed in detail, and how the genomic RNA (gRNA) is packaged, and the virions are assembled and released from the cell.

Coronavirus is a class of viruses that have single-stranded positive-sense RNA genomes in their envelopes [D. Kim *et al.*]. The virus contains a 29.7 kB positive-sense RNA genome flanked by 5' and 3' untranslated regions of 265 and 342 nucleotides, respectively [E. J. Snijder *et al.*] that contain cis-acting secondary RNA structures essential for RNA synthesis [N. C. Huston *et al.*]. The genome just prior to the 5′ end contains the transcriptional regulatory sequence leader (TRS-L) [C.J. Budzilowicx *et al.*]. The SARS-CoV genome is polycistronic and contains 14 open reading frames (ORFs) that are expressed by poorly understood mechanisms [E. J. Snijder *et al.*]. Preceding each ORF there are other TRSs called the body TRS (TRS B). The 5′ two-thirds of the genome contains two large, overlapping, nonstructural ORFs and the 3′ third contains the remainder ORFs [H. Di *et al.*]. Genome expression starts with the translation of two large ORFs of the 5’ two-thirds: ORF1a of 4382 amino acids and ORF1ab of 7073 amino acid that occurs via a programmed (- 1) ribosomal frameshifting [E. J. Snider *et al.*], yielding pp1a and pp1ab. These two polyproteins are cleaved into 16 subunits by two viral proteinases encoded by ORF1a, nsp3, and nsp5 that contain a papain-like protease domain and a 3C-like protease domain [M. D. Sacco *et al.*]. The processing products are a group of replicative enzymes, named nsp1-nsp16, that assemble into a viral replication and transcription complex (RTC) associated with membranes of endoplasmic reticulum (ER) with the help of various membrane-associated viral proteins [S. Klein *et al.*, E. J. Snijder *et al.,* P. V'Kovski, *et al.*]. Besides replication, which yields the positive-sense gRNA, the replicase also mediates transcription leading to the synthesis of a nested set of subgenomic (sg) mRNAs to express all ORFs downstream of ORF1b that encode structural and accessory viral proteins. These localize to the 3′ one-third of the genome, as stated above, and result in a 3′ coterminal nested set of 7–9 mRNAs that share ~70–90 nucleotide (nt) in the 5′ leader and that is identical to the 5′ end of the genome [P. Liu, and J. Leibowitz]. sgRNAs encode conserved structural proteins (spike protein [S], envelope protein [E], membrane protein [M], and nucleocapsid protein [N]), and several accessory proteins. SARS-CoV-2 is known to have at least six accessory proteins (3a, 6, 7a, 7b, 8, and 10). Overall the virus is predicted to express 29 proteins [D. Kim *et al.*]. The gRNA is packaged by the structural proteins to assemble progeny virions.

Viral translation:

SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of ~30 kb, sequence orientation in a 5’ to 3’ direction typical of positive sense and reflective of the resulting mRNA [D. Kim *et al.*]. The SARS-CoV-2 genome contains a 5’-untranslated region (UTR; 265 bp), ORF1ab (21,289 bp) holding two overlapping open reading frames (13,217 bp and 21,289 bp, respectively) that encode two polyproteins [D. Kim *et al.*]. Other elements of the genome include are shown below [V. B. O'Leary *et al.*]. **The first event upon cell entry is the primary translation of the ORF1a and ORF1b gRNA to produce non-structural proteins (nsps).**

This is completely dependent on the translation machinery of the host cell. Due to fewer rare “slow-codons”, SARS-CoV-2 may have a higher protein translational rate, and therefore higher infectivity compared to other coronavirus groups [V. B. O'Leary *et al.*]. The ORF1a produces polypeptide 1a (pp1a, 440–500 kDa) that is cleaved into nsp-1 through nsp-11. A -1 ribosome frameshift occurs immediately upstream of the ORF1a stop codon, to allow translation through ORF1b, yielding 740–810 kDa polypeptide pp1ab, which is cleaved into 15 nsps [D. Kim *et al.*]. Two overlapping ORFs, ORF1a and ORF1b, generate continuous polypeptides, which are cleaved into a total of 16 so-called nsps [Y Finkel *et al.*]. Functionally, there are five proteins from pp1ab (nsp-12 through nsp-16) as nsp-1-11 are duplications of the proteins in pp1a due to the ORF overlap. The pp1a is approximately 1.4–2.2 times more expressed than pp1ab. After translation, the polyproteins are cleaved by viral proteases nsp3 and nsp5. Nsp5 protease can be referred to as 3C-like protease ($3CL^{pro}$) or as main protease (M^{pro}), as it cleaves the majority of the polyprotein cleavage sites. [H.A. Hussein *et al.*] Nsp1 cleavage is quick and nsp1 associates with host cell ribosomes and results in host cellular shutdown, suppressing host gene expression [M. Thoms *et al.].* Fifteen proteins, nsp2–16 constitute the viral RTC. They are targeted to defined subcellular locations and establish a network with host cell factors. Nsp2–11 remodel host membrane architecture, mediate host immune evasion and provide cofactors for replication, whilst nsp12–16 contain the core enzymatic functions involved in RNA synthesis, modification and proofreading [P. V'Kovski *et al.*]. nsp-7 and nsp-8 form a complex priming the RNA-dependent RNA polymerase (RdRp or RTC) - nsp-12. nsp14 provides a 3′–5′ exonuclease activity providing RNA proofreading function. Nsp-10 composes the RNA capping machinery nsp-9. nsp13 provides the RNA 5′-triphosphatase activity. Nsp-14 is a *N*7-methyltransferase and nsp-16 the 2′-*O*-methyltransferase. Many of the nsps have multiple functions and many viral proteins are involved in innate immunity inhibition. Nsp-3 is involved in vesicle formation along with nsp-4 and nsp-6 where viral replication occurs. Interactions between SARS-CoV-2 proteins and human RNAs thwart the IFN response upon infection: nsp-16 binds to U1 and U2 splicing RNAs to suppress global mRNA splicing; nsp-1 binds to 40S ribosomal RNA in the mRNA entry channel of the ribosome to inhibit host mRNA translation; nsp-8 and nsp-9 bind to the 7SL RNA to block protein trafficking to the cell membrane [A. K. Banerjee *et al.*]. Xia et al. [H. Xia *et al.*] found that nsp-6 and nsp-13 antagonize IFN-I production via distinct mechanisms: nsp-6 binds TANK binding kinase 1 (TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation, and nsp-13 binds and blocks TBK1 phosphorylation.

Viral transcription and replication:

Viral transcription and replication occur at the viral replication organelle (RO) [E. J. Snijder *et al.*]. The RO is specifically formed during infection by reshaping ER and other membranes, giving rise to small spherular invaginations, and large vesiculotubular clusters, consisting of single- and/or double-membrane vesicles (DMV), convoluted membranes (CM) and double-membrane spherules invaginating from the ER [S. Klein *et al.,* E. J. Snijder *et al.*]. There is some evidence that DMV accommodate viral replication which is based on radiolabelling viral RNA with nucleoside precursor ([5-^{3}H]uridine) and detection by EM autoradiography [E. J. Snijder *et al.*].

Viral replicative proteins and specific host factors are recruited to ROs [E. J. Snijder *et al.*]. RNA viral genome is transcribed into messenger RNA by the viral RTC [P. Ahlquist *et al.*]. Viral RTC act in combination with other viral and host factors involved in selecting template RNAs, elongating RNA synthesis, differentiating genomic RNA replication from mRNA transcription, modifying product RNAs with 5’ caps or 3’ polyadenylate [P. Ahlquist *et al.*]. Positive-sense (messenger-sense) RNA viruses replicate their genomes through negative-strand RNA intermediates [M. Schwartz *et al.*]. The intermediates comprise full-length negative-sense complementary copies of the genome, which functions as templates for the generation of new positive-sense gRNA, and a nested set of sg mRNAs that lead to the expression of proteins encoded in all ORFs downstream of ORF1b. The transcription of coronaviruses is a discontinuous process that produces nested 3′ and 5′ co-terminal sgRNAs. Of note, the synthesis of sg mRNAs is not exclusive to the order *Nidovirales* but a discontinuous minus-strand synthesis strategy to produce a nested set of 3′ co-terminal sg mRNAs with a common 5′ leader in infected cells are unique features of the *coronaviruses* and *arteriviruses* [W. A. Miller and G. Koev.]. Of note, the produced genomic RNA represents a small fraction of the total vRNA [N. S. Ogando *et al.*].

The discontinuous minus-strand synthesis of a set of nested sg mRNAs happens during the synthesis of the negative-strand RNA, by an interruption mechanism of the RTC as it reads the TRS-B preceding each gene in the 3′ one-third of the viral genome [I. Sola, F. Almazan *et al.,* I. Sola, J. L. Moreno, *et al.*]. The synthesis of the negative-strand RNA stops and is re-initiated at the TRS-L of the genome sequence close from the 5′ end of the genome [H. Di *et al.*]. Therefore, the mechanism by which the leader sequence is added to the 5' end requires that the RTC switches template by a jumping mechanism. This interruption process involves the interaction between complementary TRSs of the nascent negative-strand RNA TRS-B and the positive-strand gRNA at the positive-sense TRS-L. The TRS-B site has a 7-8 bp conserved core sequence (CS) that facilitates RTC template switching as it hybridizes with a near complementary CS in the TRS-L [I. Sola, F. Almazan *et al.* I. Sola, J. L. Moreno, *et al.*]. Upon re-initiation of RNA synthesis at the TRS-L region, a negative-strand copy of the leader sequence is added to the nascent RNA to complete the synthesis of negative-strand sgRNAs. This means that all sg mRNAs as well as the genomic RNA share a common 5' sequence, named leader sequence [X. Zhang et al.]. This programmed template switching leads to the generation of sg mRNAs with identical 5' and 3' sequences, but alternative central regions corresponding to the beginning of each structural ORF [I. Sola *et al.* 2015, S. G. Sawicki *et al.*, Y. Yang *et al.*]. Of note, the existence of TRSs also raises the possibility that these sites are at the highest risk of recombining through TRS-B mediated template switching [Y. Yang]. The set of sg mRNAs is then translated to yield 29 identified different proteins [F. Wu *et al.*], although many papers have identified additional ORFs [D. Kim *et al.*. Y. Finkel *et al.,* A. Vandelli *et al.*]. The translation of the linear single-stranded RNA conducts to the generation of the following proteome: 4 are structural proteins, S, N, M, and E; 16 proteins nsp: the first 11 are encoded in ORF1a whereas the last 5 are encoded in ORF1ab. In addition, 9 accessory proteins named ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10 have been identified [F. Wu *et al.*]. At the beginning of infection, there is the predominant expression of the nsp that result from ORF1a and ORF1ab, however, at 5 hpi, the proteins encoded by the 5′ last third are found in higher amounts, and the nucleoprotein is the protein found in higher levels [Y. Finkel *et al.*].

Viral assembly:

The final step of viral production requires virion assembly and this process is not well explored for SARS-CoV-2. For example, the role of the structural proteins of SARS-CoV-2 in virus assembly and budding in not known. In general, all beta-coronavirus structural proteins assemble at the endoplasmic reticulum (ER)-to-Golgi compartment [J. R. Cohen *et al.,* A. Perrier *et al.*] and viral assembly requires two steps: Genome packaging which is a process in which the SARS-CoV-2 gRNA must be coated by the viral protein nucleoprotein (N) protein, forming viral ribonucleoprotein (vRNPs) complexes, before being selectively packaged into progeny virions [P. V'Kovski *et al.*], a step in which vRNPs bud into the lumen of the ER and the ER-Golgi intermediate compartment (ERGIC) [N. S. Ogando *et al.*]. This results in viral particles enveloped with host membranes containing viral M, E, and S transmembrane structural proteins that need to be released from the cell.

SARS-CoV-2 gRNA packaging involves the N protein. The N protein of human coronaviruses is highly expressed in infected cells. It is considered a multifunctional protein, promoting efficient sub-genomic viral RNA transcription, viral replication, virion assembly, and interacting with multiple host proteins [P. V'Kovski *et al.*, D. E. Gordon *et al.*, R. McBride, and M. van Zyl, B. C.]. In relation to transcription and replication, the N protein could provide a cooperative mechanism to increase protein and RNA concentrations at specific localizations S. Alberti, and S. Carra, S. F. Banani *et al.*], and this way organize viral transcription. Five studies have shown that N protein undergoes liquid-liquid phase separation (LLPS) *in vitro* [A. Savastano *et al.*, H. Chen *et al.*, C. Iserman *et al.*, T. M. Perdikari *et al.*, J. Cubuk *et al.*], dependent on its C-terminal domain (CTD) [H. Chen *et al.].* It has been hypothesised that N could be involved in replication close to the ER and in packaging of gRNA into vRNPs near the ERGIC where genome assembly is thought to take place [A. Savastano *et al.*], but so far this is still speculative. Phosphorylation of N could adjust the physical properties of condensates differentially in ways that could accommodate the two different functions of N: transcription and progeny genome assembly [A. Savastano *et al.*, C. Iserman *et al.,* C. R. Carlson *et al.*].

