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The role of IFI16 in modulating the immune response upon HCoV infection

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Statement of originality:

I hereby declare that I Kanza Nadeem,

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The present thesis was conducted at the Laboratory of Molecular Virology, Department of Medical Biotechnology, University of Piemonte Orientale, Novara, during the academic year 2023–2024.

"And say: My Lord increases me in knowledge." *(Qur'an, Ta-Ha 20:11)*

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1. SUMMARY

Over millions of years, bats have evolved unique defense mechanisms that allow them to coexist with a wide range of viruses, including coronaviruses (CoV), without showing signs of illness, making them ideal reservoirs for viruses that can be transmitted to humans. Notably, bats lack the entire locus encoding PYHIN family of proteins, which includes the interferon-γ-inducible protein 16 (IFI16). IFI16 is known to function in the sensing and restriction of several DNA viruses, also recently it has been reported to sense and restrict RNA viruses, including influenza A virus (IAV), porcine reproductive respiratory syndrome virus 2 (PRRSV2) and Chikungunya virus (CHIKV). Despite the emerging evidence of IFI16 displaying a role in the control of RNA virus replication, it remains largely unexplored whether it could modulate also CoV infection. According to this background, the aim of this thesis was to investigate the role of IFI16 in modulating human CoV (HCoV) replication, using two viral strains: the highly pathogenic beta-CoV SARS-CoV-2 and the low pathogenic alpha-CoV NL63. The experiments were carried out in IFI16 knock-out (IFI16KO) and trasfected control (TC) LLC-MK2 cells

Our findings reveal a dual role for IFI16 in modulating the replication of HCoVs, depending on the viral strain. In the context of SARS-CoV-2, IFI16 acts as a restriction factor, limiting viral replication, as evidenced by the increased viral load and faster protein expression kinetics observed in IFI16-depleted cells. Conversely, in NL63 infection, IFI16 appears to support viral replication, with reduced viral RNA levels and N protein expression in IFI16-deficient cells.In addition, we demonstrated that IFI16 activity on HCoVs is IFN-independent, as suggested by the lack of significant induction of IFN- β and its downstream targets (Mx1and IFIT1) in both IFI16KO and TC cells infected with the two viruses.

These results highlight the complexity of host-virus interactions and suggest that IFI16 differentially influences HCoV infections. Further investigation into the mechanism by which IFI16 modulates the replication of SARS-CoV-2 and NL63 are needed.

Overall, this study has the potential to deepend our understanding of the role of IFI16 in controlling HCoV replication and help develop novel therapeutic approaches for HCoV-related diseases.

2. INTRODUCTION

2.1 Coronaviruses

2.1.1 General features and classification of coronaviruses

Coronaviruses are enveloped, positive-sense, single-stranded RNA (+ssRNA) viruses that infect a wide range of mammalian and avian species, including humans (Hartenian et al. 2020a). They are known for their large genome and distinctive spike proteins that facilitate host cell entry. Coronaviruses belong to the order *Nidovirales*, within the family *Coronaviridae*, and subfamily *Coronavirinae* (King et al. 2020). Based on their phylogenetic relationships and genomic organization, coronaviruses are classified into four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus*, and *Deltacoronavirus* (Woo et al. 2009) **Figure 1.** Among these, *Alphacoronaviruses* and *Betacoronaviruses* exclusively infect mammals, whereas *Gammacoronaviruses* and *Deltacoronaviruses* predominantly infect avian species (de Wit et al. 2016). To date, only seven coronaviruses have been identified as capable of infecting humans. (Banerjee et al. 2021), these human coronaviruses (HCoVs) are categorized based on their pathogenicity into low or highly pathogenic HCoVs. Low pathogenic HCoVs (HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1), typically cause mild to moderate upper-respiratory tract illness, such as common cold, in healthy individuals (Su et al. 2016). Conversely, highly pathogenic HCoV (SARS-CoV, MERS-CoV, and SARS-CoV-2) infect the lower respiratory tract and are associated with severe illnesses, including acute respiratory distress syndrome (ARDS) and severe pneumonia (Hu et al. 2015a; Ye et al. 2020a). Notably, the highly pathogenic HCoVs are all included into the *Betacoronavirus* genus, which is further divided into five subgenera: *Embecovirus, Sarbecovirus, Merbecovirus, Nobecovirus*, and *Hibecovirus* (Hu et al. 2021).

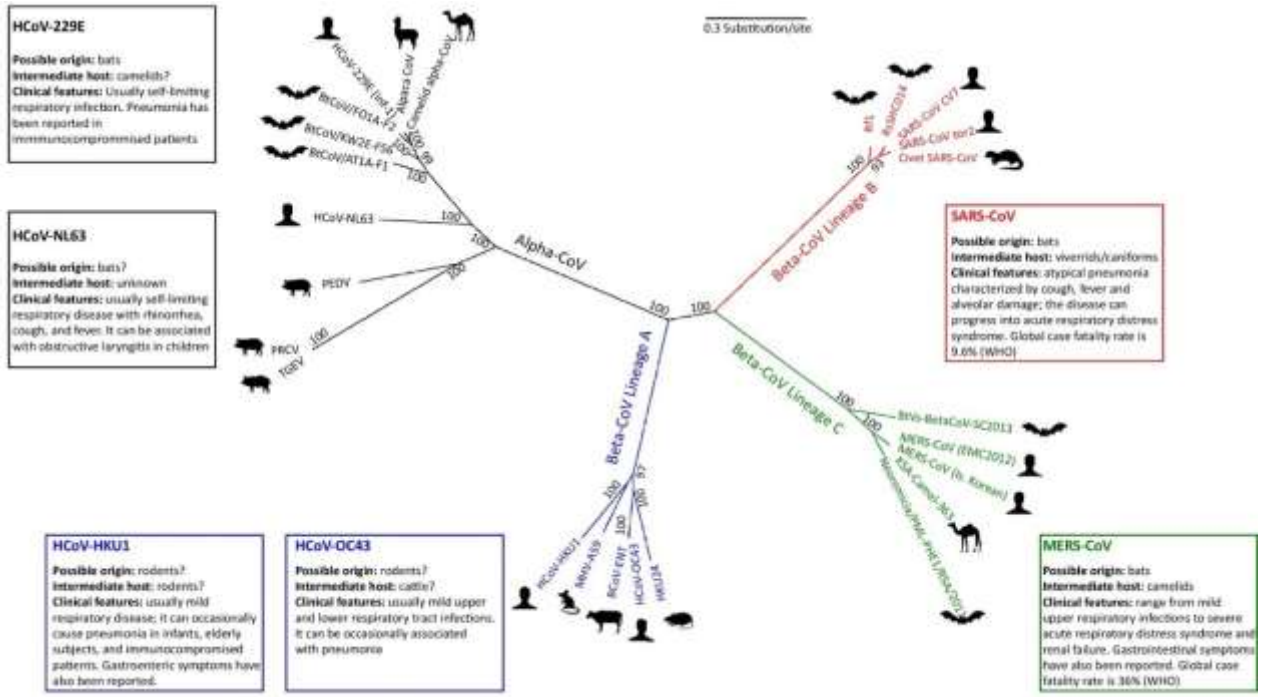

Figure 1 Phylogenetic relationships and genome organization of human and animal coronaviruses (CoVs).
The phylogenetic tree of complete genome sequences of HCoVs and selected mammalian CoVs was obtained with RAxML 8.2.4. Numbers indicate bootstrap support. CoVs are colored according to genus and lineage. Information about origin, intermediate host, and clinical presentation is reported for the six HCoVs (Adapted from Forni et al. Cell 2017).

HCoV-229E and HCoV-OC43 were the first human coronaviruses to be isolated, in 1965 and in 1967, respectively (Banerjee et al. 2021; Hu et al. 2021). Before 2002, these were the only known HCoVs circulating in the human population and were considered relatively harmless, causing mild illness. However, in 2002, a novel coronavirus emerged in southern China and was responsible for severe respiratory infections (hence, the name Severe Acute Respiratory Syndrome [SARS]-CoV) with a fatality rate of approximately 11% (Cui, Li, and Shi 2019a). Subsequently, in 2004 and 2005, two new strains of HCoV were identified in individuals with upper respiratory tract infections, HCoV-NL63 and HCoV-HKU1 (Ye et al. 2020a). In 2012, another highly pathogenic coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), was detected in Saudi Arabia, causing severe lower respiratory tract infections with a fatality rate of approximately 35% (Banerjee et al. 2021; Hu et al. 2015a). Unlike SARS-CoV, which disappeared a year after its emergence, MERS-CoV has continued to cause outbreaks in the Middle East and South Korea. In late 2019, a novel coronavirus resembling SARS-CoV was discovered in the Hubei Province of

China and was named SARS-CoV-2. This virus, responsible for coronavirus disease 2019 (COVID-19), rapidly spread worldwide, leading the World Health Organization (WHO) to declare a pandemic in March 2020 (Su et al. 2016; Ye et al. 2020a).

All coronaviruses circulating in humans have animal origins **Figure 2**: SARS-CoV, MERS-CoV, SARS-CoV-2, HCoV-NL63 and HCoV-229E are believed to have originated in bats, whereas HCoV-OC43, HCoV-HKU1, are thought to have originated from rodents (Banerjee et al. 2021; Ye et al. 2020a). In addition, before being able to infect humans, HCoVs first exploited intermediate hosts, thus they are transmitted to humans through zoonotic spill over events. Masked palm civets and dromedary camels have been identified as intermediate hosts for SARS-CoV and MERS-CoV, respectively (Hu et al. 2015b). Whereas the intermediate host for SARS-CoV-2 remains under investigation, with studies suggesting potential candidates such as Malayan Pangolins, although definitive evidence is still lacking (T. Zhang, Wu, and Zhang 2020). For HCoV-229E and HCoV-OC43, alpacas and bovines have been proposed as potential intermediate hosts respectively, (Corman et al. 2014).

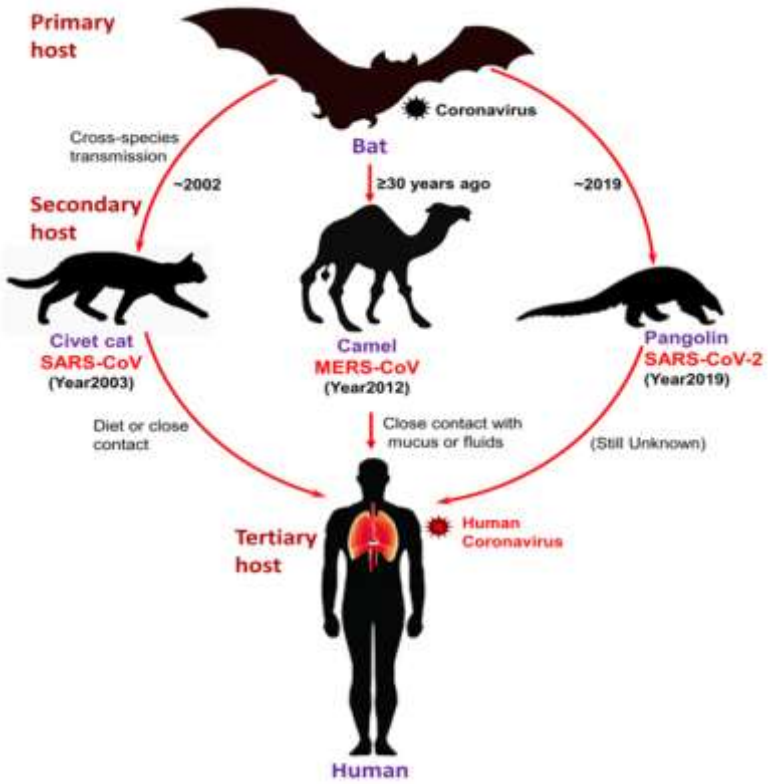

Figure 2 The schematic representation illustrating the transmission of the three highly pathogenic HcoVs: SARS-CoV, MERS-CoV, and SARS-CoV-2 from bats to humans via intermediate hosts (Kirtipal, Bharadwaj, and Kang 2020).