The ERGIC constitutes the main assembly site of coronaviruses [S. Klein *et al.,* E. J. Snijder *et al.*, L. Mendonca *et al.*] and budding events have been seen by EM studies. For SARS-CoV-2, virus-budding was mainly clustered in regions with a high vesicle density and close to ER- and Golgi-like membrane arrangements [S. Klein *et al.,* E. J. Snijder *et al.*, L. Mendonca *et al.*]. The ectodomain of S trimers were found facing the ERGIC lumen and not induce membrane curvature on its own, therefore proposing that vRNPs and spike trimers [S. Klein *et al.*].

Finally, it has been shown that SARS-CoV-2 virions de novo formed traffic to lysosomes for unconventional egress by Arl8b-dependent lysosomal exocytosis [S. Ghosh *et al.*]. This process results in lysosome deacidification, inactivation of lysosomal degradation enzymes, and disruption of antigen presentation [S. Ghosh *et al.*].

How it is Measured or Detected

Viral translation:

SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation [Schubert *et al.* 2020]

- Sucrose pelleting binding assay to verify Nsp1–40S complex formation
- In vivo translation assay
- Transient expression of FLAG-Nsp1 in HeLa cells and puromycin incorporation assay

SARS-CoV-2 disrupts splicing, translation, and protein trafficking [Banerjee *et al.* 2020]

- SARS-CoV-2 viral protein binding to RNA
- Interferon stimulation experiments
- Splicing assessment experiments
- IRF7-GFP splicing reporter, 5EU RNA labeling, capture of biotinylated 5EU labeled RNA

Membrane SUnSET assay for transport of plasma membrane proteins to the cell surface

Viral transcription:

The mRNA transcripts are detected by the real-time reverse transcription-PCR (RT-PCR) assay. Several methods targeting the mRNA transcripts have been developed, which includes the RT-PCR assays targeting RdRp/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 [Chan *et al.*]. RT-PCR assays detecting SARS-CoV-2 RNA in saliva include quantitative RT-PCR (RT-qPCR), direct RT-qPCR, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) [Nagura-Ikeda M, *et al.*]. The viral mRNAs are reverse-transcribed with RT, followed by the amplification by PCR.

Viral replication:

viral replication is measured by RT-qPCR in infected cells, formation of liquid organelles is assessed in vitro reconstitution systems and in infected cells. Labelling by radioactive nucleosides.

Viral production:

Plaque assays, infectivity assays, RT-qPCR to detect viral RNA in released virions, sequencing to detect mutations in the genome, electron microscopy.

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Event: 1848: Toll Like Receptor (TLR) Dysregulation

Short Name: TLR Activation/Dysregulation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:378 - Dysregulated underperforming Toll Like Receptors (TLRs) leading to high pathogen load	MolecularInitiatingEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent

Stressors

Name
Danger Associated Molecular Patters (DAMPs)
Pathogen Associated Molecular Patterns (PAMPs)
cell free mitochondrial DNA (mtDNA)
SARS-CoV
Sars-CoV-2

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
humans	Homo sapiens	High	NCBI
mice	Mus sp.	High	NCBI
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Birth to < 1 month	High
Old Age	High
All life stages	High

Sex Applicability

Sex	Evidence
Mixed	Moderate
Male	High

Cell applicability: TLRs are broadly expressed on various cell types. Examples include: epithelial cells, macrophages, neutrophils, platelets, dendritic cells, NK cells, Tcells, Bcells, neurons, Adipocytes.

Tissue/organ level : TLRs are broadly expressed in all vital tissues/organs: lung, heart, liver, spleen, kidney, brain, muscle, gut, skin

Taxonomic Applicability: TLRs are well conserved across species but between species variations are reported in terms of sensitivity towards stressors. For instance certain CpG-ODNs have a stronger TLR9 activating potential in mice than in human and vice versa.

Life Stages: TLRs are expressed in all life stages but age variation of level of TLR activation/dysregulation are reported. In elderly immunoscenescence and inflammation are both linked to TLR dysregulation

Sex Applicability: Male and female subjects both express functionally active TLRs but sex differences have been reported. For instance certain TLR gain and/or loss of function polymorphisms have higher prevalence in men. Example of TLR7 loss of function (van der Made et al 2020) and TLR9 gain of function (Gao et al 2018, Traub et al 2012, Elsherif et al 2019). Higher testosterone in men has also been linked to higher TLR4 expression.

TLR7 is located in a region on the X-chromosome which have a high chance of escaping inactivation leading to higher expression levels in women. Estrogens trigger TLR7, which is higher in women. Exposure of Peripheral blood mononuclear cells (PBMC) to TLR7 ligands will cause a higher production of type I IFN (IFN-a) in female cells than male cells. (Kovats, 2015; Takahashi and Iwasaki, 2021; Libert et al., 2010; Scully et al., 2020)

Key Event Description

Background

Toll-like receptors (TLRs) are a family of 13 conserved transmembrane receptors that are at the forefront of directing innate and adaptive immune responses against invading bacteria, fungi, viruses and parasites (Akira 2003, Takeda, Akira 2004, Pasare, Medzhitov 2005, Tal, Adini et al. 2020, van der Made, Simons et al. 2020). Upon activation TLRs initiate overlapping and distinct signaling pathways in various cell types such as macrophages (MP), conventinal DC (cDC), plasmacytoid DC (pDC), lamina propria DC (LPDC), and inflammatory monocytes (iMO). Engagement of TLR with specific stressors (e.g. PAMPs and DAMPs) induces conformational changes of TLRs that allow homo- or heterophilic interactions of TLRs and recruitment of adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM to control intracellular signalling pathways leading to the synthesis and secretion of appropriate cytokines and chemokines by cells of the immune system. TLRs have various biological roles both in pathogen combat and tissue homeostasis.

This KE is first developed in context of COVID-19 CIAO project.

The key gatekeepers in detecting and combating viral infections are TLR3, TLR7, TLR8 and TLR9 and these are predominantly localized in intracellular compartments. In the setting of COVID-19, multiple TLRs are likely relevant in viral combat. Literature covering TLR triggering via SARS-CoV-2 derived PAMPS (Pathogen Associated Molecular Patterns) include:

- TLR7 and TLR8 (+TLR3, TLR4, TLR6) (Khanmohammadi and Rezaei, 2021)
- TLR1, TLR4 and TLR6 activated by SARS-CoV-2 spike proteins (Choudhury A *et al*, 2020)
- TLR9: Less CpG suppression in coronavirus compared to other viruses, for SARS-CoV-2 in the Envelope (E) open reading frame (E-ORF) and ORF10 (Ng et al., 2004; Digard et al. 2020) and multidisciplinary links described in Bezemer and Garssen, 2021

TLR dysregulation can be multi-fold:

1. Underperformance of TLR function leading to poor pathogen combat. This is covered in AOP 378

    - COVID-19 patients having poor TLR function (due to polymorphisms) could potentially have less viral clearance capability and more adverse events leading to more severe disease and mortality. This has been shown for TLR7 loss of function polymorphisms (van der Made et al 2020). Knowledge Gap:

it is not known if loss of function of other TLRs has a worse outcome in COVID-19 patients.

2. Overperformance of TLR function contributing to exaggerated immune response/cytokine storm/thrombosis/progression into ARDS and MOD. This is covered in AOP377

- TLR7 and TLR9 expression, measured by RNAseq gene analysis, is more elevated in black Americans than white Americans, which is proposed to explain in part the racial disparity in Covid-19 mortality rates via TLR mediated DC activation (Tal et al. 2020)
- genetic mutations leading to TLR9 gain of function in human is associated with immune-mediated disease and with a higher incidence of ICU acquired infection (Chatzietal.,2018;Ng et al.,2010).
- Higher presence of host derived TLR stressors in vulnerable patients can contribute to TLR overstimulation/dysregulation. (Bezemer and Garssen, 2021)

Different classes of "stressors" act on TLR activation/dysregulation

1. Pathogen associated molecular patterns (PAMPs). TLRs can sense PAMPS during infection or upon exposure to stressors containing micro-organisms or fragments thereof (e.g. cigarette smoke, bioaerosols, house dust mite)

- TLR1 is activated by bacterial Lipopeptides
- TLR2 is activated by bacterial lipoproteins and glycolipids, TLR2 can form conformations with TLR1 and TLR6 to distinguish between diacyl and triacyl lipopeptides.
- TLR3 is activated by viral double stranded RNA(dsRNA)
- TLR4 is activated by Bacterial LPS
- TLR5 is activated by Bacterial flagellig
- TLR6 is activated by Bacterial lipopeptides and Fungal zymosan
- TLR7 and 8 recognize viral single stranded RNA(ssRNA) and bacterial RNA.
- TLR9 recognizes RNA and DNAmotifs that are rich in unmethylated Cytosine-phosphate-Guanine (CpG) sequences. CpG-motifs are higher expressed in the bacterial and viral genome compared to the vertebrate genome (Hemmi et al., 2000).

2. host derived Damage-Associated Molecular Patterns (DAMPS). Note that in the context and nomenclature of AOP these DAMPS cannot be labeld as "stressors" since they are derived from inside and not from outside, however these "pseudostressors" do act on the TLR receptors in similar way as PAMPs

- TLR2 and TLR4 are activated by heat shock proteins 60 and 70 (HSP60 and HSP70); extracellular matrix components (ECM); oligosaccharides of hyaluronic acid (HA) and heparan sulfate (HS) (Piccinini AM and Midwood KS, 2010).
- high-mobility group protein B1 (HMGB1) triggers TLR2, TLR4 and TLR9
- Oxidative injury/Oxidized phospholipids triggers TLR4 mediated NET formation
- Human mitochondrial DNA (mtDNA), evolutionary derived from endosymbiont bacteria, contains unmethylated CpG-motifs and triggers inflammatory responses directly via TLR9 during injury and/or infection (Zhang et al., 2010).
- Altered self-ligands, called carboxy-alkyl-pyrroleprotein adducts (CAPs), that are generated during oxidative stress, are known to aggravate TLR9/MyD88 pathway activation (Zhanget al., 2010;Panigrahi et al., 2013). CAPs have been shown to promote platelet activation, granule secretion, and aggregation in vitro and thrombosis in vivo (Panigrahi et al., 2013).

3. synthetic TLR triggers/blockers (agonists/antagonists) for therapeutic purposes. Examples include CpG-ODNs triggering TLR9 for vaccin adjuvants/cancer treatment/immuno-modulation

Several Modulating factors can contribute to TLR activation/dysregulation

- Co-infection and Trauma (for instance ventilator induced damage) can induce increased levels of TLR9 stressor, mtDNA, which is known to drive worse outcome at ICU in setting of other disorders.
- High levels of Visceral Fat, can increase TLR9 expression levels ánd circulating mtDNA
- Aging triggers both immunosenescence and inflammaging in part via impaired TLR function versus inappropriate triggering via increases of circulating DAMPS (Shaw et al 2011)
- Genetic polymorphisms can lead to TLR dysregulation (TLR9 gain of function and TLR7 loss of function with worse outcome at ICU Chatzi et al 2018, van der Made et al 2020, Chen et al 2011,)
- Circulating DAMPS such as mtDNA levels increase with age which is a familiar trait contributing to chronic inflammation, so called“inflamm-aging”in elderly people (Pinti et al., 2014).
- Vitamin D inhibits expression levels of TLR9
- Men, higher testosterone, higher TLR4

How it is Measured or Detected

Patient specific Ex vivo analysis

- Levels of TLR specific stressors (for instance for TLR9, cell free DNA/RNA, mtDNA) are measurable in biological samples (serum, plasma)
- TLR gain of function and loss of function polymorphisms are measurable
- TLR expression levels on different cell types and different tissues are measurable by mRNA analysis and by protein analysis
- TLR function in response to stressors is measurable by analysing components of downstream cascades and read outs of inflammatory mediators (IL6, IL8, IL10, Il17, INF, TNFalpha, etc). This can be done by ex vivo stimulations of cells isolated from patients for instance PBMCs.