Given the occurrence of these spill over events and the extensive distribution of coronaviruses among various animal populations, it is plausible that coronaviruses will continue to pose public health threats (Cui, Li, and Shi 2019b). Factors such as increased human-animal interactions resulting from habitat encroachment, changes in agricultural practices, urbanization, deforestation, and climate change are likely to elevate the risk of emerging zoonotic diseases, including those caused by coronaviruses (Allen et al. 2017; Jones et al. 2008).

2.1.2 Virus structure and genome organization

Coronavirus particles are typically spherical in shape, ranging from 80 to 120 nm in diameter, and are composed of four major structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins **Figure 3** (F. Wu et al. 2020; Zhixing Zhu et al. 2020). These proteins are essential for various steps in the viral replication cycle, while also maintaining the structural integrity of the virus. The viral envelope is made up of a lipid bilayer in which the M, E, and S proteins are anchored. Inside the virion, the helical nucleocapsid, consists of multiple copies of the N protein which binds to the RNA genome, stabilizing its structure (Zhixing Zhu et al. 2020).

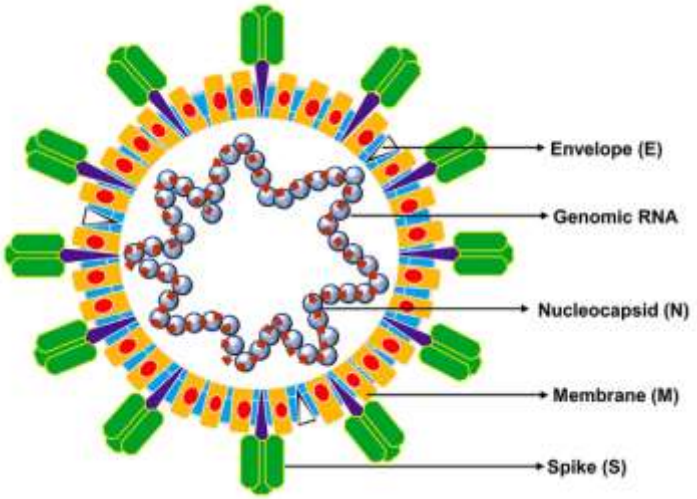

Figure 3 Schematic diagram of a CoV virion with the four structural proteins:
spike (S) glycoprotein, membrane (M), glycoprotein, envelope (E) protein and nucleocapsid (N) phosphoprotein which encapsidates the ssRNA genome (Kirtipal, Bharadwaj and Kang, 2020).

The genome of coronaviruses is one of the largest among RNA viruses, varying in size from 26 to 32 kilobases, depending on the genus. CoV genome is +ssRNA molecule, structurally similar to eukaryotic mRNAs as it features a 5’ cap and a 3’ poly-A tail (Fu, Cheng, and Wu 2020; Shi et al. 2020a; Zhenglin Zhu et al. 2021).

The genome organization of coronaviruses follows a conserved order of genes: 5'-replicase-S-E-M-N-3', which includes multiple open reading frames (ORFs) that encode both structural and non-structural proteins (NSPs), as well as additional genus-specific accessory proteins **Figure 4**. At the 5' end of the genome there are two overlapping ORFs, ORF1a and ORF1b, which encode the two polyproteins pp1a and pp1ab (Gordon et al. 2020). These polyproteins are cleaved by viral proteases into 16 NSPs, which assemble to form the replicase-transcriptase complex (RTC). The RTC is responsible for amplifying the genomic RNA (gRNA) and synthesizing sub-genomic mRNAs (sgRNAs) (Shi et al. 2020b). The 3' end of the genome is transcribed into sgRNAs, which encode for the structural proteins S, E, M, and N. The 3' end also contains ORFs encoding for the accessory proteins (Artika, Dewantari, and Wiyatno 2020; Kirtipal, Bharadwaj, and Kang 2020).

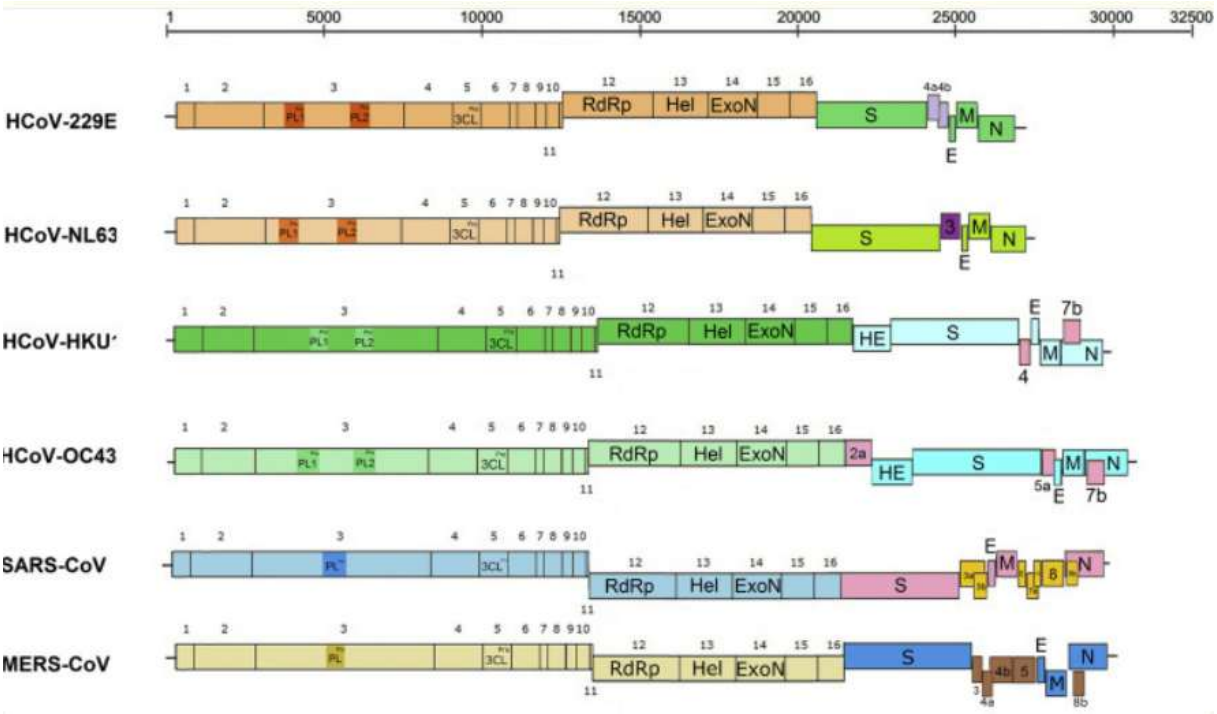

Figure 4 Schematic structure of HCoVs genome (Li et al., 2019).

2.1.3 Coronavirus life cycle

CoVs are named for their characteristics crown-like appearance under an electron microscope, which is due to the presence of S proteins forming trimeric projections on the viral envelope (Bar-On et al. 2020). The S protein is responsible for viral attachment and entry into host cells- Indeed, the infection process of CoVs begins with the interaction between the S protein and different

cellular receptors, such as angiotensin-converting enzyme 2 (ACE2), dipeptidyl peptidase 4 (DPP4) or amino peptidase N (APN, CD13) (Jackson et al. 2021; Woo et al. 2012). Following receptor binding, host proteases such as transmembrane serine protease 2 (TMPRSS2) or lysosomal cathepsins cleave the S protein, triggering the fusion of the viral membrane and cellular membranes, leading to the release of the viral RNA into the cytoplasm (Hoffmann, Kleine-Weber, and Pöhlmann 2020). Subsequently, ORF1a and ORF1b are translated encoding for the two polyproteins pp1a and pp1ab, which are then cleaved by viral proteases to generate NSPs that will assemble to form the RTC, essential for viral replication (V'kovski et al. 2021). Among NSPs, RNA-dependent RNA polymerase (RdRp) plays a key role in viral genome replication and transcription (Snijder et al. 2020; V'kovski et al. 2021).

CoVs employ a unique mechanism of replication that involves the synthesis of a complementary full-length negative-strand RNA intermediate. This intermediate serves as a template for generating new positive-strand progeny genomes, which will be packaged into newly assembled virions (D. Kim et al. 2020). In addition to full-length genome replication, coronaviruses utilize a process called discontinuous transcription to generate sgRNAs, which encode structural and accessory proteins. During transcription, the viral RdRp complex synthesizes minus strand sgRNAs, which contains a 5' leader sequence derived from the 5'-end of the genome. This leader sequence is joined to a downstream transcription regulatory sequence (TRS) located upstream of structural and accessory genes, enabling the formation of a nested set of sgRNAs. These sgRNAs serve as templates for the production of viral proteins, including S, E, M, and N(Sexton et al. 2016).

Following protein translation, viral structural proteins are processed in the endoplasmic reticulum (ER) and transported to the ER-Golgi intermediate compartment (ERGIC), where they interact with newly synthesized genomic RNA encapsidated by N protein. This interaction promotes virion assembly, leading to the formation of virus particles that bud into the lumen of secretory vesicles (Schoeman and Fielding 2019). The mature virions are then transported to the plasma membrane via the secretory pathway and released from the cell through exocytosis, allowing for further spread of the virus **Figure 5** (Astuti and Ysrafil 2020).

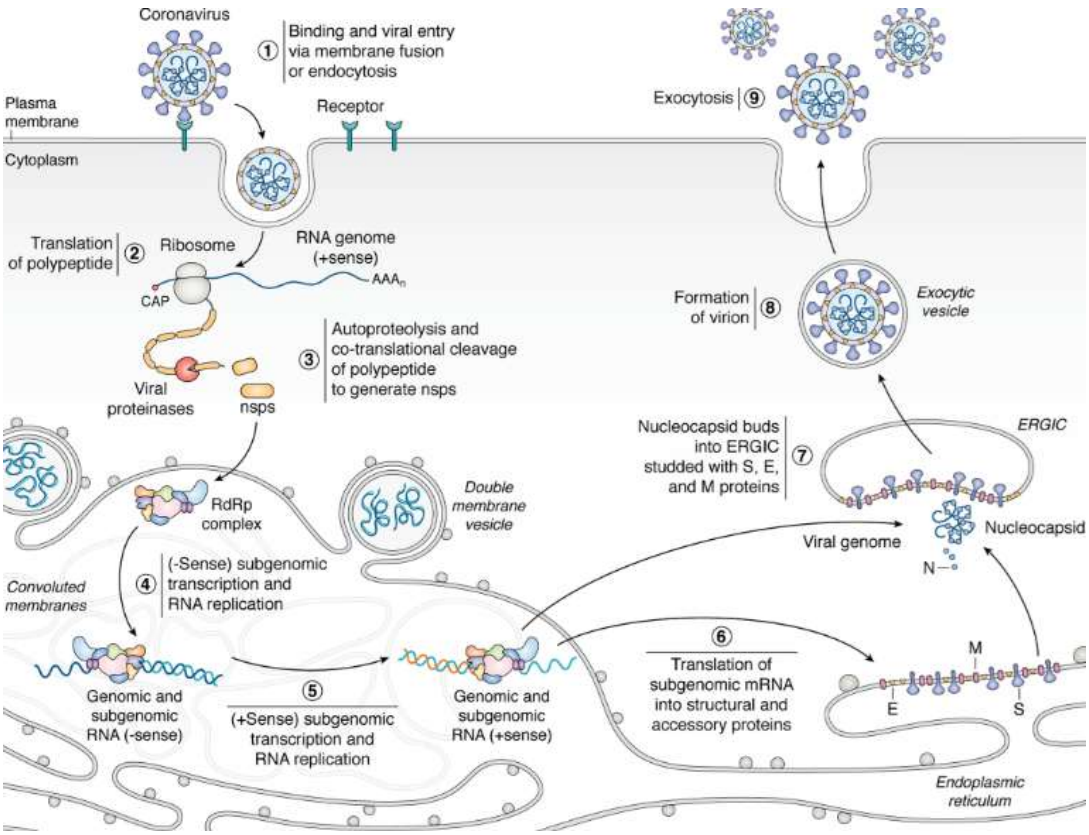

Figure 5 Schematic diagram of coronavirus life cycle:
Coronaviruses initiate infection by binding to host cell receptors, leading to viral genome entry via endocytosis or membrane fusion (step 1). After, the viral RNA is uncoated and ORF1a and ORF1b are translated to produce polyproteins pp1a and pp1ab, which are cleaved into the 16 NSPs that form the RTC (step 2 – 3). The RdRp complex synthesizes negative-sense RNA intermediates that serves as templates for genome replication and sgRNA transcription (step 4 – 5). These sgRNAs are then translated into structural and accessory proteins (step 6). Subsequently, genomic RNA is encapsidated by the nucleocapsid N protein and assembles with the other structural proteins (S, E, and M) in the (Step 7). Finally, fully formed virions are transported through the secretory pathway and released via exocytosis (step 8 – 9) (Adapted from Hartenian et al. 2020b).

2.1.4 HCoV-NL63

Human coronavirus NL63 (HCoV-NL63) is an *Alphacoronavirus* that was first identified in 2003 in a 7-month-old child who presented with symptoms of bronchiolitis and conjunctivitis. Since then, HCoV-NL63 infections have been reported globally, confirming their widespread presence. Although it has been established that HCoV-NL63 originated from bats, the potential intermediate host remains unidentified (Ye et al. 2020b).

HCoV-NL63, alongside with HCoV-229E and HCoV-OC43, is one of the primary causes of common cold (Santacroce et al. 2021). While HCoV-NL63 typically affects the upper respiratory tract, leading to mild symptoms such as fever, sore throat, rhinitis, and cough, it can cause more

severe illness in vulnerable populations such as young children, the elderly, and individuals with compromised immune systems (Chitre et al. 2024; Pyrc, Berkhout, and van der Hoek 2007b).

The virus has a capped and polyadenylated ssRNA genome that spans 27,553 nucleotides. The genome structure follows the order: 5'-ORF1a-ORF1b-S-ORF3-E-M-N-poly-T-3' **Figure 6** (Abdul-Rasool and Fielding 2010). The viral genome encodes seven distinct ORFs from six separate mRNAs, including the full-length genomic RNA and five sgRNAs. These sgRNAs encode the viral structural and accessory proteins: S, ORF3, E, M, and N proteins (Santacroce et al. 2021). Except for ORF3, a common TRS is present upstream of all other ORFs, which regulates the formation of sgRNAs (Gaunt et al. 2010).

Figure 6 HCoV- NL63 genome organization (Castillo et al. 2023; Pyrc et al. 2004b).

HCoV-NL63 shares certain similarities with other coronaviruses, including SARS-CoV and MERS-CoV, in terms of its ability to infect the human respiratory system (van der Hoek et al. 2005). However, unlike SARS-CoV and MERS-CoV, HCoV-NL63 infection is typically self-limiting and resolves with supportive care (Weil et al. 2022).

Unlike other *Alphacoronaviruses*, which utilize APN as their receptor to enter cells, HCoV-NL63 uses ACE2, a receptor that is also employed by some *Betacoronaviruses*, including SARS-like coronaviruses (van Dorp et al. 2021). ACE2, a type I membrane protein, is predominantly expressed in the lungs, heart, kidney, and intestines. It is a homologue of the ACE protein, and both play critical roles in the renin-angiotensin system (RAS). ACE2 serves as a negative regulator of this system by inactivating angiotensin II (Ang II), acting as an antagonist of ACE's vasoconstrictive effects (Liu, He, and Huang 2021).

The initial steps of HCoV-NL63 infection involve binding to the host cell membrane through heparan sulfate proteoglycans, which then facilitate interaction with ACE2, the primary entry receptor for the virus (Milewska et al. 2018). This recognition is primarily mediated by viral S protein. Recent studies have suggested that the entry of HCoV-NL63 into cells occurs via clathrin-mediated endocytosis, with binding of the virus to ACE2 triggering the recruitment of clathrin and

the subsequent formation of clathrin-coated pits (Chen and Hao 2020). Alternatively, the virus can bypass this endocytic pathway and use the priming protease TMPRSS2 to directly enter the cell from the surface (Milewska et al. 2018).

A unique feature of HCoV-NL63 is its spike protein, which contains a distinct 179 amino acid domain in its N-terminal receptor binding domain (RBD) that is not found in other coronaviruses. This domain is the most variable region in the HCoV-NL63 genome and may play a significant role in the virus's ability to evade the host immune response (Pyrc, Berkhout, and van der Hoek 2007c).

Interestingly, the interaction between the HCoV-NL63 S protein and ACE2 is weaker than that of the SARS-CoV S protein, which may help explain the milder pathological effects associated with HCoV-NL63 infection compared to that of SARS-CoV. In contrast, SARS-CoV infection leads to a reduction in ACE2 expression, and this downregulation has been hypothesized as a major contributor to the severe pneumonia and acute lung failure observed in infected individuals (Sakuma et al. 2007).

2.1.5 SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the coronavirus responsible for the life-threating disease known as COVID-19 (Todres and Diaz 2021). It was first identified in Wuhan, China, with the first case officially reported by the WHO on December 31, 2019. The virus rapidly spread worldwide, leading to the WHO declaring a global pandemic on March 11, 2020, (Todres and Diaz 2021).

SARS-CoV-2 belongs to the *Betacoronavirus* genus and shares approximately 79% of genomic similarity with SARS-CoV and 50% with MERS-CoV. The clinical presentation of SARS-CoV-2 varies significantly, influenced by several factors such as age and comorbidities like diabetes and hypertension. While most individuals experience mild flu-like symptoms such as fever, sore throat, fatigue, and anosmia, and ageusia, around 10-15% of cases develop into severe disease, which may lead to complications and fatal outcomes (Guan et al. 2020).

SARS-CoV-2 virions contain a large N-encapsidated +ss RNA genome, and the viral lipid envelope is embedded with three transmembrane proteins: S, M and E (Ke et al., 2021). SARS-CoV-2 genome is about 29.9 kb in length and contains approximately 15 ORFs **Figure 7**. At the 5' end, the ORF1a and ORF1b regions encode large polyproteins that are proteolytically cleaved into 16 NSPs, which form the viral replicase machinery. The 3' end encodes for the four structural proteins:

S, E, M and N, along with nine accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8b, ORF9a, ORF9b, and ORF10) (C. Yin 2020).

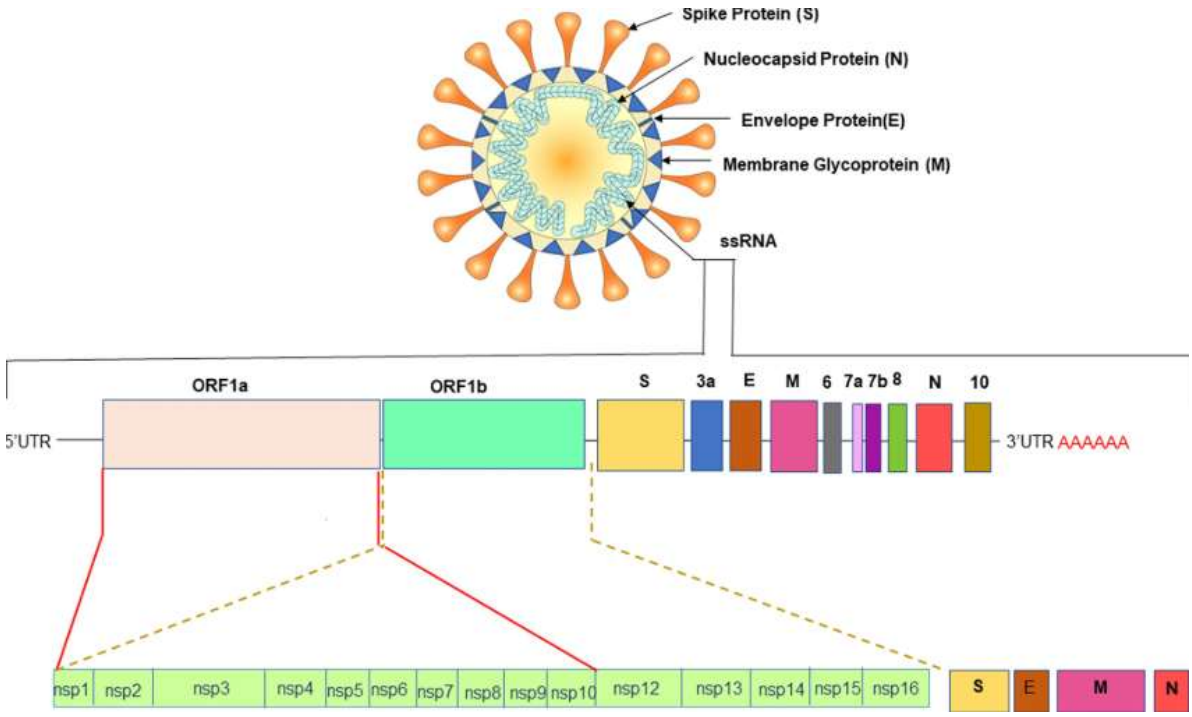

Figure 7 Schematic representation of the SARS-CoV-2 virus particle and genome structure.
The virion has an outer lipid envelope embedded with three structural proteins S, M and E. The envelope contains the +ssRNA genome containing approximately 15 ORFs that encode for 16 NSPs and for the structural proteins (Rastogi et al., 2020).