In vitro/ in vivo models

- TLR Reporter assays
- TLR knock-out mice

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Event: 1496: Increased, secretion of proinflammatory mediators

Short Name: Increased proinflammatory mediators

Key Event Component

Process	Object	Action
cytokine production involved in inflammatory response	Cytokine	increased
chemokine secretion	Chemokine	increased
complement activation		increased
	Interleukin	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:173 - Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:392 - Decreased fibrinolysis and activated bradykinin system leading to hyperinflammation	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:39 - Covalent Binding, Protein, leading to Increase, Allergic Respiratory Hypersensitivity Response	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	KeyEvent
Aop:237 - Substance interaction with lung resident cell membrane components leading to atherosclerosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rats	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Human, mouse, rat

Cytokines are the common pro-inflammatory mediators secreted following inflammogenic stimuli. Cytokines can be defined as a diverse group of signaling protein molecules. They are secreted by different cell types in different tissues and in all mammalian species, irrespective of gender, age or sex. A lot of literature is available to support cross species, gender and developmental stage application for this KE. The challenge is the specificity; most cytokines exhibit redundant functions and many are pleotropic.

Key Event Description

Pro-inflammatory mediators are the chemical and biological molecules that initiate and regulate inflammatory reactions. Pro-inflammatory mediators are secreted following exposure to an inflammogen in a gender/sex or developmental stage independent manner. They are secreted during inflammation in all species. Different types of pro-inflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas and Hughes, 2004). Cell-derived pro-inflammatory mediators include cytokines, chemokines, and growth factors. Blood derived pro-inflammatory mediators include vasoactive amines, complement activation products and others. These modulators can be grouped based on the cell type that secrete them, their cellular localisation and also based on the type of immune response they trigger. For example, members of the interleukin (IL) family including IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, IL-3, IL-5 and Granulocyte-macrophage colony stimulating factor (GM-CSF) are involved in the adaptive immune responses. The pro-inflammatory cytokines include IL-1 family (IL-1α, IL-1β, IL-1rα, IL-18, IL-36α, IL-36β, IL-36γ, IL-36Rα, IL-37), IL-6 family, Tumor necrosis factor (TNF) family, IL-17, and Interferon gamma (IFN-γ) (Turner et al., 2014). While IL-4 and IL-5 are considered T helper (Th) cell type 2 response, IFN-γ is suggested to be Th1 type response.

Different types of pro-inflammatory mediators are secreted during innate or adaptive immune responses across various species (Mestas and Hughes, 2004). However, IL-1 family cytokines, IL-4, IL-5, IL-6, TNF-α, IFN-γ are the commonly measured mediators in experimental animals and in humans. Similar gene expression patterns involving inflammation and matrix remodelling are observed in human patients of pulmonary fibrosis and mouse lungs exposed to bleomycin (Kaminski, 2002).

Literature evidence for its perturbation:

Several studies show increased proinflammatory mediators in rodent lungs and bronchoalveolar lavage fluid, and in cell culture supernatants following exposure to a variety of carbon nanotube (CNT) types and other materials. Poland et al., 2008 showed that long and thin CNTs (>5 µm) can elicit asbestos-like pathogenicity through the continual release of pro-inflammatory cytokines and reactive oxygen species. Exposure to crystalline silica induces release of inflammatory cytokines (TNF-α, IL-1, IL-6), transcription factors (Nuclear factor kappa B [NFκB], Activator protein-1 [AP-1]) and kinase signalling pathways in mice that contain NF-κB luciferase reporter (Hubbard et al., 2002). Boyles et al., 2015 found that lung responses to long multi-walled carbon nanotubes (MWCNTs) included high expression levels of pro-inflammatory mediators Monocyte chemoattractant protein 1 (MCP-1), Transforming growth factor beta 1 (TGF-β1), and TNF-α (Boyles et al., 2015). Bleomycin administration in rodents induces lung inflammation and increased expression of pro-inflammatory mediators (Park et al., 2019). Inflammation induced by bleomycin, paraquat and CNTs is characterised by the altered expression of pro-inflammatory mediators. A large number of nanomaterials induce expression of cytokines and chemokines in lungs of rodents exposed via inhalation (Halappanavar et al., 2011; Husain et al., 2015a). Similarities are observed in gene programs involving pro-inflammatory event is observed in both humans and experimental mice (Zuo et al., 2002).

How it is Measured or Detected

The selection of pro-inflammatory mediators for investigation varies based on the expertise of the lab, cell types studied and the availability of the specific antibodies.

Real-time reverse transcription-polymerase chain reaction (qRT-PCR) – will measure the abundance of cytokine mRNA in a given sample. The method involves three steps: conversion of RNA into cDNA by reverse transcription method, amplification of cDNA using the PCR, and the real-time detection and quantification of amplified products (amplicons) (Nolan et al., 2006). Amplicons are detected using fluorescence, increase in which is directly proportional to the amplified PCR product. The number of cycles required per sample to reach a certain threshold of fluorescence (set by the user – usually set in the linear phase of the amplification, and the observed difference in samples to cross the set threshold reflects the initial amount available for amplification) is used to quantify the relative amount in the samples. The amplified products are detected by the DNA intercalating minor groove-binding fluorophore SYBR green, which produces a signal when incorporated into double-stranded amplicons. Since the cDNA is single stranded, the dye does not bind enhancing the specificity of the results. There are other methods such as nested fluorescent probes for detection, but SYBR green is widely used. RT-PCR primers specific to several pro-inflammatory mediators in several species including mouse, rat and humans, are readily available commercially.

Enzyme-linked immunosorbent assays (ELISA) – permit quantitative measurement of antigens in biological samples. The method is the same as described for the MIE. Both ELISA and qRT-PCR assays are used *in vivo* and are readily applicable to *in vitro* cell culture models, where cell culture supernatants or whole cell homogenates are used for ELISA or mRNA assays. Both assays are straight forward, quantitative and require relatively a small amount of input sample.

Apart from assaying single protein or gene at a time, cytokine bead arrays or cytokine PCR arrays can also be used to detect a whole panel of inflammatory mediators in a multiplex method (Husain et al., 2015b). This method is quantitative and especially advantageous when the sample amount available for testing is scarce. Lastly, immunohistochemistry can also be used to detect specific immune cell types producing the pro-inflammatory mediators and its downstream effectors in any given tissue (Costa et al., 2017). Immunohistochemistry results can be used as weight of evidence; however, the technique is not quantitative and depending on the specific antibodies used, the assay sensitivity may also become an issue (Amsen and De Visser, 2009).

Cell models - of varying complexity have been used to assess the expression of pro-inflammatory mediators. Two dimensional submerged monocultures of the main fibrotic effector cells – lung epithelial cells, macrophages, and fibroblasts – have routinely been used *in vitro* due to the large literature base, and ease of use, but do not adequately mimic the *in vivo* condition (Sharma *et al.,* 2016; Sundarakrishnan *et al.,* 2018). Recently, the EpiAlveolar *in vitro* lung model (containing epithelial cells, endothelial cells, and fibroblasts) was used to predict the fibrotic potential of MWCNTs, and researchers noted increases in the pro-inflammatory molecules TNF-α, IL-1β, and the pro-fibrotic TGFβ using ELISA (Barasova *et al.,* 2020). A similar, but less complicated co-culture model of immortalized human alveolar epithelial cells and idiopathic pulmonary fibrosis patient derived fibroblasts was used to assess pro-fibrotic signalling, and noted enhanced secretion of Platelet derived growth factor (PDGF) and Basic fibroblast growth factor (bFGF), as well as evidence for epithelial to mesenchymal transition of epithelial cells in this system (Prasad et al., 2014). Models such as these better capitulate the *in vivo* pulmonary alveolar capillary, but have lower reproducibility as compared to traditional submerged mono-culture experiments.

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Event: 1750: Increased inflammatory immune responses

Short Name: Increased inflammatory immune responses

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Event: 1748: Increase, the risk of acute respiratory failure

Short Name: Increase, the risk of acute respiratory failure

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent

Biological Context

Level of Biological Organization

Level of Biological Organization

Organ

List of Adverse Outcomes in this AOP

Event: 351: Increased Mortality

Short Name: Increased Mortality

Key Event Component

Process	Object	Action
mortality		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:96 - Axonal sodium channel modulation leading to acute mortality	AdverseOutcome
Aop:104 - Altered ion channel activity leading impaired heart function	AdverseOutcome
Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality	AdverseOutcome
Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality	AdverseOutcome
Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality	AdverseOutcome
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:186 - unknown MIE leading to renal failure and mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	AdverseOutcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	AdverseOutcome
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	AdverseOutcome
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	AdverseOutcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	AdverseOutcome
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	AdverseOutcome
Aop:410 - GSK3beta inactivation leading to increased mortality via defects in developing inner ear	AdverseOutcome
Aop:450 - Inhibition of AChE and activation of CYP2E1 leading to sensory axonal peripheral neuropathy and mortality	AdverseOutcome
Aop:536 - Estrogen receptor agonism leading to reduced survival and population growth due to renal failure	KeyEvent

Biological Context

Level of Biological Organization

Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

All living things are susceptible to mortality.

Key Event Description

Increased mortality refers to an increase in the number of individuals dying in an experimental replicate group or in a population over a specific period of time.

How it is Measured or Detected

Mortality of animals is generally observed as cessation of the heart beat, breathing (gill or lung movement) and locomotory movements. Mortality is typically measured by observation. Depending on the size of the organism, instruments such as microscopes may be used. The reported metric is mostly the mortality rate: the number of deaths in a given area or period, or from a particular cause.

Depending on the species and the study setup, mortality can be measured:

- in the lab by recording mortality during exposure experiments
- in dedicated setups simulating a realistic situation such as mesocosms or drainable ponds for aquatic species
- in the field, for example by determining age structure after one capture, or by capture-mark-recapture efforts. The latter is a method commonly used in ecology to estimate an animal population's size where it is impractical to count every individual.

Regulatory Significance of the AO

Increased mortality is one of the most common regulatory assessment endpoints, along with reduced growth and reduced reproduction.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2056: Binding to ACE2 leads to SARS-CoV-2 cell entry

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	High
SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	adjacent		
SARS-CoV-2 infection leading to hyperinflammation	adjacent		
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent	High	High
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Moderate
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	High	Moderate
Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

This KER deals with the evidence supporting the individual weight that the surface protein of SARS-CoV-2 spike needs to bind:ACE2, and of being cleaved in two different sites, for viral entry to occur. Viral entry is essential for initiating a cascade of events leading to COVID19.

Evidence Supporting this KER

Binding of SARS-CoV-2 S protein to ACE2 receptors present in the brain (endothelial, neuronal and glial cells) :

The highest ACE2 expression level in the brain was found in the pons and medulla oblongata in the human brainstem, containing the medullary respiratory centers, and this may in part explain the susceptibility of many COVID-19 patients to severe respiratory distress (Lukiw et al., 2020). High ACE2 receptor expression was also found in the amygdala, cerebral cortex and in the regions involved in cardiovascular function and central regulation of blood pressure including the sub-fornical organ, nucleus of the tractus solitarius, paraventricular nucleus, and rostral ventrolateral medulla (Gowrisankar and Clark 2016; Xia and Lazartigues 2010). The neurons and glial cells, like astrocytes and microglia also express ACE-2, thus highlighting the vulnerability of the nervous system to SARS-CoV-2 infection. Additionally, they also express transmembrane serine protease 2 (TMPRSS2) and furin, which facilitate virus entry into the host (Jakhmola et al.

2020).

Once inside the brain, the virus can infect the neural cells, astrocytes, and microglia. These cells express ACE-2, thus initiating the viral budding cycle followed by neuronal damage and inflammation (Jakhmola et al. 2020). Specifically in the brain, ACE2 is expressed in endothelium and vascular smooth muscle cells (Hamming et al., 2004), as well as in neurons and glia (Gallagher et al., 2006; Matsushita et al., 2010; Gowrisankar and Clark, 2016; Xu et al., 2017; de Morais et al., 2018) (from Murta et al., 2020).

Astrocytes are the main source of angiotensinogen and express ATR1 and MasR; neurons express ATR1, ACE2, and MasR, and microglia respond to ATR1 activation (Shi et al., 2014; de Morais et al., 2018).

Binding of S protein to ACE2 receptors present in the intestines

Biological Plausibility

Upon binding of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) to angiotensin-converting enzyme 2 (ACE2) on the surface of the host cells, SARS-CoV-2 enters inside the cells with an internalization mechanism.