SARS-CoV-2, like SARS-CoV and HCoV-NL63, utilizes the ACE-2 receptor to facilitate entry into host cells. After binding to the receptor, the virus enters via two distinct pathways depending on the availability of host proteases (K. Wang et al. 2020). If membrane-bound proteases, such as TMPRSS2, are present, the virus enters the cells through the cellular membrane (early fusion pathway) (Qianqian Zhang et al. 2021). In the absence of these proteases, the virus enters through the endocytic pathway (late fusion pathway). In this later case, the virus is internalized within endosomes, where the viral genome is released into the cytosol following the fusion of the viral and endosomal membranes. This process is facilitated by the endosomal protease cathepsin L, which cleaves the viral S protein. Variation in the activity of host proteases can influence the efficiency of SARS-CoV-2 entry into ACE2-expressing cells. Notably, this dependence on Cathepsin activity is not observed in HCoV-NL63, where viral entry does not rely on the activity of Cathepsins (Kung et al. 2022).

SARS-CoV-2 induces the formation of double-membrane vesicles in infected cells, which contain RTCs (Fehr and Perlman 2015b). These RTCs comprise various viral proteins, including the RNA-dependent RNA polymerase (nsp12), helicase (nsp13), RNA cap-modifying methyltransferases (nsp14 and nsp16), and exoribonuclease (nsp14) (Slanina et al. 2021). The RTCs synthesize both progeny genomes and subgenomic mRNAs, utilizing negative-strand intermediates. Structural and accessory proteins are translated by ribosomes attached to ER-membrane, and they are transported to the ERGIC (T. Tang et al. 2020). There, they are encapsidated by the N protein, along with progeny genomes and other ER membrane-bound components, leading to virion formation. These vesicles containing the viral particles eventually fuse with the plasma membrane, enabling the release of new virions into the extracellular space (I.-C. Huang et al. 2006)

2.2 Coronaviruses and the innate immune system

2.2.1 Innate immune response against coronaviruses

Innate immunity represents an antigen-nonspecific mechanism used by the host as the first line of defense against invading pathogens and harmful stimuli (C. Wu et al. 2020). This immune response relies on the activation of pattern recognition receptors (PRRs), which are expressed on both the cell membrane and within the cytoplasm (S.-C. Yang et al. 2021). These receptors include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene-like receptor (RLRs), and AIM2-like receptors (ALRs) (C. Huang et al. 2020). PRRs are predominantly found on innate immune cells, such as dendritic cells, macrophages, monocytes, neutrophils, and epithelial cells (Grommes and Soehnlein 2011). These receptors recognize two main categories of molecular pattern: (1) pathogen-associated molecular pattern (PAMPs), highly conserved microbial structures that help identify viral, bacterial, or fungal pathogens or. (2) Damage-associated molecular patterns (DAMPs), molecules released from damaged or dying host cells due to infection or inflammation, including reactive oxygen species (ROS), uric acid, heat shock proteins, DNA, and RNA (Carissimo et al. 2020; W. Wang et al. 2020). As RNA viruses, coronaviruses are primarily detected by RNA-sensing PRRs, including RLRs and TLRs (Akira, Uematsu, and Takeuchi 2006). Among the TLR family, TRL3, TLR7, and TLR8 are known to recognize viral RNA in endosomal compartments. Specifically, TLR3 detects double-stranded RNA (dsRNA), whereas TLR7 and TLR8 sense ssRNA (Hadjadj et al. 2020). In contrast, cytoplasmic RLRs, such as RIG-I and melanoma differentiation-associated gene 5 (MDA5), recognize intracellular non-self RNA molecules that possess distinct secondary structures or

modifications. Upon ligand recognition, PRRs trigger antiviral defense mechanism (Zheng et al. 2021), indeed the TLR pathway involves signal transduction through adaptor proteins, such as myeloid differentiation primary response 88 (MYD88) and TIR-domain-containing adapter-inducing IFN-β (TRIF) (Hadjadj et al. 2020; Zheng et al. 2021).Activation of RLRs induces confirmational changes in these receptors, leading to the exposure of their caspase activation and recruitment domains (CARDs). This allows them to interact with the mitochondrial antiviral signaling protein (MAVS), a key a **Figure 8** adaptor protein that mediates downstream immune response. Adaptor proteins MYD88, TRIF and MAVS facilitate the recruitment of ubiquitin ligases, including TNF receptor-associated factor 3 (TRAF3) and TRAF6 (Menezes et al. 2021). These ligases initiate intracellular signaling cascades that culminate in the activation of key transcription factors: IRF3 and IRF7, which drive the production of type I interferon (IFN-α/β), and NF- κB, a transcription factor responsible for inducing the expression of proinflammatory cytokines that contribute to immune system activation (Solanich et al. 2021; Qian Zhang et al. 2020).

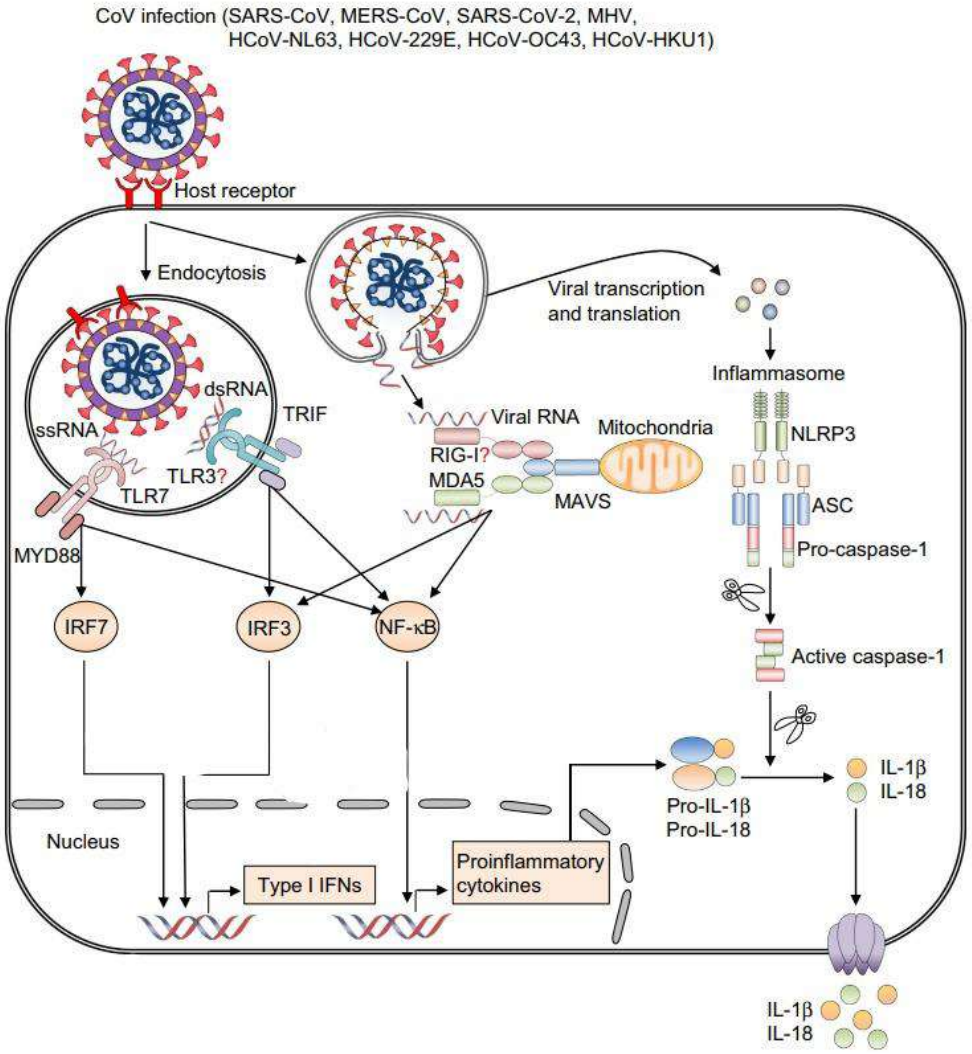

Figure 8 Innate immune sensing pathways in CoV infection.
Upon CoV infection, dsRNA and ssRNA are recognized by TLR3 and TLR7, respectively. Signaling downstream these TLRs induces activation of NF-κB to produce proinflammatory cytokines and phosphorylation of IRF3 and IRF7 to drive type I IFN production. Viral RNA in the cytosol is recognized by RIG-I and MDA-5, which then associate with MAVS, leading to activation of NF-κB and phosphorylation of IRF3. CoVs-induced inflammasome activation is also shown *(*adapted from Lee et al., 2020).

Type I IFN (IFN-α and IFN-β) play a crucial role in establishing an "antiviral state", restricting viral replication in infected cells and preventing viral spread to uninfected cells (Lei et al. 2020). These interferons exert their effects through autocrine and paracrine signalling, leading to: transcription of interferon stimulated genes (ISGs) that produce antiviral proteins interfering with viral replication and supressing viral protein synthesis; induction of apoptosis in infected cells, effectively limiting viral dissemination; activation and maturation of dendritic cells, which

subsequently initiate the adaptive immune response by stimulating T and B cells **Figure 9** (Bermejo-Jambrina et al. 2018; Paludan et al. 2021).

In addition to IFN signalling, another critical inflammatory pathway activated during coronavirus infections is the inflammasome response. Inflammasomes are multiprotein complexes responsible for the caspase-dependent release of pro inflammatory cytokines such as IL-1β and Il-18, which amplify immune responses (Mogensen 2009). Their assembly can be triggered by PAMPs or DAMPs (Kawai and Akira 2010). (Bermejo-Jambrina et al. 2018; Mogensen 2009; Paludan et al. 2021)

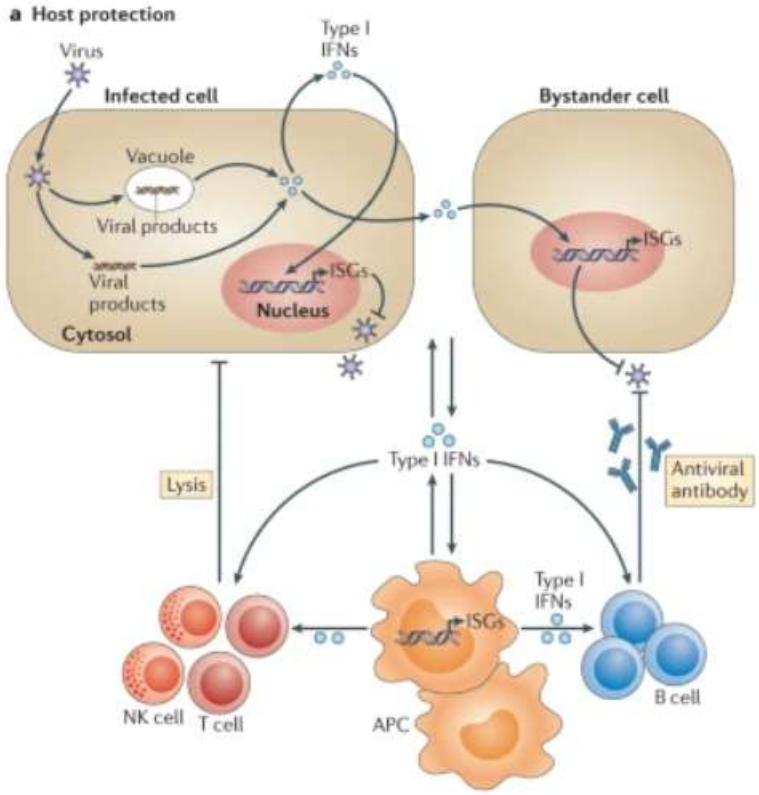

Figure 9 Production of type I IFNs in response to viral infection.
When vertebrates' cells get infected by a virus, they produce type I interferons (IFNs) in response. These IFNs trigger the activation of interferon-stimulated genes (ISGs) in both infected and nearby cells, helping to stop viral replication. IFNs also act on immune cells like antigen-presenting cells (APCs), boosting their ability to present antigens and enhance the immune response. Additionally, they strengthen adaptive immune cells B-cells, T-cells, and natural killer (NK)-cells helping them fight infection through antibody production and cytotoxic activity (Adapted from McNab et al. 2015).

2.2.2 Innate immune evasion of coronaviruses

Despite the robust innate immune response, SARS-CoV-2 has developed multiple strategies to evade immune detection and suppress antiviral signaling. Several viral proteins antagonize IFN

induction and signaling, thereby delaying the immune response and allowing efficient viral replication **Figure 10** (Asano et al. 2021). Notably, SARS-CoV-2 is more sensitive to type I IFNs than other pathogenic coronaviruses, making immune evasion a crucial aspect of its survival strategy. By targeting various components of the IFN pathway, including PRRs, adaptor proteins, and downstream signaling molecules, SARS-CoV-2 reduces IFN production and inflammatory cytokine responses. This dampening of immune activation enables viral persistence and efficient replication, ultimately contributing to disease pathogenesis (Lokugamage et al. 2020; Pfaender et al. 2020).

SARS-CoV-2 employs a range of strategies to suppress the IFN response, primarily through its NSPs and accessory proteins (Chowdhury et al. 2020). Several NSPs have been identified as key players in interfering with IFN signaling. For instance, NSP1 of SARS-CoV-2 can block the phosphorylation and nuclear translocation of IRF3, thereby impairing IFN-I transcription. Additionally, NSP1 of SARS-CoV-1/2 and MERS-CoV inhibit STAT1/2 phosphorylation, thereby blocking IFN-mediated antiviral signaling (Liao et al. 2020; Shi et al. 2020c).

Similarly, NSP3 suppresses IFN-β production by disrupting the assembly and stability of stimulator of interferon genes (STING) dimers, which are essential for downstream antiviral signaling (Jo et al. 2016; Schoggins et al. 2014). SARS-CoV-2 PL^{pro}, a component of NSP3, also directly inhibits MDA5 activation by de-ISGylating CARD (García-Sastre 2017).

In addition to NSPs, various SARS-CoV-2 accessory proteins also contribute to IFN suppression through distinct mechanisms. Indeed, ORF3a inhibits STAT1 phosphorylation and downregulates type I IFN receptor (IFNAR1) (Minakshi et al. 2009), whereas ORF3b prevents the nuclear translocation of IRF3, impairing IFN-1 induction (Konno et al. 2020). ORF4b of MERS-CoV is able to suppress NF- κB translocation into the nucleus, reducing proinflammatory cytokine production (Canton et al. 2018).

Another crucial strategy employed by coronaviruses to evade the host immune response consists in the masking of viral RNA to prevent detection by PRRs (Zhao, Wei, and Tao 2021).

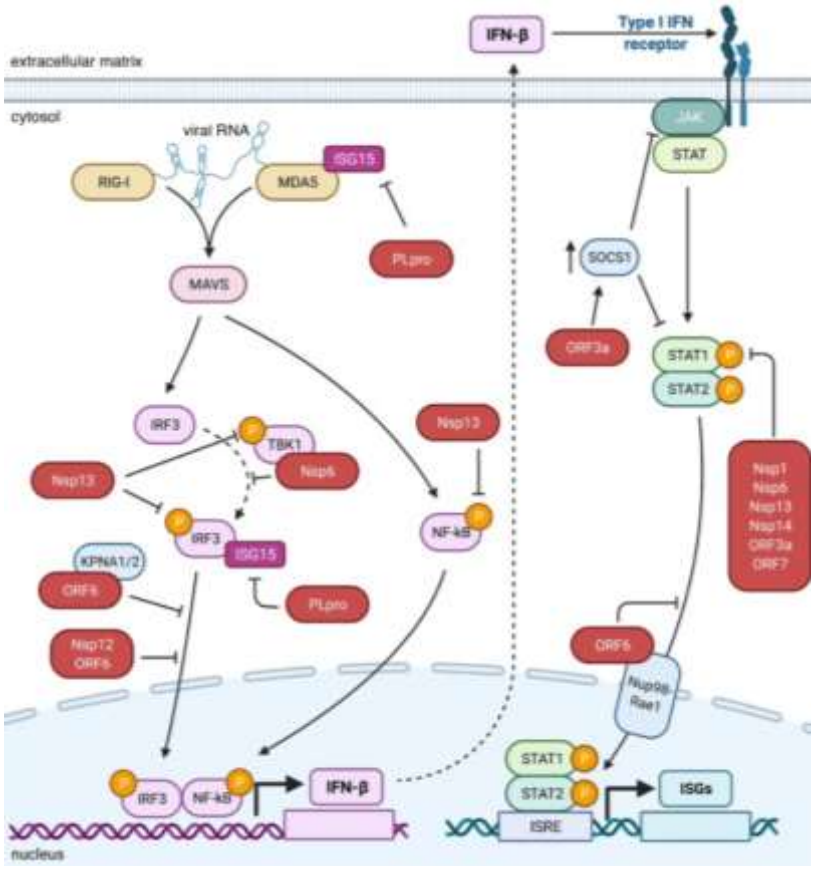

Figure 10 The diagram depicting SARS-CoV-2 inhibition of IFN induction and signaling.
The key components involved in IFN induction and signaling are depicted. Several SARS-CoV-2 proteins (highlighted in red) have been identified as inhibitors of distinct steps within this pathway (Adapted from Madden and Diamond 2022).

A suppressed or delayed IFN response is thought to contribute significantly to the severity of SARS-CoV-2 infections (Minkoff and tenOever 2023). Insufficient IFN production during the early stages of infection prevents effective viral clearance, allowing the virus to be replicated unchecked (Kang et al. 2021a; Minkoff and tenOever 2023). This ultimately leads to high viral titers that drive an excessive inflammatory response, commonly referred to as a cytokine storm (CS). This hyperinflammatory state is characterized by elevated levels of proinflammatory cytokines, including IL-1, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-17 and IL-18 as well as TNF-α, IFN- γ granulocyte colony-stimulating factor (G-CSF), and monocyte chemoattractant protein-1 (MCP-1) (Kang et al. 2021b). Excessive immune activation results in widespread infiltration of innate and adaptive immune cells into inflamed tissues potentially leading to severe tissue damage.

Additionally, systemic inflammation disrupts coagulation and vascular homeostasis, contributing to conditions such as capillary leak syndrome, thrombosis, disseminated intravascular coagulation. Ultimately, these events can lead to acute respiratory distress syndrome (ARDS), multi organ failure, and death (Chowdhury et al. 2020).

2.3 The PYHIN protein family

Pyrin and hematopoietic interferon-inducible nuclear (HIN) domain-containing (PYHIN) protein family represents a unique class of PRRs involved in innate immune responses (Erdemci-Evin et al. 2024). PYHIN proteins are IFN-inducible factors expressed exclusively in mammals. Humans encode four PYHIN proteins: Absent in Melanoma 2 (AIM2), γ-IFN-Inducible protein 16 (IFI16), IFN-Inducible protein X (IFIX), and Myeloid Nuclear Differentiation Antigen (MNDA) (Bosso and Kirchhoff 2020; Erdemci-Evin et al. 2024).

Structurally, PYHIN proteins share two key functional domains: an N-terminal pyrin domain (PYD) and at least one C-terminal hematopoietic interferon-inducible nuclear protein 200 (HIN200) domain. The PYD belongs to the larger superfamily of death domain (DD), which are defined by their characteristic alpha helical folding (Jin et al. 2012). These domains facilitate homo or heterotypic interactions with other PYD-containing proteins, regulating diverse cellular processes such as inflammation, immune signaling, apoptosis, and cell cycle progression **Figure 11** (Bosso and Kirchhoff 2020).

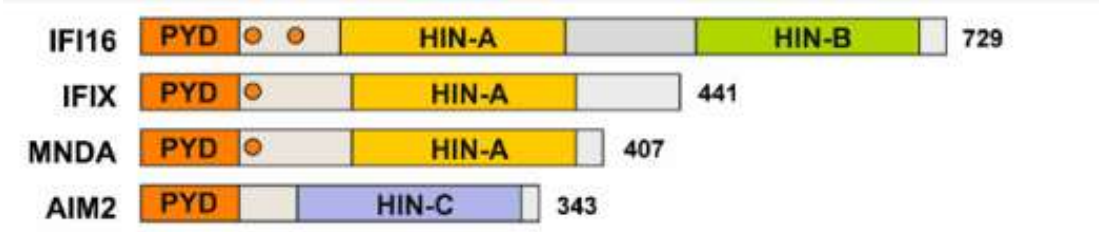

Figure 11 The human PYHIN protein family.
Each PYHIN family member possesses an N-terminal PYD domain and one or more HIN domains at the C-terminal. With the exception of AIM2, all PYHIN proteins harbor at least one nuclear localization signal (NLS) depicted by the orange circle (Adapted from Erdemci-Evin et al. 2024).

The HIN domain is exclusive to PYHIN family members and enables sequence-independent DNA binding through tandem oligonucleotide/oligosaccharide-binding (OB) folds. This binding occurs via electrostatic interactions between positively charged amino acid residues within the HIN domain and the phosphate backbone of DNA. (Jin et al. 2012; Kumar et al. 2022).

AIM2 is the most well-characterized member of the human PYHIN family and primarily resides in the cytoplasm, where it functions as a sensor for cytosolic dsDNA. Upon dsDNA recognition, AIM2 triggers a signaling cascade leading to inflammasome formation, caspase-1 activation, and the subsequent release of pro-inflammatory cytokines such as IL-18 and IL-1β, ultimately promoting apoptotic cell death Vanaja et al., 2015). Similarly, pathogen-derived nucleic acids can also be detected by IFI16 in both the nucleus and the cytoplasm. It has been proposed that IFI16 can differentiate between loosely packed to foreign DNA in the nucleus and the tightly packed chromatin structure of the host DNA **Figure 12** (Lamkanfi and Dixit 2014). Additionally, IFI16 has been shown to translocate to the cytoplasm upon viral infection, where it interacts with cyclic guanosine monophosphate adenosine monophosphate (cGAMP) synthase (cGAS) to activate the STING pathway, leading to IFN production (Vanaja, Rathinam, and Fitzgerald 2015).