Empirical Evidence

Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is initiated by virus binding to the ACE2 cell-surface receptor (Nature 579, 270–273, 2020 ; J. Virol. 94, e00127-20; Nature 588, 327–330). The SARS-CoV-2 surface spike (S) protein mediates the binding to the receptor and requires 2 cleavage steps for viral entry to occur, as follows. The spike protein contains 1273 aminoacids divided into two subunits, S1 and S2. The subunits are cleaved by furin-like enzymes, as spike of sars-cov-2 contains an insertion ^{680}SPRRAR↓SV^{687} forming a cleavage motif RxxR for furin-like enzymes at the boundary of S1/S2 subunits. In addition, there is a second cleavage site ^{808}PSKPS**KR**|SFIEDL^{822} just before the fusion peptide that needs to occur for viral entry. The S1 subunit contains a receptor-binding domain (RBD) encompassing the receptor-binding motif (RBM) that binds ACE2. The S2 contains a fusion peptide (FP), that penetrates into cell membranes and mediates fusion between the viral and host membranes to release viral proteins and genome.

Uncertainties and Inconsistencies

When TMPRSS2 is not available, spike it is hypothethised that the virus may use alternative proteases to get in the cells either by fusion with the plasma membrane or entry via endosomes and fusion with endocytic membranes at low pH, when proteases for priming become active, but evidence is less robust.

Quantitative Understanding of the Linkage

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Chemicals (weak evidence)	PFAS (PFOS, PFOA, PFNA, PFHxS, and GenX)	Short-term (10 days), high dose (20 mg/kg/day) exposure to PFOA leads to about 1.6 fold upregulation of the pulmonary mRNA level of *Ace2* and to about 1.5 upregulation of the pulmonary mRNA level of *Tmprss2* in CD1 mice. [1] Long-term (12 weeks) of an environmentally relevant PFAS mixture (PFOS, PFOA, PFNA, PFHxS, and GenX; each in 2 mg/l concentration) exposure leads to downregulation of pulmonary mRNA expression of Ace2 2.5-fold in C57BL/6 J male mice. A similar decreasing trend was observed in PFAS-exposed male mice for *Tmprss2*. [2]	1. doi: 10.1016/j.toxrep.2021.11.014 2. doi: 10.1016/j.taap.2022.116284
Sex (strong evidence)	female sex (XX chromosomes)	ACE2 localizes to the X sex chromosome and displays a sex-dependent expression profile with higher expression in female than in male tissues [1,2]. Estradiol inhibits TMPRSS2, needed to facilitate SARS-CoV-2 entry into the cell [3]. Estrogen therapy has been shown to mitigate endoplasmic reticulum stress induced by SARS-CoV-2 invasion through activation of cellular unfold protein response and regulation of inositol triphosphate (IP3) and phospholipase C [4]. Different studies have also illustrated that estradiol increases the expression of ADAM17, leading to high-circulating soluble ACE2 potentially neutralizing SARS-CoV-2 and preventing its binding to mACE2. [5] Thus, Estradiol might reduce SARS-CoV-2 infectivity through modulation of cellular ACE2/TMPRSS2/ADAM17 axis expression.	1. doi: 10.1177/1933719115597760 2. doi: 10.1016/j.mce.2015.11.004 3. doi: 10.1007/s11033-021-06390-1 4.doi: 10.1016/j.mehy.2020.110148 5. doi: 10.2217/pgs-2020-0092
	Male sex (XY chromosomes)	Androgen receptors (ARs) play a key role in increasing transcription of TMPRSS2. This may explain the predominance of males to COVID-19 fatality and severity. [6]	6. doi: 10.1073/pnas.2021450118
Age	Young/old people	ACE2 protein expression is increased with aging in several tissues [1], including lungs and particularly in patients requiring mechanical ventilation [2]. During aging, telomere dysfunction activates a DNA damage response leading to higher ACE2 expression. Thus, telomere shortening could contribute to make elderly more susceptible to SARS-CoV-2 infection [3].	1. doi: 10.1016/j.exger.2021.111507 2. doi: 10.1371/journal.pone.0247060 3. doi: 10.15252/embr.202153658

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Lipids	Atherogenic dyslipidemia	Lipids, as important structural components of cellular and sub-cellular membranes, are crucial in the infection process [1]. Changes in intracellular cholesterol alter cell membrane composition, impacting structures such as lipid rafts, which accommodate many cell-surface receptors [2], including ACE2 and TMPRSS2 [3, 4]. **In COVID-19.** In an *in vitro* study, the depletion of membrane-bound cholesterol in ACE2-expressing cells led to a reduced infectivity of SARS-CoV [3]. In vitro, higher cellular cholesterol increased uptake of SARS-CoV-2 S protein; this effect was decreased with Methyl-beta-cyclodextrin, a compound which extracts cholesterol from cell membranes [5]. HDL scavenger receptor B type 1 (SR-B1), a receptor found in pulmonary and many other cells, could facilitate ACE2-dependent entry of SARS-CoV-2 [6].	1. doi: 10.1001/jama.2020.12839 2. doi: 10.3389/fcell.2020.618296 3. doi: 10.1016/j.bbrc.2008.02.023 4. doi: 10.1096/fj.202000654R 5. doi: 10.1101/2020.05.09.086249 6.doi: 10.1038/s42255-020-00324-0 7.doi: 10.1016/j.bbalip.2020.158849 8.doi: 10.1016/j.obmed.2020.100283 9. doi: 10.3390/ijms21103544 10.doi: 10.1101/2020.04.16.20068528
	Obesity	**In COVID-19.** ACE2 is highly expressed in adipose tissue, thus excess adiposity may drive more infection (8). Obese patients have more adipose tissue and therefore more ACE2-expressing cells [9]. SARS-CoV-2 dysregulates lipid metabolism in the host and the effect of such dysregulated lipogenesis on the regulation of ACE2, specifically in obesity [10]. Lung epithelial cells infected with SARS-CoV-2 showed upregulation of genes associated with lipid metabolism [11], including the SOC3 gene. A mouse model of diet-induced obesity showed higher Ace2 expression in the lungs, which negatively correlated with the expression sterol response element binding proteins 1 and 2 (SREBP) genes. Suppression of Srebp1 showed a significant increase in Ace2 expression in the lung. Lipids, including fatty acids, could interact directly with SARS-CoV-2 influencing spike configuration and modifying the affinity for ACE2 and thus its infectivity [12]. The dysregulated lipogenesis and the subsequently high ACE2 expression in obese patients might be one mechanism underlying the increased risk for severe complications [10].	
Vitamin D (moderate evidence)	Vitamin D deficiency	Vitamin D administration enhanced mRNA expression of VDR and ACE2 in a rat model of acute lung injury [1]. In particular, vitamin D upregulates the soluble ACE2 form [2]. Thus, low vitamin D status may impair the trapping protective mechanism of soluble ACE2 [3]. Furthermore, vitamin D deficiency has been shown to reduce the expression of antimicrobial peptides (-defensin, cathelicidin), which act against enveloped viruses [4,5]. **In COVID-19.** Decreased sACE2 and cellular viral defense might be some mechanisms explaining how low vitamin D modulate SARS-CoV-2 infectibility.	1. doi: 10.1016/j.injury.2016.09.025 2. doi: 10.1152/ajplung.00071.2009 3. doi: 10.3390/ijms22105251 4. doi: 10.1007/s11154-021-09679-5 5. doi: 10.1080/14787210.2021.1941871
Gut microbiota	Gut dysbiosis (alteration of gut microbiota)	Some evidence shows that gut microbiota influences Ace2 expression in the gut. Colonic Ace2 expression decreased significantly upon microbial colonization in mice and rats [1,2]. *Coprobacillus* enrichment was associated with severe COVID-19 in patients [3] and was shown to upregulate colonic ACE2 in mice [4]. The abundance of *Bacteroides* species was associated with reduced ACE2 expression in the murine gut [4] and negatively correlated with fecal SARS-CoV-2 load [3,5]. Thus, gut dysbiosis might lead to higher levels of ACE2 in the gut, potentially increasing the ability of SARS-CoV-2 to enter enterocytes.	1. doi: 10.1080/19490976.2021.1984105 2. doi: 10.1161/HYPERTENSIONAHA.120.15360 3.doi: 10.1053/j.gastro.2020.05.048 4.doi: 10.1016/j.cell.2017.01.022 5. doi: 10.1016/j.tifs.2020.12.009

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Genetic factors		Polymorphisms inducing amino acid residue changes of ACE2 in the binding interface would influence affinity for the viral S protein. Evidence exists that K353 and K31 in hACE2, the main hotspots that form hydrogen bonds with the main chain of N501 and Q493 in receptor-binding motif respectively, play a role in tightly binding to the S protein of SARS-CoV-2 [1]. Around the twenty natural ACE2 variants, three alleles of 17 variants were found to affect the attachment stability [2]. Thus, the ACE2 variants modulating the interaction between the virus and the host have been reported to be rare, consistently with the overall low appearance of ACE2 polymorphisms. In this context, it is key to approach both the ACE2 genotypes and the clinical descriptions of the phenotypes in a population-wide manner, in order to better understand how ACE2 variations are relevant in the susceptibility for SARS-CoV-2 infection [3]. In addition, since ACE2 is X-linked, the rare variants that enhance SARS-CoV-2 binding are expected to increase susceptibility to COVID-19 in males [4]. On the other hand, the X-chromosome inactivation of the female causes a “mosaic pattern”, which might be an advantage for females in terms of reduced viral binding [5]. TMPRSS2 single-nucleotide polymorphisms (SNPs) were associated with a frequent “European haplotype” [6], which not observed in Asians, is suggested to upregulate TMPRSS2 gene expression in an androgen-specific way. Thus, there is a need for in vitro validation studies to assess the involvements of population-specific SNPs of both ACE2 and TMPRSS2 in susceptibility toward SARS-CoV-2 infection. The occurrence of a pandemic is related to the genetics of the infecting agent. In the case of SARS-CoV-2, through genomic surveillance it is possible to track the spread of SARS-CoV-2 lineages and variants, and to monitor changes to its genetic code that can influence viral entry and production. Consequently, genomic surveillance is crucial to understand how mutations occurring on SARS-CoV-2 genome influence and drive the pandemic [7]. For example, a recent study [8] highlights that through genomic surveillance it is possible to trace co-infections by distinct SARS-CoV-2 genotypes, which are expected to have a different impact on factors modulating COVID-19. Genomic surveillance of SARS-CoV-2 is able to reveal tremendous genomic diversity [9], and coupled with language models and machine learning approaches, contributes to predicting the impact of mutations (such as those occurring in the spike protein), and thus can better address challenging aspects, like an estimation of the efficacy of therapeutic treatments [10].	[1] doi: 10.1080/07391102.2020.1796809 [2] doi: 10.1002/jmv.26126 [3] doi: 10.1038/s42003-021-02030-3 [4] doi: 10.1101/2020.04.05.026633 [5] doi: 10.3390/ijms21103474 [6] doi: 10.18632/aging.103415 [7] doi: 10.1038/s41588-022-01033-y [8] doi: 10.1038/s41598-022-13113-4 [9] doi:10.1371/journal.pone.0262573 [10] doi: 10.3389/fgene.2022.858252
Therapeutic intervention against COVID-19	Casirivimab, Imdevimab and Sotrivimab	Are monoclonal antibodies designed to recognize and attach to two different sites of the Receptor-Binding Domain (RBD) of the S protein of SARS-CoV-2, blocking the virus to enter cells [1,2,3].	1) 10.1056/NEJMoa2035002 2) EMA Starts Rolling Review of REGN-COV2 Antibody Combination (Casirivimab / Imdevimab). EMA 2021. Available online: https://www.ema.europa.eu/en/news/ema-starts-rolling-review-regn-cov2-antibody-combination-casirivimab-imdevimab (accessed on 12 May 2022) 3) EMA Starts Rolling Review of Sotrovimab (VIR-7831) for COVID-19. EMA 2021. Available online: https://www.ema.europa.eu/en/news/ema-starts-rolling-review-sotrovimab-vir-7831-covid-19 (accessed on 12 May 2022)
	Heparin	Interacts directly with viral particles and has been shown to bind to the SARS-CoV-2 S1 Spike RBD, causing significant protein architecture alteration, impacting infectivity [1,2].	1) 10.3389/fmed.2021.615333 2) 10.1055/s-0040-1721319

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Air pollution		Air pollution induces Increased expression of ACE2 which may result in increased viral entry and coronavirus production. Increased ACE2 expression has been reported in the respiratory system in response to air pollution exposure (1-4). Increased expression may affect susceptibility to SARS-CoV-2 infection. Similarly, some constituents of air pollution (PM, ozone) have been reported to increase the expression of TMPRSS2 (3, 5-6).	1) https://doi.org/10.1186/s12989-015-0094-4 2) 10.1016/j.burns.2015.04.010 3) 10.1016/j.envres.2021.110722 4) 10.3390/ijerph17155573 5) 10.1186/s12989-021-00404-3 6) https://doi.org/10.1038/s41598-022-04906-8
Pre-existing heart failure		ACE2 mRNA and protein levels, as well as enzymatic activity, were shown to be upregulated in explanted hearts from patients with end-stage HF, as well as in the HF rat model [1-3]. Myocytes, fibroblasts, vascular smooth muscle cells, pericytes [4] and endothelial cells of the coronaries [5] express ACE2, while myocytes in patients suffering from heart disease exhibit higher ACE2 expression [6]. Pericytes - the mural cells lining microvasculature, interacting with endothelial cells notably to maintain microvascular stability - exhibited the strongest ACE2 expression in HF patients [7], rendering these cells involved in the coronary vasculature of the myocardium, more susceptible to infection. Furthermore, SARS-CoV-2 infects and replicates in pericytes, and a decrease in their numbers follows [8]. Patients with pre-existing HF showed increased ACE2 levels in myocytes and pericytes, having thereby higher risk of heart injury [7, 9]. In addition, sACE2 levels are higher in HF patients [10, 11] and sACE2 activity is increased in HF [12]. In contrast to a protective role of sACE2, it has been proposed that viral binding to circulating sACE2 forms SARS-CoV-2/sACE2 complexes, which might mediate infection of cells in distal tissues [13]; hence, pre-existing HF might disseminate SARS-CoV-2 infection. Interestingly, the increase in sACE2 activity is associated with HF with reduced ejection fraction (HFrEF) but not with HF with preserved ejection fraction (HFpEF), suggesting (i) a rather complex role of HF in regulating ACE2-mediated infection by SARS-CoV-2 [10] and (ii) the potential of sACE2 activity to be used as a biomarker to distinguish between the two HF types. Lastly, it is noteworthy that Khoury et al. provided evidence in a different direction, by showing that ADAM17 and TMPRSS2 [14] expression levels are downregulated in a HF rat model, thus potentially conferring a protective role against infection by SARS-CoV-2 in HF [3].	1: https://doi.org/10.1186/1741-7015-2-19 2: https://doi.org/10.1161/01.CIR.0000094734.67990.99 3: https://onlinelibrary.wiley.com/doi/10.1111/jcmm.16310#:~:text=https%3A//doi.org/10.1111/jcmm.16310 4: https://doi.org/10.1161/CIRCULATIONAHA.120.047911 5: https://doi.org/10.1152/ajpheart.00331.2008 6: https://doi.org/10.1093/eurheartj/ehaa311 7: https://doi.org/10.1093/cvr/cvaa078 8: https://doi.org/10.21203/rs.3.rs-105963/v1 9: https://doi.org/10.1016/j.jacbts.2020.06.007 10: https://doi.org/10.1177/1470320316668435 11: https://doi.org/10.1093/eurheartj/ehaa697 12: https://doi.org/10.1002/jmv.27144 13: https://doi.org/10.1002/rmv.2213 14: https://doi.org/10.1016/j.cell.2020.02.052

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Diet	Chemicals in foods affect ACE3 expression	• Geranium and lemon oils were found to reduce in vitro ACE2 activity and expression, as well as ACE2 and TMPRSS2 mRNA levels [207]. • Several molecular modelling and docking studies indicate the potential for compounds found in garlic [208], turmeric (curcumin) [209], thyme and oregano (carvacrol) [210], green tea [211] and other plant foods (quercetin) [212] to inhibit binding of SARS-CoV-2. • Pelargonidin, found in red and black berries, was shown to dose-dependently block SARS-CoV-2 binding to ACE2, reduce SARS-CoV-2 replication in vitro and reduce ACE2 expression [213]. • Quercetin and related compounds inhibit recombinant human ACE2 activity [214] at physiologically relevant concentrations in vitro. • In a human crossover study, 30-day supplementation with resveratrol decreased ACE2 in adipose tissue [216], potentially attenuating an increased risk for infection and viral replication in humans with obesity. In vitro, resveratrol inhibited the replication of SARS-CoV-2 [217].	• 207: http://doi.org/10.3390/plants9060770 • 208: http://doi.org/10.1021/acsomega.0c00772 • 209: http://doi.org/10.1007/s13337-020-00598-8 • 210: http://doi.org/10.1080/07391102.2020.1772112 • 211: http://doi.org/10.1080/07391102.2020.1779818 • 212: http://doi.org/10.18632/aging.103001 • 213: http://doi.org/10.1016/j.bcp.2021.114564 • 214: http://doi.org/10.1021/acs.jafc.0c05064 • 216: http://doi.org/10.1080/21623945.2021.1965315 • 217: http://doi.org/10.1002/ptr.6916

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Relationship: 2351: SARS-CoV-2 production leads to TLR Activation/Dysregulation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	Moderate	Not Specified

Relationship: 2303: TLR Activation/Dysregulation leads to Increased proinflammatory mediators

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	Not Specified

Key Event Relationship Description

The engagement of TLR with Pathogen-Associated Molecular Patterns (PAMPs) and host derived damage-associated molecular patterns (DAMPs) induces conformational changes of TLRs that allow recruitment of adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM to control intracellular signaling pathways, including ERK, p38 and NF-B, driving the synthesis and secretion of cytokines and chemokines [Kawai, T.; Akira, S. Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. 2007, 13, 460–469] [DOI:https://doi.org/10.1016/j.molmed.2007.09.002]. In a healthy state the amount and type of proinflammatory mediators are appropriate to required circumstances (e.g. defence against invading pathogens) and resolution of inflammation is promoted to reestablish homeostasis. Dysregulated TLR Activation can result from e.g. overabundance of PAMPS/DAMPS or over-, or under-expression of TLR protein intra- and/or extracellularly or over- or under-expression of downstream proteins. These circumstances can be modulated by a number of factors including biological/intrinsic factors (e.g. age, sex, genetic factors), pre-existing co-morbidities, lifestyle factors, environmental factors and therapeutic interventions. In context of an adverse outcome the resulting dysregulated over- or under-activation of TLRs contributes to dysproportional amounts of proinflammatory mediators (overproduction or underproduction) halting back defence and homeostasis.

Quantitative Understanding of the Linkage

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Chemicals (weak evidence)	PFAS (PFOA)	PFOA exposure reduces TLR2 and Myd88 expression in zebrafish and induces a dose-dependent increase in IFN and B-cell-activating factor (BAFF) mRNA levels [1]. The TLR/MyD88/NF-kB pathway could be a mechanism through which PFOA interferes with BAFF and IFN expression [2]. Increasing TLR2 expression in zebrafish exposed to PFOA showed a linear correlation with increased levels of MyD88, IL-1β, and IL-21 mRNA levels [3].	1) doi: 10.1016/j.chemosphere.2020.126200 2) doi: 10.1186/2045-7022-3-S3-O5 3) doi: 10.1016/j.jhazmat.2014.08.043
SEX	male sex (XY chromosome)	Male and female subjects both express functionally active TLRs, but sex differences have been reported. TLR3, TLR4 and TLR7 are coded by the X chromosome. Thus, certain TLR gain and/or loss of function polymorphisms have higher clinical prevalence in men. Particularly, TLR7 localizes to an area of the X chromosome known to escape X-chromosome inactivation [1]. TLR7 is more highly expressed at the protein level by female immune cells than by male ones [1]. A study showed that specific TLR7 loss of function variants lead to poor outcome of SARS-CoV-2, which could be explained via the role of TLR7 in responding to SARS-CoV-2 mRNA recognition by inducing the production of the antiviral cytokine interferon-a (IFN-a) [2]. An X-linked recessive TLR7 deficiency was found present in approximately 1% of men under 60 years old with life-threatening COVID-19) [2]. Plasmacytoid dendritic cells of men with these TLR7 variants produce less IFN-a ex vivo, which could explain their poor defence against SARS-CoV-2 [2]. Sex-specific associations between TLR polymorphisms and poor lung function have been reported [3]. Examples include gain of function polymorphisms of rs187084 in the TLR9 gene displaying significantly lower lung function in male swine operators than those with wild type. Additionally, the gain of function polymorphisms TLR9-1237T/C, rs5743836 is a risk for severe sepsis in pediatric critical care patients, with males having a higher risk and a more pronounced allele frequency of TLR9-1237T/C than females [4]. The sex hormone, testosterone, can reduce TLR4 expression and sensitivity, which is proposed to explain in part the less optimal defence during infection in male compared to female [5].	1) doi: 10.1126/sciimmunol.aap8855 2) doi: 10.1126/sciimmunol.abl4348 3) doi: 10.1080/15287394.2018.1544523 4) Elsherif et al., Int J Clin Exp Med 2019;12(4):4381-4386 5) doi.org/10.1095/biolreprod.107.063545
Age	Young/old people	In older people, there are studies indicating both an increase and decrease in TLR expression and signalling [**1**,**2**,**3**]. Renshaw et al. [**4**] showed a decline in TLR expression and function in aged mice, explanatory for increased susceptibility to infections and poor adaptive immune responses in aging. On the other hand, Olivieri et al. [**5**] reported that the effect of age on signalling events downstream of TLRs is greater than the effect of age on TLR levels. They suggested that inflammaging can be triggered by an impairment of miRNAs/TLR signalling interaction (in endothelial and immune system cells), leading to activation of immune cells over time. Inflammaging is a higher basal inflammatory state in older subjects, which is a major driving force of frailty and common severe age-related diseases. Other complex age-dependent TLRs signalling mechanism include the decreased ability of aged macrophages to fight pathogens, the accumulation of senescent cells in aged subjects, and the increased release of endogenous TLRs ligands from senescence cells.	1) 10.1007/s40520-018-1064-0 2) 10.4049/jimmunol.0901022 3) 10.1016/j.mad.2005.07.009 4) 10.4049/jimmunol.169.9.4697 5) 10.1186/1742-4933-10-11
Lipids	Obesity	Obesity influences TLR9 expression, which is higher in visceral compared to subcutaneous adipose tissue depots in mice and obese patients [1]. Obesity induced cell-free DNA fragments released from adipocytes stimulate chronic adipose tissue inflammation and insulin resistance via TLR9 activation [2]	1. doi: 10.1530/JOE-18-0326 2. doi: 10.1126/sciadv.1501332
Vitamin D (moderate evidence)	Vitamin D deficiency	Vitamin D status modulates cytokine production, at least partly through the differential modulation of TLRs. Vitamin D3 down-regulates TLR9 in human monocytes but not TLR3, which resulted in less secretion of IL-6 in response to TLR9 challenge [1]. High-dose oral supplementation of vitamin D3 (4000 IU/day) in human decreased TLR9 protein levels and mRNA expression of TLR3, TLR7, and TLR9 [2].	[1] doi:10.1093/rheumatology/keq124 [2] doi: 10.1007/s11010-019-03658-w
Genetic factors		It is well-documented that the TLR expression is determined by genetic variation within the TLR genes [1]. In addition to the already mentioned sex differences in certain TLR polymorphisms, TLR genes also exhibit a distinct population distribution pattern and are the target of selection pressure. Ethnicity disparity in COVID-19 mortality rates were suggested to be explained in part by elevated gene expression of TLR7 and TLR9. In addition, allelic variation in the TLR adaptor protein, Ticam2, influences susceptibility to SARS-CoV infection in mice as Ticam2$^{-/-}$ mice had high susceptibility to SARS-CoV-2 infection [2].	[1] doi: 10.1096/fj.202001115R [2] doi: 10.1016/j.clim.2020.108481

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Pre-existing heart failure		TLRs are expressed in the myocardium, with TLR4 being the most abundantly expressed, and TLR2 and TLR3 being present to a lesser extent [1]. TLR4 is upregulated in failing hearts [2-4]. The higher expression of TLR4 in HF patientscould predispose them towards pro-inflammatory responses. Evidence shows that the S proteins of SARS-CoV-2 can bind to TLR4 directly or activate it via DAMP- and PAMP-mediated pathways, and thus, induce pro-inflammatory mediators, such as IL-1, IL-6 and TNF-α [5]. The mediator of this TLR-induced activation appears to be NF-κB [6], an essential transcription factor involved in various cardiovascular pathologies [7]. In addition, following infection by SARS-CoV-2 of adult rat cardiac tissue resident macrophage-derived fibrocytes, TLR4 was further activated with a dual effect: it caused the upregulation of ACE2 and induced a pro-inflammatory M1 polarization of macrophages [8], which can further enhance the pro-inflammatory factors secretion. However, the involvement of pre-existing HF in the modulation of COVID-19 via TLRs is still not fully elucidated but deserves further investigation.	1: https://doi.org/10.1248/bpb.28.886 2: https://e-century.us/files/ijcem/12/4/ijcem0080845.pdf 3: https://doi.org/10.1172/JCI6709 4: 10.1016/j.ijcard.2006.12.024 5: https://doi.org/10.1155/2021/8874339 6: https://doi.org/10.1155/2018/9874109 7: https://doi.org/10.3390/ijms20071599 8: doi.org/10.1101/2021.08.11.455921
Diet	High-fat diets impact TLR-mediated inflammation	• In mice, expression of TLR2 and TLR4 in circulating macrophages is upregulated by circulating free fatty acids, which are increased with consumption of high fat diets [339,340]. Circulating free fatty acids also activate the NF-kB signaling directly or by activating cellular surface TLR in the hypothalamus, in mice and primary human myotube and adipose cells, leading to increased expression of some pro-inflammatory mediators [341,342]. • A high-fat diet was also shown to modulate inflammation via TLR9, as mice lacking TLR9 or receiving a TLR7/9 antagonist had reduced upregulation of specific pro-inflammatory cytokines compared to controls upon high fat diet [343].	• 339: http://doi.org/10.1371/journal.pone.0012191 • 340: http://doi.org/10.3389/fimmu.2018.02649 • 341: http://doi.org/10.1155/2010/823486 • 342: http://doi.org/10.1111/j.1749-6632.2011.06388.x • 343: http://doi.org/10.1172/JCI83885