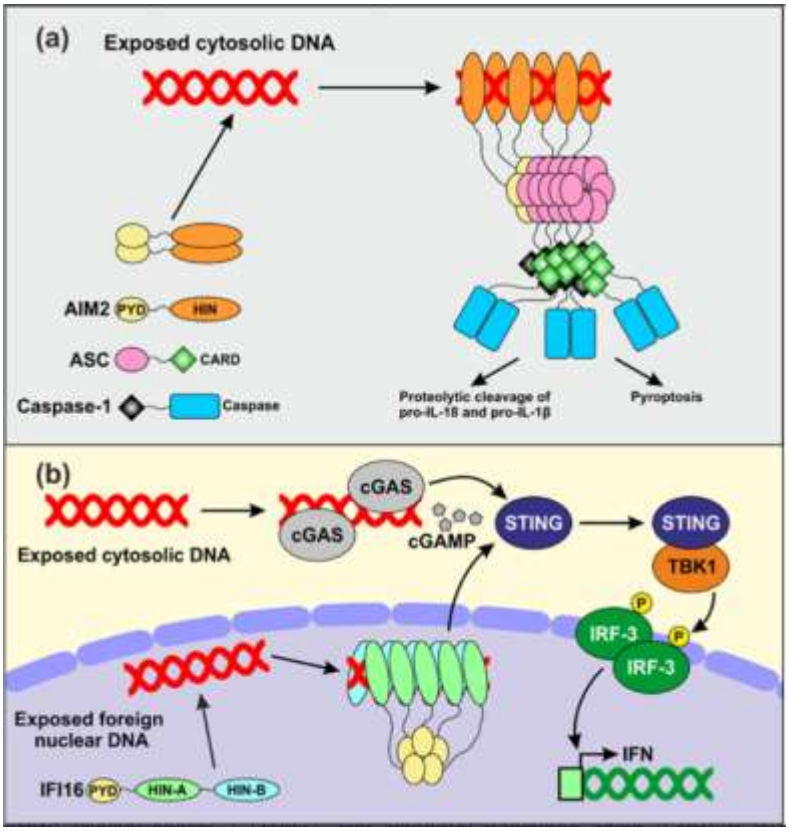

Figure 12 The diagram proposing the mechanisms of DNA sensing AIM2 and IFI16.
a) AIM2 forms inflammasome filament upon binding dsDNA, leading to caspase-1 activation, cytokine maturation, and pyroptosis. b) IFI16 and cGAS cooperate in detecting nuclear and cytosolic DNA, activating STING-TBK1, IRF3 signaling for IFN transcription (Adapted from Bosso and Kirchhoff 2020).

Beyond their role as innate DNA sensors, PYHIN proteins have been increasingly recognized for their ability to restrict viral replication through multiple mechanisms. IFI16 has been demonstrated

to inhibit viral transcription across several virus families, including herpesviruses, retroviruses, papillomaviruses, cytomegaloviruses, and hepatitis viruses. These antiviral effects are achieved through epigenetic modifications and interference with transcription factors such as SP1 (Bosso and Kirchhoff 2020).

Interestingly, bats exhibit genetic or functional loss of key components of the innate immune system, including the PYHIN gene family and the interferon inducible gene IFI16. It has been hypothesized that the loss of PYHIN locus occurred as an evolutionary adaptation to the high metabolic demands of flight (Tsagkogeorga et al. 2013). The intense energy expenditure required for sustained flight generates large amounts of reactive oxygen species (ROS), which can damage DNA and trigger excessive inflammation. To counteract this, the bat immune system has evolved to limit inflammasome activation, thereby preventing hyperinflammatory responses. This immune tolerance has allowed bats to coexist with numerous highly pathogenic viruses, including filoviruses (Ebola and Marburg), paramyxoviruses (Hendra and Nipah), and severe acute respiratory syndrome (SARS) related coronaviruses (SARS-CoV, MERS-CoV and SARS-CoV-2) (Ratsimandresy, Dorfleutner, and Stehlik 2013). Despite harboring high viral loads in their tissues and sera, bats typically remain asymptomatic, whereas the same viruses often provoke severe immune dysregulation in humans (B. Wang et al. 2020).

The loss of PYHIN genes in bats may explain their ability to act as reservoirs for various viruses without experiencing overt diseases (Ahn et al. 2016). While bats retain other cytosolic DNA sensors, PYHIN proteins uniquely drive inflammasome activation, a key process in controlling inflammation during viral infection (Ahn et al. 2016). Consequently, the deletion of PYHIN locus may contribute not only to the high viral diversity observed in bats but also to their ability to remain asymptomatic upon infection (B. Wang et al. 2020).

2.3.1 Interferon-γ- inducible protein 16

Interferon-γ- inducible protein 16 (IFI16) is a member of the PYHIN protein family, involved in the regulation of several biological processes including DNA damage responses, apoptosis, cell growth, and regulation of cell differentiation (X. Fan, Jiao, and Jin 2022a). Structurally, IFI16 contains two HIN domains (A and B) separated by a spacer region, allowing it to bind both dsDNA and ssDNA, as well as RNA in a sequence independent manner **Figure 13**. Additionally, IFI16 possesses an N terminal PYRIN domain that facilitates protein-protein interactions, thereby influencing immune signaling pathways (Caneparo et al. 2018).

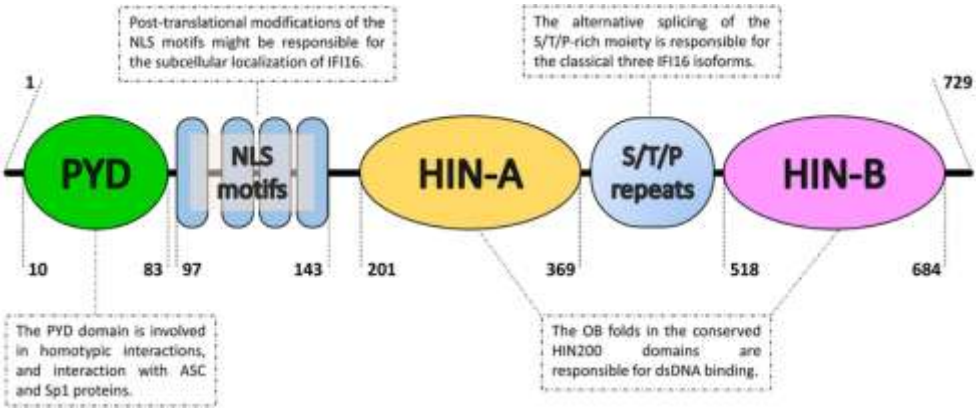

Figure 13 Schematic illustration of IFI16 protein structure.
Residues 1-83 constitute the N-terminal PYD domain, whereas residues 201-369 and 518-684 form the HINA and HINB domains, respectively. At the N-terminal the NLS motif is present and between the two HIN domains there is a S/T/P-rich seven amino acid repeat motifs (Adapted from Caneparo et al. 2018).

IFI16 was originally classified as a nuclear protein due to the presence of a NLS. However, there is now evidence that IFI16 can shuttle between the nucleus and cytoplasm, through the precise mechanisms regulating this redistribution remain incompletely understood (Xiaobo Chang et al. 2019a). Studies have shown that IFI16 translocate to the cytoplasm in response to various stimuli. For instance, ultraviolet B (UVB) exposure induces IFI16 relocalization in Keratinocytes, while viral infections, including those caused by Epstein bar virus (EBV), herpes simplex virus type 1 (HSV-1), and cytomegalovirus (CMV), trigger its cytoplasmic accumulation. Furthermore, during Kaposi sarcoma associated herpes virus (KSHV) infection, IFI16, along with ASC and procaspase-1, migrates to the cytoplasm, where it assembles into an inflammasome complex, leading to caspase-I activation and IL-1β processing (F. Zhang, Yuan, and Ma 2021).

While the role of IFI16 in sensing DNA viruses is well established, its function during RNA virus infections remains less defined (F. Zhang, Yuan, and Ma 2021). Recent evidence suggests that IFI16 can inhibit influenza A virus (IAV) replication by interacting with retinoic acid inducible gene I (RIG-I), a cytosolic RNA sensor belonging to RIG-I-like receptor (RLR) family. Upon recognizing PAMPs during RNA virus infection, RIG-I triggers an innate immune response characterized by IFN-I production and antiviral gene activation. Notably, IFI16 has been shown to enhance RIG-I transcription and directly bind the viral RNA genome during IAV infection, thereby amplifying the antiviral response. Moreover, IFI16 expression is upregulated following IAV infection and has been implicated in pyroptosis induction in alveolar epithelial cells, a mechanism that potentially limits viral dissemination (Choubey 2022; Z. Fan et al. 2023).

Beyond IAV, IFI16 has been reported to restrict the replication of other RNA viruses through distinct mechanisms. In chikungunya virus (CHIKV) infection, IFI16 interacts directly with the viral genomic RNA inhibiting its replication and maturation, functioning as an antiviral restriction factor (Veeranki and Choubey 2012). Similarly, in porcine reproductive and respiratory syndrome virus 2 (PRRSV2) infection, IFI16 facilities antiviral signaling by binding MAVS, thereby promoting MAVS-mediated IFN-I production (Choubey 2022; Z. Fan et al. 2023). These findings highlight the multifaceted role of IFI16 in antiviral immunity, not only in DNA virus infections but also in modulating host responses to RNA viruses **Figure 14.**

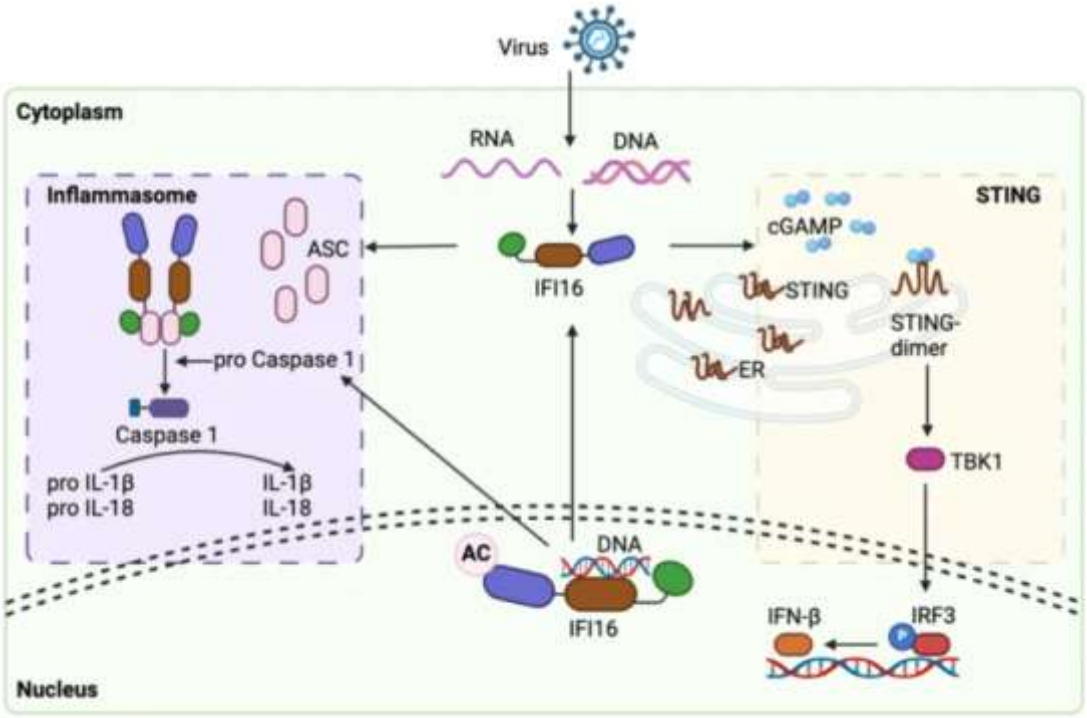

Figure 14 The diagram showing the mechanisms of action of IFI16.
Upon sensing DNA or RNA, the NLS of IFI16 is acetylated and the protein translocate to the cytoplasm, where it assembles into an inflammasome complex, leading to caspase-I activation and IL-1β processing. Also, in the cytoplasm IFI16 interacts with cGAS to activate the STING-TBK1, IRF3 pathway, leading to IFN production (Adapted from Xindi Chang et al. 2024)

3.OBJECTIVES OF THE THESIS

Due to their ability to fly great distances, bats have evolved over time a unique immune system to limit collateral damage caused by by-products of elevated metabolic rate (Zhang et al., 2012). These changes have contributed to make bats natural reservoirs for various zoonotic viruses, including coronaviruses, without exhibiting severe disease symptoms (Ratsimandresey, Dorfleutner, and Stehlik 2013b). One key aspect of bats immune adaptation is the loss of the PYHIN gene protein family, which encompasses sensors of intracellular self and foreign DNA and activator for the inflammasome and IFN response. Among PYHIN proteins, the interferon-γ-inducible protein 16 (IFI16) plays a role in the innate immune response by acting as a DNA sensor in inflammasome signaling and as viral restriction factor for DNA viruses (B. Yang et al. 2018). In addition, emerging evidence suggests IFI16 in the sensing and restriction of RNA viruses (Z. Li et al. 2024).