Relationship: 2061: Increased proinflammatory mediators leads to Increased inflammatory immune responses

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	Low

Relationship: 2062: Increased inflammatory immune responses leads to Increase, the risk of acute respiratory failure

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	Moderate	Low

Relationship: 2135: Increase, the risk of acute respiratory failure leads to Increased Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	Moderate	Not Specified

Relationship: 2496: SARS-CoV-2 cell entry leads to IFN-I response, antagonized

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Not Specified
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent	Low	
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Moderate
Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Sex and age applicability

It has been shown that in human populations males are more likely to suffer severe infections and deaths due to COVID-19 than females. However, in the viral entry and infection phase, one study found that women of working age had higher infection rates than men, but the suggested cause was higher contact rates among women (Doerre and Doblhammer, 2022). Contact rate increase is an important transmission factor but would not constitute a gender-based biological difference in viral entry or IFN-I pathway antagonism. A biological basis for females having higher levels of Type I IFN has been proposed concerning Toll-like receptor (TLR) 7. TLR7 is expressed in plasmacytoid dendritic cells (pDCs), an immune cell type that on infection with SARS-CoV-2 migrates from peripheral blood into the respiratory tract epithelium. TLR7 stimulates higher IFN-I production in pDCs in women than in men (Van der Sluis et al. 2022). It is proposed that this is due to the TLR7 gene being on the X chromosome, and that X inactivation in males is incomplete regarding the TLR7 gene, creating a double gene-dose effect in females (Spiering and de Vries, 2021). In a mouse SARS-CoV model, XY males had more adverse outcomes than XX females and XXY males (Gadi et al. 2020). Additionally, loss-of-function TLR7 mutations have been identified that are associated with increased COVID-19 severity (Szeto et al. 2021). However, these results focus on disease outcome as the endpoint, where factors beyond the initial antiviral response could be involved. Also note that the nasal and upper respiratory tract (URT) epithelial cells express ACE2 receptors for SARS-CoV-2 entry while the pDCs do not, relying on viral endocytosis (Van der Sluis et al. 2022). There is not a clear picture in the literature of the timing of pDC arrival in the epithelium after exposure, and the role of TLR7 in sex differences is currently hypothetical (Spiering and de Vries, 2021).

Taxonomic applicability

Generally, most mammals are likely susceptible to the SARS-CoV-2 virus based on reports of naturally and experimentally infected animals (See AO 1939). No infections have been reported in other classes of vertebrates. Other than bioinformatic studies on the ACE2 sequence across vertebrates however, there have been few studies on the mechanisms of susceptibility to infection of non-human hosts. Three studies were found on protein targets in the IFN-I innate immune response pathway that included other vertebrates. Rui et al. (2021) showed that SARS-CoV-2 3CLpro and ORF3a inhibit vertebrate (human, mouse, and chicken) STING ability to induce IFNβ promoter activity in a dose-dependent manner in HEK293T cells transfected with IFNβ-luciferase reporter plasmid vectors, together with tagged STING and cGAS vectors and increasing amounts of the SARS-CoV-2 3CLpro or ORF3a expression vectors. This study shows that the vulnerability of the host IFN-I pathway protein components to inhibition by SARS-CoV-2 protein stressors is not limited to humans, however Rui et al. (2021) did not determine the specific amino acids involved in the STING-ORF3a or STING-3CLpro interactions. Mostaquil et al. (2020) studied the cleavage site of IRF3 by PLpro (SARS-CoV-2 NSP3) and compared sequences across mammals. They determined that the IRF3 cleavage site in mammalian species in the taxonomic orders of primates, carnivora, artiodactyla, chiroptera (bats) and a few other mammals was conserved and would generally be susceptible to cleavage, and therefore IFN-I antagonism, but rodentia IRF3 would likely not be susceptible. Hameedi et al. (2022) compared molecular dynamic simulations of 3CLpro cleavage of NEMO in humans and mice showing a decrease in the average number of contacts between mNEMO and 3CLpro compared to hNEMO. Also, hNEMO may be more strongly bound to the catalytic site, and the mNEMO/3CLpro interaction appears more prone to destabilization (Hameedi et al., 2022).

Key Event Relationship Description

Upon entry of a virus into the host cell (KE1738), the virus is unpackaged from the structural nucleocapsid (N), envelope (E), and membrane (M) proteins. The viral RNA is detected by Pattern Recognition Receptor (PRR) proteins including RIG-I and MDA5 but the M proteins can interact with these PRRs directly, and block this initial host reaction (Fu et al., 2021). The viral genomic RNA can then be translated directly at the host ribosomes. The viral proteins are processed through cleavage by viral protease enzymes. This releases a repertoire of non-structural proteins (NSPs) and accessory open reading frame (ORF) proteins that has evolved, for example in the SARS-CoV-2 virus, to bind and block the proteins in the interferon I (IFN-I) antiviral cascade (KE1901). The normal function of the host's IFN-I response to other viruses is the expression of IFN-I which in turn stimulates the expression of many interferon-stimulated gene (ISG) proteins with antiviral functions. The SARS-CoV-2 antagonism of the IFN-I pathway delays or curtails the expression of IFN-I and ISG proteins.

Evidence Supporting this KER

Empirical evidence supporting this relationship is described below.

Biological Plausibility

This relationship is concerned with how entry of the virus into the host cell and subsequent release and transcription of viral proteins affects the downstream innate immune response. In particular, literature suggests the main pathway antagonized is the expression of type I interferons (IFN-I), consisting primarily of IFNα and IFNβ, and IFN-I stimulated genes (ISGs) (Banerjee et al., 2020; Blanco-Melo et al., 2020; Cheemarla et al., 2021; Xia et al., 2020; Sharif-Askari et al., 2022). Although there are few studies with evidence for cell entry leading directly to reduced IFN expression (Xia et al., 2020; Hatton et al. 2021), several studies demonstrate individual viral protein interactions with and blocking of host proteins in the IFN-I pathway or ISG proteins (Schubert et al. 2020; Thoms et al. 2020; Rui et al. 2021; Shin et al. 2020; Liu et al. 2021; Mostaqil et al., 2021; Xia et al. 2020; Quarleri and Delpino, 2021; Xia and Shi, 2020; Miorin et al. 2020; Kato et al. 2020; Fu et al. 2020; Chen et al. 2020; Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al 2020; see below and also key event 1901). These studies provide the biological rationale that SARS-CoV-2 entry into the host cell causes interactions between viral proteins and known protein components of the host IFN-I antiviral response, resulting in inhibition of IFN-I and ISG expression.

Empirical Evidence

Empirical evidence in support of temporal concordance comes from patient reports, showing that interferon expression is delayed by SARS-CoV-2 compared to other viruses like influenza, which is also described as an untuned or imbalanced response between interferons being initially low in moderate to severe cases (Banco-Melo et al. 2020; Galani et al., 2021; Hadjadj et al., 2020; Hatton et al., 2021; Rouchka et al., 2021). This indicates that SARS-CoV-2 stressors are suppressing the interferon response and highlights an important point regarding the difference between SARS-CoV-2 and other viruses in the stressors produced upon viral entry. Other viruses, as well as non-viral compounds used in research (e.g., polyinosinic:polycytidylic acid or poly[I:C]) enter the cell and stimulate the normal functional operation of the immune response, while SARS-CoV-2 blocks the response at multiple points, acting as a true prototypical stressor.

Hatton et al. (2021) used human nasal epithelium differentiated at the air-liquid interface (ALI) cultures (organoids) with several cell types. Secretory cells were the cell type with the highest expression of viral transcripts, with ciliated and deuterosomal cells also showing expression. The SARS-CoV-2-infected secretory and ciliated cells also had many downregulated ISGs. Compared to SARS-CoV-2, influenza A virus induced significantly higher levels of IFN-I (IFNβ) and IFN-III (IFNλ1) at 6 and 24 hours post infection, as well as ISGs Ubiquitin specific peptidase 18 (USP18), radical s-adenosyl methionine domain containing 2 (RSAD2), and ubiquitin-like protein ISG15 at 24 hours post infection (Hatton et al., 2021).

Individual stressors from the virus were investigated by Xia et al. (2020) using an IFN-β promoter luciferase assay. HEK293T cells were co-transfected with luciferase reporter plasmids, the specific viral protein expressing plasmid, and stimulator plasmid RIG-I (2CARD). Of the viral proteins tested (NSPs 1, 2, 4-16, S, N, E, M, and ORFs 3a, 3b, 6, 7a, 7b, 8, and 10), four proteins (NSPs 1, 6, and 13 and ORF6) significantly reduced INF-β induction compared to the control (empty vector). A similarly conducted ISRE-promoter luciferase assay showed significant inhibition of the IFN-I signaling pathway (normally resulting in induction of ISGs) by NSPs 1, 6, 7, 13 and 14, ORFs 3a, 6, 7a and 7b, and M protein (Xia et al., 2020). See Xia et al. (2020) and Xia and Shi (2020) for schematics depicting the actions of the SARS-CoV-2 proteins on the protein components of the IFN-I antiviral response pathway.

SARS-CoV-2 stressor proteins and the IFN-I pathway responses were investigated individually in the following studies:

Viral protein stressor	Host protein	Crystal Structure PDB	KER findings: Binding, Stressor/IFN-I or ISG expression
N (nucleocapsid)	**RIG-I**: Retinoic acid-inducible gene I	Not available (NA)	Significant reductions in IFNβ mRNA induction were seen when SARS-CoV-2 N protein was co-transfected into A549 cells with RIG-I, MAVS, or TBK1, and similar transfections resulted in IFNβ promoter activity reduction in poly(I:C)-stimulated HEK293T cells (Chen et al., 2020).
NSP3 Papain-like protease (Plpro)	**MDA5**: Melanoma differentiation-associated gene 5	NA	Sun et al. (2022) determined that SARS-CoV-2 and avian coronavirus infectious bronchitis virus (IBV) NSP3 PLpro N-terminal domain directly interacts with MDA5 to inhibit IFNβ expression when co-transfected in HEK293T cells.
M (membrane)	**MAVS**: Mitochondiral antiviral signaling protein	NA	Fu et al. (2020) found M interaction with MAVS (as determined by coimmunoprecipitation and in vitro pull-down assay) interferes with recruitment of downstream pathway proteins TRAF, TBK1, and IRF3, inhibiting IFNβ1 promoter, IFN-stimulated response element (ISRE), and NFκB promoter activity in a dose-dependent manner. The M protein inhibited the transcription of ISGs (ISG56, CXCL10, and TNF) based on mRNA levels, and inhibited IFNβ and TNFα secretion based on measures of these proteins in HEK293 cell culture.
NSP3 Papain-like protease (Plpro)	**ISG15**: Ubiquitin-like interferon stimulated gene 15	6YVA	Shin et al. (2020) generated a crystal structure and found that SARS-CoV-2 Plpro preferentially cleaves ISG15. ISG15 functions in antiviral immunity to directly inhibit viral replication (Perng and Lenschow, 2018).
ORF9b	**TOMM70**: Translocase of outer mitochondrial membrane	7KDT	Gordon et al. (2020) showed interaction between TOMM70 and ORF9b via affinity purification-mass spectrometry (AP-MS). TOMM70-ORF9b interaction is supported by several studies (Gao et al., 2021; Brandherm et al., 2021; Ayinde et al., 2022). Jiang et al. (2020) used a dual luciferase reporter assay to show human IFN-β promoter activity was significantly reduced in the presence SARS-CoV-2 Orf9b compared to controls.
ORF6	**Nup98-RAE1**: Nuclear pore complex 98-ribonucleic acid export 1	7VPG, 7VPH	Gordon et al. (2020) showed interaction between ORF6 and the host Nup98-RAE1 protein pair via AP-MS. The interaction was confirmed by Miorin et al., 2020 and Li et al., 2021 (see crystal structures). Miorin et al. (2020) also demonstrate that upon treatment with recombinant IFN-I in HEK293T cells, Nup98 binding to SARS-CoV-2 Orf6 blocks translocation of STAT1 into the nucleus, resulting in suppression of ISRE-dependent gene expression.
ORF6	**KPNA2**: Karyopherin subunit alpha 1	NA	Using co-immunoprecipitation, Xia et al. (2020) showed that ORF6 selectively bound with KPNA2. Expression of ORF6 blocked nuclear translocation of IRF3, suggesting that ORF6 inhibited IFN-β production by binding to KPNA2 to block IRF3 nuclear translocation.
N (nucleocapsid)	**G3BP1/2**: GTPase-activating protein SH3 domain–binding protein	7SUO	Biswal et al. (2022) solved the X-ray crystal structure of the G3BP1 N-terminal nuclear transport factor 2-like domain bound to the first intrinsically disordered region of SARS-CoV-2 N protein.
ORF9b	**NEMO**: Nuclear factor kappa-B (NF-κB) essential modulator	NA	The interaction of the N-terminus of ORF9b with NEMO upon viral infection interrupts its K63-linked polyubiquitination, thereby inhibiting viral-RNA-induced IFNβ1 activation in HEK293T cells in an ORF9b-dose-dependent manner (Wu et al., 2021)
NSP5 (3CLpro)	**NEMO**	7T2U	Hameedi et al. (2022) solved the X-ray crystal structure of 3CLpro bound to NEMO and characterized 3CLpro cleavage of NEMO.