Despite the emerging evidence of IFI16 involvement in the control of RNA virus replication, little is known about its function in coronavirus replication. According to this background, we decided to investigate the role of the IFI16 protein in human coronavirus (HCoV) infection.

To gain a boarder insight into IFI16 activity on HCoVs replication we decided to work with two viral species: the low pathogenic α-HCoV NL63 and the highly pathogenic β-HCoV SARS-CoV-2. We characterized SARS-CoV-2 and NL63 infection in a gold-standard rhesus macaque epithelial kidney cell line, named, LLC-MK2, that supports efficient replication of both viruses. Particularly, we generated LLC-MK2 cells knockout for the IFI16 gene (IFI16KO) and their transfected control (TC).

The studies described in this thesis aimed at understanding the molecular events involving IFI16 in HCoV sensing and characterizing the host response. By providing mechanistic insights into the role of IFI16 in HCoV infection, this study will contribute to a better understanding of host-pathogen interactions and may offer new therapeutic strategies for controlling viral diseases

4. MATERIALS AND METHODS

4.1 Biosafety statements and facility

All experiments with live NL63 were performed in a biosafety level 2 (BSL2) facility at the Università del Piemonte Orientale, Novara, Italy. Experiments involving live SARS-CoV-2 were performed in biosafety levels 3 (BSL3) facility at the Università degli Studi di Milano, Milan, Italy. The standard operating procedures for both BSL2 and BSL3 facilities were approved by relevant authorities in Italy. All personnel underwent comprehensive training prior to beginning work in the BSL2 and BSL3 facilities.

4.2 Cell lines and viruses

Rhesus monkey kidney epithelial cells (LLC-MK2, ATCC: CCL-7) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at 37 °C with 5% CO_2.

NL63 (NR-470 also referred to as Amsterdam I, Bei Resources) was kindly provided by Lucia Nencioni (University of Rome, La Sapienza, Rome, Italy). HCoV-NL63 was propagated and maintained in our laboratory on Caco-2 cells and titrated as plaque-forming units by immunofluorescence staining of the nucleocapsid protein.

All the experiments on SARS-CoV-2 have been performed in collaboration with Serena Delbue (University of Milan). SARS-CoV-2 was isolated from a nasal-pharyngeal swab positive for SARS-CoV-2. The isolated SARS-CoV-2 strain belongs to the B.1 lineage, carrying the characteristic spike mutation D614G. The B.1 lineage is the large European lineage, the origin of which roughly corresponds to the Northern Italian outbreak in early 2020. The complete nucleotide sequence has been deposited at GenBank and GISAID (accession Nos. MT748758.1 and EPI_ISL 584051, respectively). SARS-CoV-2 was propagated and maintained in the Laboratory of Molecular Virology at the University of Milan using Vero E6 cells. SARS-CoV-2 was titrated as plaque-forming units by plaque assay.

4.3 Generation of IFI16KO LLC-MK2 cells

IFI16KO and TC cells were generated using CRISPR/Cas9 technology, as previously described (Albertini et al. 2018; Lo Cigno et al. 2020). Briefly, vesicular stomatitis virus G (VSV-G)-pseudotyped lenti-CRISPR virions were produced by transfecting HEK293T cells with the following plasmids: CRISPR/Cas9 vector and Virapower lentiviral packaging mix (Invitrogen).

Viral supernatants were collected after 72 h and used to transduce LLC-MK2 cells by infection in the presence of 10 μg /ml polybrene. Transduced cells were selected with puromycin (10 μg/ml) at 2 days post-transduction. After two weeks a single cell suspension culture was established using limiting dilution. After three weeks individual clones were subjected to western blotting to confirm absence of the targeted gene products.

4.4 HCoV-NL63 and SARS-CoV-2 infection

Subconfluent TC and IFI16KO LLC-MK2 cells were infected with the appropriate MOI and virus absorption was allowed for 2 hours before changing media. Every infection with NL63 was performed at MOI 1, while with SARS-CoV-2 at MOI 0.5 at 34°C and 5% CO_2.

4.5 Quantitative real-time PCR

qRT-PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories), as previously described (Albertini et al. 2018). Amplification of subgenomic *N*, *E*, and *S* genes was carried out using the following reaction conditions: 2 min at 95°C, 40 cycles of 5 s at 95°C, 10 s at 55°C, 20 s at 72°C, followed by 5 min at 72°C and 10 min at 4°C. Total RNA was extracted using TRI Reagent (Sigma-Aldrich), and 1 μg was retrotranscribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Reverse-transcribed cDNAs were amplified in duplicate using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). *GAPDH* was used as a housekeeping gene for normalization of cDNA levels. The relative normalized expression after stimulation was calculated as fold change over control using the formula = 2 - Δ(ΔCT) where ΔCT = CTtarget—CTGAPDH and Δ(ΔCT) = ΔCTstimulated – ΔCTcontrol.

4.6 Droplet digital PCR

ddPCR was performed using the one-step RT-ddPCR advanced kit for probes (Bio-Rad Laboratories) according to the manufacturer's instructions on the Bio-Rad QX200 (Bio-Rad Laboratories). PCR cycling conditions were as follows: 60 min reverse transcription at 50°C, 10 min enzyme activation at 95°C, 30 s denaturation at 94°C (40 cycles or 45 cycles, for SARS-CoV-2 and NL63, respectively), 1 min annealing/extension cycle at 55°C (40 cycle for SARS-CoV-2 and OC43 or 45 cycles for NL63, respectively; ramp rate of 2-3°C/s), 10 min enzyme deactivation at 98°C and a 30-min hold at 4°C. Positive and negative droplet readings were performed using a Bio-Rad Droplet Reader (Bio-Rad Laboratories).

4.7 Immunoblotting

Whole-cell protein extracts (15 µg) were prepared, separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to immunoblot analysis as previously described (Lo Cigno et al. 2020). The following primary antibodies were used: rabbit polyclonal antibodies (pAbs) anti-IFI16 (in-house made, 1:1000) and anti-NL63 N (40641-T62; SinoBiological, 1:2000), rabbit mAbs anti-SARS-CoV-2 N (40143-R001; SinoBiological, 1:1000), mouse mAbs anti-SARS-CoV-2 S (GTX632604; GeneTex, 1:500), and anti-GAPDH (60004-1-Ig; Proteintech, 1:10000). Immunocomplexes were detected using sheep anti-mouse or donkey anti-rabbit immunoglobulin antibodies conjugated to horseradish peroxidase (HRP) (GE Healthcare Europe GmbH) and visualized by enhanced chemiluminescence (Super Signal West Pico; ThermoFisher Scientific) using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories).

4.8 Immunofluorescence

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. To enable antibodies to cross the cellular membranes, permeabilization was performed with 0.5% Triton X-100 in PBS 1X for 20 min on ice. After, to reduce unspecific binding of antibodies to non-target structures, blocking was performed with 1% Normal Goat Serum (NGS) in PBS 1X for 30 min at room temperature. This was followed by O/N incubation at 4°C with the primary antibody diluted in blocking solution. After, several washings were performed with PBS 1X + 0.05% Tween-20 to remove the unbound antibody, and then 1-hour incubation with secondary antibody in the dark was performed; in addition, 4',6-diamidino-2-phenylindole (DAPI) was added to stain cells' nuclei. After a few washes, coverslips were mounted on slides using anti-fade mounting medium and visualized using the Leica SP8 lightning confocal microscope (Leica Microsystems). The percentage of infected cells expressing N or dsRNA for each cell line were normalized to the total number of DAPI-positive cells. Image analysis was carried out using the LAS X software (Leica Microsystems), and values were expressed as mean ± SD (error bars).

The following primary antibodies were used: mouse mAbs anti-IFI16 (sc-8023; Santa Cruz, 1:100) and anti-dsRNA (MABE1134; Sigma-Aldrich), rabbit mAb anti-SARS-CoV-2 N (40143-R001; SinoBiological, 1:2000), rabbit pAb anti-NL63-N (40641-T62; SinoBiological, 1:200).

4.9 Proximity Ligation Assay (PLA)

Proximity Ligation Assay (PLA) was performed to detect protein-protein interactions between N and IFI16, in infected and mock treated LLC-MK2 cells at various time points pi. Cells grown on

coverslips were fixed with 4% PAF for 15 min, washed thrice with PBS and permeabilized with 0.5% Triton X-100 on ice for 20 mins to allow access of antibodies to intracellular targets. Coverslips were then transferred to a humidified chamber and blocked with Duolink® blocking solution at 37 °C for 1 h to minimize non specific binding. Primary antibodies against N, and IFI6 were diluted in Duolink® antibody diluent and applied to the samples, which were incubated in a humidified chamber for 24 hours. Following the washes with Duolink® wash buffer A, the sample were incubated with Duolink® PLA PLUS and MINUS probes, secondary antibodies conjugated to complementary oligonucleotides recognizing the species of the primary antibodies at 37 °C for 1 h. Ligation was then performed by incubating the samples with ligase and Duolink® ligation buffer at 37 °C for 30 min, enabling circularization of oligonucleotides only when the target proteins were in close proximity. This was followed by rolling circle amplification with Duolink® amplification buffer and polymerase at 37 °C for approximately 100 min in the dark, during which fluorescently labeled detection oligonucleotide hybridized to the amplified products. PLA signals were visualized using fluorophore: green (excitation ~ 495 nm, emission ~ 527 nm) and red (excitation ~ 594 nm, emission ~ 624 nm), corresponding to the secondary probes for mouse and rabbit primary antibodies, respectively. After amplification, coverslips were washed with Duolink® wash buffer B and counterstained with DAPI to visualize nuclei. Finally, coverslips were mounted using Duolink® in situ mounting medium with DAPI, and image using confocal microscopy. PLA signals appeared as discrete fluorescent puncta, representing sites where target proteins or modifications were in close molecular proximity, and were analyzed relative to nuclear staining to assess interaction dynamics across experimental conditions.

4.9 Statistical analysis

All statistical tests were performed using Graph-Pad Prism version 7.00 for Windows (GraphPad Software). The data are stated as mean ± standard deviation (SD). For comparisons consisting of two or more groups, means were compared using unpaired Student's t-test, two-tailed Student's t-test, Mann-Whitney test or two-way ANOVA. Differences in p-value < 0.05 were considered statistically significant.