NSP1	**POLA**1: DNA polymerase alpha 1, catalytic subunit **40S ribosomal subunit**	7OPL 6ZOJ, 6ZOK, 6ZOL	Kilkenny et al., 2021 demonstrate that components of the host DNA polymerase α (Pol α)–primase complex or primosome directly bind with SARS-CoV-2 NSP1. They also provide a cryo-electron microscopy structure of NSP1 bound to the primosome. Schubert et al. (2020) provide cryo-EM structures of NSP1 bound to the 40S ribosome subunit, inhibiting translation of host proteins.
NSP6, NSP13	**TBK1**: TANK-binding kinase 1	NA	Sui et al. (2022) show that NSP13 recruits TBK1 to an aggregation of ubiquitinated proteins (p62) for autophagic degradation, resulting in inhibition of IFNβ production, and that NSP13 impaired IRF3 luciferase reporter activity induced by TBK1 in a dose-dependent manner. Xia et al. (2020) co-transfected HEK293T cells with plasmids containing TBK1 and either nsp6 or nsp13. Only NSP13 inhibited TBK1 phosphorylation, and did so in a dose-dependent manner, but both NSP6 and NSP13 suppressed IRF3 phosphorylation. Both NSP6 and NSP13 bind TBK1, as shown by co-immunoprecipitation. NSP6 binds to TBK1 without affecting TBK1 phosphorylation but this decreases IRF3 phosphorylation, while NSP13/TBK1 binding inhibits TBK1 phosphorylation. In both cases, IFN-β production is reduced (Xia et al., 2020).
NSP5 (3CLpro), ORF3a	**STING**: Stimulator of interferon genes	NA	Rui et al. (2021) SARS-CoV-2 ORF3a and 3CLpro inhibited IFNβ promoter activity through cyclic GMP-AMP synthase (cGAS)-STING pathways, specifically through interaction with STING, as indicated by co-immunoprecipitation. 3CLpro also bound to STING and specifically inhibited K63-ubiquitin-mediated modification of STING, which is required for signaling and downstream expression of IFN-I.
NSP3 Papain-like protease (Plpro)	**IRF3**: Interferon regulatory factor 3	NA	Mostaquil et al. (2020) showed with a fluorescent-based cleavage assay that NSP3 (Plpro) cleaves IRF-3, and thereby reduces IRF-3 available for induction of IFN-I expression.
N (nucleocapsid)	**STAT1**/**STAT2**: Signal transducer and activator of transcription	NA	Mu et al. (2020) used Sendai virus (SeV)-induced ISRE-promoter activation via the luciferase reporter assay to determine that SARS-CoV-2 N protein can inhibit the phosphorylation of STAT1 and STAT2 resulting in decrease in ISG production. They also showed through co-immunoprecipitation that N interacts with both STAT1 and STAT2, and that N inhibits STAT1/2 phosphorylation by blocking interactions with kinases including JAK1.

Uncertainties and Inconsistencies

There are uncertainties based on differing disease outcomes, especially associated with timing of IFN increase or suppression under different cell culture circumstances and in different people infected with SARS-CoV-2. Effectiveness of IFN treatment is still uncertain due to some studies evaluating IFN along with other drugs (Sodeifian et al., 2021).

Interferon-induced transmembrane proteins (IFITMs 1, 2 and 3) are ISGs that have been implicated in SARS-CoV-2 entry as well as antiviral activity (Prelli Bozzo et al., 2021), in addition to the fact that the SARS-CoV-2 entry receptor ACE2 is an IFN-I stimulated gene (Ziegler et al., 2020). These are some of the paradoxes that confound transcriptomic studies that determine up- or downregulation of IFNs and ISGs in response to infection, and responses are highly dependent on the time points sampled. Efforts to address uncertainties around when and under what circumstances IFNs and ISGs either promote or supress the virus are ongoing.

Quantitative Understanding of the Linkage

The current quantitative understanding of this relationship is described below.

Response-response relationship

A specific titer of virus can be used for infection, but as shown by Hatton et al. (2021), different cell types may express different levels of the actual stressors (viral protein transcripts). Because there are many stressors from each viral particle, which might be differentially expressed and also differentially inhibit each of their targets, a consistent whole viral entry dose leading to IFN-I or ISG response is difficult to measure. However, Chen et al. (2020), Xia et al. (2020), Fu et al. (2021), Wu et al. (2021) and Sui et al., (2022) all showed that individual protein stressor components of SARS-CoV-2 reduced IFN-I expression in a dose-dependent manner.

Time-scale

In humans, the viral entry MIE and early KEs coincide with the time from exposure to symptoms, within which are the latent period, or time from exposure to infectiousness, and the serial interval, or the time interval between the onset of symptoms in the primary (infector) and secondary case (infectee). Viral entry leading to antagonism of the IFN response occurs during the latent period of the disease prior to symptom onset. Latent period calculation is based on serial interval and median pre-symptomatic infectious period: Serial interval 5.2 days (Rai et al. 2021) – 2.5 days pre-symptom infectious period (Byrne et al. 2020) equals approximately 2.7 days. The latent period was longer in asymptomatic cases (4-9 days).

Known modulating factors

Genetic mutations. Autoimmunity to IFNs has been found in some COVID-19 patients. These individuals produce autoantibodies that attack IFN (Bastard et al., 2021 and 2022), which may be associated with human leukocyte antigen (HLA) gene mutations (Ku et al., 2016; Chi et al., 2013). Zhang et al., 2020 note inborn errors (genetic mutations) in IFN-I immunity that result in severe COVID-19, but some are also genes for proteins involved in the initial response (TBK1, IRF3, NEMO, IFNAR1, IFNAR2, STAT1, and STAT2). Zhang et al. (2022) also found similar mutations (STAT2 and IFNAR1) in children with COVID-19 pneumonia.

Pollutant exposures. Most studies have been conducted with the endpoints to determine whether prior or concurrent exposure to chemical or air particulate pollutants exacerbates COVID-19 symptoms resulting in *more severe disease or higher mortality rates*. This would point to effects downstream of viral replication usually relating to antibody suppression, inflammation and organ/tissue damage. Fewer studies can be found that study pollutant effects on *susceptibility to infection*, which are relevant to this KER, specifically cell entry or interferon response antagonism.

Marques et al., (2022) reviews associations between COVID-19 and outdoor air pollutants including $PM_{2.5}$, PM_{10}, O_3, NO_2, SO_2 and CO, reporting that environmental air pollution increases both disease incidence and severity. Physiological mechanism is not investigated for most studies. One relevant study estimated significant odds ratios for increased risk of severe COVID-19 and gene transcriptional analysis showing downregulation of genes associated with the IFN-I pathway in patients with high short-term NO_2 exposure (Feng et al., 2023).

Per- and polyfluoroalkyl substances (PFAS) are a large group of contaminants of current concern, due to their potential for toxicity, ubiquitous presence in the environment and consumer products, as well as their resistance to degradation. Although most community exposure to PFAS is through diet and drinking water, airborne and dermal exposures may also occur, especially in the workplace (CDC/NIOSH 2022). Statistical links between high measured serum or urine concentrations of specific PFAS compounds or mixtures and higher rates of COVID-19-positive cases have been found. One study in Sweden calculated a sex- and age-Standardized Incidence Ratio (SIR) for the town of Ronneby that had highly PFAS-contaminated drinking water compared to a demographically matched town with background PFAS levels (Nielsen et al. 2021). Serum PFAS concentrations were previously measured in 2014-15 for 3507 participants (Xu et al. 2021), after the Ronneby drinking water contamination issue was identified in 2013. Ronneby residents had higher infection risk, with a SIR of 1.19 [95% CI: 1.12-1.27]. Ji et al. (2021) measured urine and serum in a smaller study in China with 160 subjects. They reported statistically significant odds ratios for infection of 1.94 [95% CI: 1.39–2.96] for perfluorooctane sulfonate (PFOS), 2.73 [1.71–4.55] for perfluorooctanoic acid (PFOA), and 2.82 [1.97–3.51] for Σ (12) PFASs, after controlling for age, sex, body mass index (BMI), comorbidities, and urine albumin-to-creatinine ratio (UACR). These odds of infection were clearly higher even though the PFAS-exposed subjects in China had serum concentrations lower than in the Ronneby study participants. Additionally, the risk of infection was similar for residents in a significantly more contaminated section of Ronneby compared with a less contaminated section, so there was no dose-response relationship (Nielsen et al. 2021). However, these associations warrant more study to determine causality. Ji et al. (2021) also found elevated PFAS to be associated with altered mitochondrial metabolism. A potential consideration is that inhibition of mitochondrial oxidative phosphorylation impairs MAVS-mediated induction of IFNs, indicating the coordination between antiviral response and mitochondrial metabolism (Yoshizumi et al., 2017). Another study proposes modulation of ACE2 and TMPRSS2 expression in the lungs of PFAS-exposed mice may play a role in PFAS-associated immune suppression (Yang et al. 2022). Houck et al., (2022) report testing 147 PFAS substances in screening platforms including the BioMAP® Diversity PLUS panel, which is used to model complex tissue adverse effects of pharmaceuticals and environmental chemicals. Toxicity Signatures within the BioMAP profile indicated the Skin Rash (MEK-Associated) Signature for PFOA, with IFNα/β as one of the target mechanisms. While not specific to COVID-19, one study found that exposure to aryl hydrocarbons and dioxins may block IFN production (Franchini and Lawrence, 2018).

Known Feedforward/Feedback loops influencing this KER

SARS-CoV-2 uses the host ACE2-receptor for entry, upon which the host IFN response could upregulate ACE2 to enhance infection (Ziegler et al., 2020), a positive feedback loop for viral entry, while the IFN response also induces antiviral protein expression to help restore homeostasis as a positive feedback loop to KE 1901.

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Relationship: 2497: IFN-I response, antagonized leads to SARS-CoV-2 production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Not Specified
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent	Low	
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Moderate
Binding of SARS-CoV-2 to ACE2 leads to hyperinflammation (via cell death)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
mammals	mammals	Not Specified	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Sex. In a large study modelling URT viral load dynamics drawn from measurements in 605 human subjects, variations over 5 orders of magnitude in URT viral load from the time of symptom onset was not explained by age, sex, or severity of illness. Additionally, these variables did not explain modelling results concerning control of viral load by immune responses in the early (innate) or late (adaptive) phases (Challenger et al. 2022). Other sources also support that rate of infection and measured viral load does not differ by gender (e.g., Arnold et al. 2022; Qi et al. 2021; Cheemarla et al. 2021). This evidence suggests that the components of the early antiviral response are not influenced by gender specific differences such as sex hormone levels or sex chromosomes to the extent of affecting viral load.

Life Stage. To apply to this KER, studies would need to show differences in IFN or ISGs correlated with viral load and differing by age. Saheb Sharif-Askari et al. (2022) reported that children had higher expression of IFN-I and associated ISGs than adults, but did not measure viral loads. Euser et al. (2021) found that SARS-CoV-2 viral loads increase with age, but did not measure IFN or ISGs. Literature that connects the two factors for age in humans was not found.

Taxonomic. No non-mammalian vertebrates have been found to become infected with SARS-CoV-2. Many mammals have tested positive and several are known to shed and transmit the virus, however the prevalent aspects of non-human mammalian infection and transmission found in the literature are ACE2 binding capacity and measures of viral load. For the few species for which IFN is mentioned in the literature (Mostaquil et al., 2020; Rui et al., 2021; Hameedi et al., 2022), the potential IFN antagonism is not linked to resulting increase in viral replication, except in the golden hamster, *Mesocricetus auratus* (Hoagland et al., 2021). The hamsters were Infected with SARS-CoV-2 resulting in high levels of virus in the upper and lower respiratory tracts and an IFN-I response that was not sufficient to control COVID-19 progression. Direct contact resulted in inoculated hamsters transmitting the virus to naïve hamsters. When intranasal IFN-I was administered to the hamsters, viral replication was reduced and transmission was prevented (Hoagland et al., 2021). For bats, IFN and ISGs are constitutively expressed and therefore may contribute to immune tolerance and lack of replication of SARS-CoV-2 in many bat species (Irving et al., 2021). Differential susceptibility and viral shedding has been found across mammalian species (EFSA/Nielson et al., 2023), and it is likely that differences in IFN-I response may be involved. Therefore, more studies are needed in diverse taxa to assess the tDOA for IFN-I antagonism leading to increase in SARS-CoV-2 replication across the potentially susceptible species.

Key Event Relationship Description

The normal function of the host's innate immune response to viruses is the expression of interferons (IFN) which in turn stimulates the expression of many interferon-stimulated gene (ISG) proteins with antiviral functions (Amor et al., 2020; Harrison et al., 2020). ISGs generally function to inhibit viral replication (Yang and Li, 2020). The SARS-CoV-2 antagonism of the IFN-I pathway delays or curtails the expression of IFN-I and ISG proteins. This results in the downstream event, SARS-CoV-2 production, increased. The increase in SARS-CoV-2 viral production can be measured as viral load, which can contribute to both transmission to new hosts and more severe disease. This KER details the specific ISGs that inhibit viral replication, and demonstrates the difference in how SARS-CoV-2 negates the function of these proteins or delays their expression compared to other viruses to successfully increase its numbers.

Evidence Supporting this KER

See below.

Biological Plausibility

The functional relationships between the upstream IFN-I antagonism and downstream increase in SARS-CoV-2 viral replication is biologically plausible via the suppression of IFN through interaction inhibition of the host pathway proteins by viral proteins. This in turn would lead to suppressing the expression of ISGs that have been demonstrated to inhibit replication. The effects of ISGs on viral replication has been demonstrated for several viruses (Schoggins et al., 2011). SARS-CoV-2 replication may be impacted by different ISGs than other families of viruses. A gain-of-function analysis evaluating the impacts of ISGs on SARS-CoV-2 viral replication (Martin-Sancho et al., 2021) showed that a specific subset of ISGs when stably overexpressed in cultured human cells infected with SARS-CoV-2 controlled viral infection, including RNA binding proteins that suppress viral RNA synthesis and ISGs inhibiting viral assembly and egress. Therefore, the lack of these ISGs due to antagonism of the IFN-I pathway leads to increased viral replication.

Empirical Evidence

Evidence from patients who contracted COVID-19 supports the relationship between IFN antagonism and viral production:

- Busnadiego et al. (2020) found that different IFNs upregulate ACE2 to differing degrees, but all IFNs elevated ISGs and inhibited SARS-CoV-2 replication in a dose-dependent manner. Some people have developed autoimmunity toward their own IFN proteins (Bastard et al., 2021; Lopez et al., 2021). They produce autoantibodies that block even exogenously administered IFN, and this has resulted in more severe COVID-19 disease in these patients. Also, Zhang et al., (2020) note inborn errors (genetic mutations) in IFN-I immunity that result in severe COVID-19.
- Cheemarla et al. (2021) used patient nasopharyngeal samples and airway epithelial organoids. COVID-19 patient samples had upregulated ISG RNAs in the upper respiratory tract. SARS-CoV-2 replicated exponentially when unchecked, doubling in 6 h. ISGs rose with viral replication and peaked as viral load declined. Rhinovirus infection before SARS-CoV-2 exposure caused ISG induction to accelerate and stopped SARS-CoV-2 replication, while blocking ISG induction increased viral replication.
- Hadjadj et al. (2020) report a phenotype in severe COVID-19 patients with no IFNβ, low IFNα, persistent blood viral load and exacerbated inflammatory response.
- Hatton et al. (2021) use human nasal epithelium differentiated at the air-liquid interface (ALI) cultures (organoids) to show delayed induction of IFN-I and -III in SARS-CoV-2 compared to influenza A virus. They found that exogenous IFNs administered pre-exposure or early in infection controlled SARS-CoV-2 replication.

Uncertainties and Inconsistencies

Schuhenn et al. (2022) found that differential immune signatures of IFNα subtypes suppress SARS-CoV-2 infection by treating primary human airway epithelial cells (hAEC) with different IFNα subtypes during SARS-CoV-2 infection. The most effective antiviral subtype was IFNα5, against both in vitro and in vivo infected mice, and additive effects with the antiviral drug remdesivir in cell culture.

Rouchka et al., (2021) found that there is not only wide variation in nasopharyngeal viral loads in COVID-19 patients early in infection, but also that viral loads were strongly correlated with host gene expression associated with IFNα-inducible cellular antiviral response genes (ISGs). Also, patients with mild symptoms were often found to have a higher viral load than those with severe disease, indicating lack of correlation between susceptibility to severe disease, and susceptibility to viral replication.

In review articles, Yang and Li (2020) and Samuel (2023) discuss the relationship between the IFN antiviral response and viral replication. Yang et al. focus on ISGs with multiple mechanisms that inhibit viral replication by sensing, degrading, or repressing expression of viral RNA. These ISGs may use a variety of co-factors, which indicates the highly complex nature of the type I IFN response. Samuel et al. report that overall genetic variability of both SARS-CoV-2 and the human host affect the IFN response, and viral replication is in turn sensitive to variation in IFN antiviral action.

These studies point out inconsistencies in quantity and type of IFN expression or administration in patients and COVID-19 disease outcome, but confirming the link between IFN-I response and viral replication. There is uncertainty in the fact that several IFN-I pathway components have been variously implicated. Because many different IFN subtypes and subsequently many different ISGs and cofactors may be involved, not only the specific repertoire of ISGs expressed may differ among individuals, but also the quantity of each ISG may influence viral production.

Quantitative Understanding of the Linkage

The current quantitative understanding of this relationship is described below.

Response-response relationship

Busnadiego et al., (2020) found an inverse, linear relationship between IFNβ or IFNλ1 concentration and viral titer, measured as plaque forming units (PFU) in primary human bronchial epithelial cells (BEpCs) differentiated and grown at an air-liquid interface (ALI). However, the upstream event of IFN antagonism is not represented by administered IFN but by antagonism of the IFN response, and does not answer the question of what dose of antagonist results in increased viral replication in a host system, where viral replication is not normal biology. Comparatively, difference in IFN expression between cells infected with influenza A virus vs. SARS-CoV-2 showed significantly higher IFNβ and IFNλ1 for influenza at both 6 and 24 hours post-infection, but this was not tied to relative viral production

(Hatton et al., 2021).

The key event of IFN-I response antagonism encompasses a broad range of stressors and targets: 1) viral proteins interacting with pathway proteins leading to IFN expression, 2) the IFN subtypes that induce the expression of ISGs, and 3) the variation in type and amount of ISGs expressed, which also varies with cell/tissue type. Viral replication related to these factors is also dependent on the dose of virus to which the individual host is exposed and the genetic make-up and overall condition of that individual. These factors may explain the variable results in IFN dose-viral production response determination, and why the actual response-response relationship for this KER, between the viral dose resulting in antagonism and viral replication increase, have not been determined. Saheb Sharif-Askari et al., 2022 concluded that more mechanistic studies are needed to quantify the amount of early IFN required to overcome SARS-CoV-2 antagonism and prevent replication. Polyinosinic:polycytidylic acid [poly(I:C)] is a synthetic analog of double-strand RNA (dsRNA) that can stimulate IFN production. The use of poly(I:C) administered before and during SARS-CoV-2 infection in mice increased ISGs and lowered viral loads (Tamir et al., 2022) but was administered at different time points rather than at different dose concentrations. Poly(I:C) dosing may be a potential method to quantify the IFN stimulation needed to overcome SARS-CoV-2 antagonism.

Time-scale

The viral entry MIE and early KEs coincide with the time from exposure to symptoms, within which are the latent period, or time from exposure to infectiousness, and the serial interval, or the time interval between the onset of symptoms in the primary (index) and secondary (contact) case. Pre-symptomatic transmission occurs from about 3 days after exposure to symptom onset at about day 5-7, viral load peaks from about day 5-7 to day 9-11, and the host can remain infectious to symptom clearance or death (Byrne et al. 2020). IFN administered prior to exposure or within the latent period window can stop replication (Sodeifian et al., 2021). In a study using a primary nasal cell model (differentiated at air-liquid interface), the virus did not proliferate beyond the limit of assay detection if treated with IFN beta or lambda 16 hours prior to infection, and virus was significantly reduced in cultures treated 6h post-infection compared to untreated cultures. Treatments 24h post infection were not significantly different from untreated controls for either type of IFN (Hatton et al., 2021). This would suggest that viral antagonism of IFN occurring during the first 24h post viral entry allows viral loads to be generated likely concurrently, reaching transmissible levels within 72h post viral entry.

Known modulating factors

IFN has been the subject of studies for potential therapeutic value to enhance the antiviral response. However, IFN administered too late, in the inflammatory stage (post-symptom onset), led to long-lasting harm and worsened disease outcome (Sodeifian et al., 2021). Therapeutics used in COVID-19 patients tend to target either the ACE2 binding, downstream inflammatory response, or viral replication via inhibition of the viral RNA-dependent, RNA polymerase to block viral genome replication (i.e., Remdesivir) (Narayanan and Parimon, 2022). No other therapeutics were found to be relevant to this KER, i.e., specifically targeted to IFN components or ISGs leading to supressed viral replication (see WHO 2021 and Terracciano et al., 2021).

It is known that per- and poly-fluorinated alkyl substances (PFAS), air pollutants, and other environmental chemicals are implicated in SARS-CoV-2 susceptibility and COVID-19 disease severity (Marques et al., 2022; Nielsen et al., 2021; Xu et al., 2021). However, it is currently unknown whether or how the mechanisms of action are related to blocking IFN components or ISGs, leading to viral replication.

Genetic factors are of importance to this KER: Autoantibodies against IFN, as noted, block even exogenously administered IFN, resulting in more severe disease (Quarleri and Delpino, 2021; Bastard et al., 2021; Busnadiego et al., 2020; Lopez et al., 2021). There are 15 known clinically recessive and inborn errors of type I IFN immunity (Zhang et al., 2022). Four of these including X-linked recessive TLR7 deficiency, and autosomal recessive IFNAR1, STAT2, or TYK2 deficiencies were found in children with moderate to critical pneumonia due to COVID-19. Zhang et al. (2022) also reported enhanced SARS-CoV-2 replication measured as expression of viral nucleocapsid (N-protein) in STAT2- and TYK2-deficient patients' cells.

Known Feedforward/Feedback loops influencing this KER

RIG-Like Receptors (RLRs) including MDA5 are Pattern Recognition Receptors (PRRs) that recognize Pathogen-Associated Molecular Patterns (PAMPs) like viral RNA and start signalling cascades to express IFNs. These PRRs and other proteins in the pathway, including STAT1 and STAT2 involved in transcription of the ISGs, are also regulated by IFN, and therefore are themselves ISGs (Yang and Li, 2020). As RNA from most viruses is detected, signalling to express more ISGs increases, and more IFN is expressed (Michalska et al., 2018). However, SARS-CoV-2 inhibits these and other components of the IFN pathway to delay expression of ISGs, and viral production goes unchecked, actually disrupting the normal antiviral positive feedback loop. In fact, SARS-CoV-2 can co-opt another ISG, interferon-induced transmembrane protein 2 (IFITM2), for efficient replication in human lung, heart, and gut cells (Nchioua et al., 2022), which might also be considered a positive feedback loop (i.e., the more IFITM2 is expressed, the more the virus replicates). However, IFITM2 and 3 have also shown antiviral activity toward SARS-CoV-2 (Shi et al., 2021), therefore the conflicting results require more research.

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