5. RESULTS

5.1 LLC-MK2 cells support both NL63 and SARS-CoV-2 replication

We screened a panel of cell lines to identify one that is permissive to both SARS-CoV-2 and NL63. Among these, we selected LLC-MK2, a rhesus macaque epithelial kidney-derived cell line, as it supports infection by both viruses, which utilize the ACE-2 receptor for cell entry.

As SARS-CoV-2 replicates within approximately 24 h, whereas NL63 takes around 72 h, we chose time points to capture early, intermediate, and late stages for each virus (*i.e.*, 16, 24, and 48 h for SARS-CoV-2 and 24, 72, and 144 h for NL63). Viral replication was assessed by measuring viral genome copy numbers in the culture supernatants of SARS-CoV-2-infected LLC-MK2 cells by droplet digital (dd)PCR **Figure 15a**. At 16 hours post infection (hpi), viral RNA was detectable at low levels, but by 24 hpi, a noticeable rise was observed, which became even more pronounced at 48 hpi **Figure 15a**. , indicating the release of new virions. Similarly, LLC-MK2 cells also support the replication of NL63, albeit with a longer replication timeline compared to SARS-CoV-2. Indeed, infections with NL63 revealed a progressive and substantial increase in viral RNA levels in the supernatants overtime, as quantified by ddPCR targeting the N gene **Figure 15b.** At 24 hpi, viral RNA was present at modest levels, but this increased significantly by 72 hpi and continued to rise sharply by 144 hpi **Figure 15b**. In parallel, intracellular viral gene mRNA levels were examined by qRT-PCR targeting the genomic *ORF1ab* and the subgenomic *N*, *E*, and *S* **Figure 15c, d**. In SARS-CoV-2-infected cells, intracellular viral RNA levels increased as early as 16 hpi and remained stable up to 48 hpi **Figure 15c**.

In contrast, NL63-infected cells showed a delayed but prolonged increase in intracellular viral RNA levels, starting at 72 hpi and continuing until 144 hpi **Figure 15d**. Collectively, these findings establish that LLC-MK2 cells are permissive to both SAR-CoV-2 and NL63, allowing for robust viral replication, transcription of multiple viral genes, and release of infectious particles, providing a valuable *in vitro* model for studying HCoV infections.

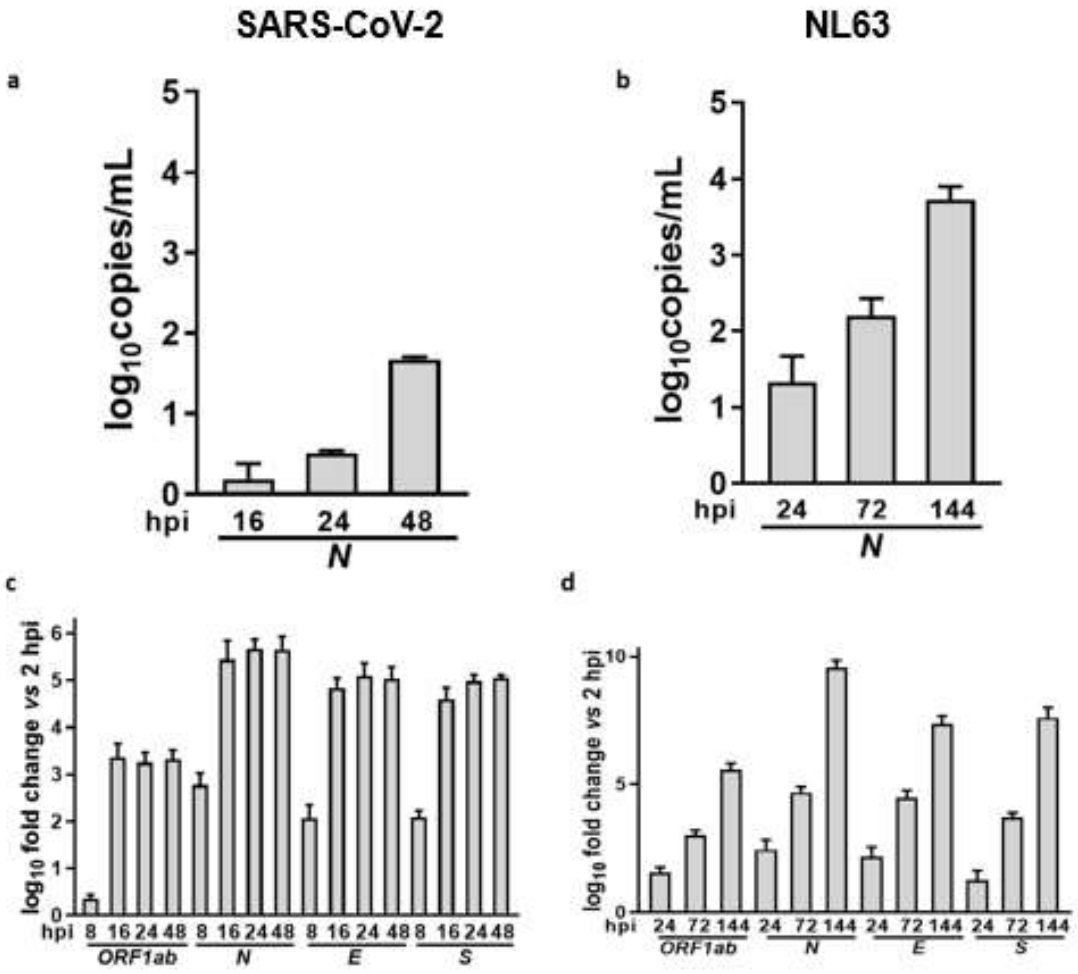

Figure 15 LLC-MK2 cells support both SARS-CoV-2 and NL63 viral replication.
a, b LLC-MK2 cells were infected with SARS-CoV-2 **(a)** or NL63 **(b)** at an MOI of 0.5 and 1, respectively. Cell-free supernatants were harvested at the indicated hpi, and the absolute quantification of viral RNA (N) was determined by ddPCR. Data are presented as mean ± SD (two-way ANOVA, n = 3 biological replicates). **c, d** LLC-MK2 cells were infected with SARS-CoV-2 **(c)** or NL63 **(d)** at an MOI of 0.5 and 1, respectively. Cell monolayers were harvested at the indicated hpi, and total RNA was extracted. Transcripts of the indicated viral genes were assessed by qRT-PCR. The data are normalized against GAPDH levels and presented as a fold-change relative to 2 hpi. Data are presented as mean ± SD (two-way ANOVA, n = 3 biological replicates).

5.2 IFI16 restricts SARS-CoV-2 and supports NL63 replication

To investigate the role of IFI16 during HCoV replication, we generated LLC-MK2 clones with stable knockout of the *IFI16* gene, referred to as LLC-MK2 IFI16KO cells (IFI16KO), as well as control cells transfected with non-targeting sgRNAs, named LLC-MK2-transfected control (TC cells) **Figure 16**.

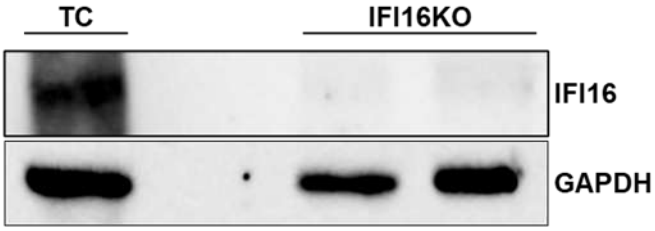

Figure 16 Knock-out of IFI16 in LLC-MK2 cells.
Total cell extracts from TC and two independent clones of IFI16KO cells were subjected to immunoblot analysis using an anti-IFI16 polyclonal antibody. GAPDH immunodetection was used as a loading control. One representative Western blot from three independent experiments is shown.

In the absence of IFI16, we observed an increase in the expression levels of SARS-CoV-2 structural proteins. Indeed, the N protein was detectable as early as 8 hpi, with its expression markedly increasing at 16 hpi and continuing to rise at later time points **Figure 17a**. Of note, SARS-CoV-2-infected TC cells displayed detectable levels of the N protein starting from 16 hpi **Figure 17c**. As shown in **Figure 17a**, the S protein was already detectable at 16 hpi and to a higher extent at 24 and 48 hpi in SARS-CoV-2-infected IFI16KO cells, while in TC cells similarly infected **Figure 17c** it could only be detected at 48 hpi. When we examined infected NL63 cells, the N protein expression levels were reduced in IFI16KO compared to TC cells **Figure 17b, d**. Unfortunately, due to the lack of suitable antibodies against the S protein of NL63, we could not assess its expression levels in NL63-infected cells. Intriguingly, IFI16 protein expression levels in TC cells remained unchanged upon infection with either SARS-CoV-2 or NL63 **Figure 17c, d**.

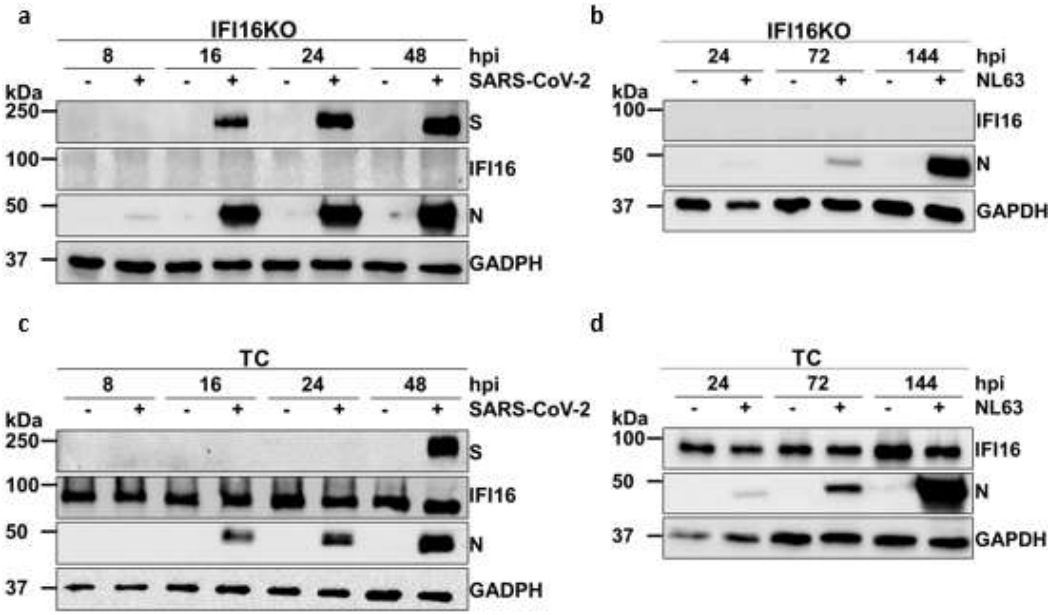

Figure 17 IFI16-depleted cells display enhanced SARS-CoV-2 and reduced NL63 replication rates.
a,b IFI16KO cells were mock-infected (-) or infected (+) with SARS-CoV-2 **(a)** or NL63 **(b)** at an MOI of 0.5 and 1, respectively. At the indicated hpi, total cell extracts were subjected to immunoblot analysis for the detection of the indicated proteins (n = 3 biological replicates). **c, d** TC cells were infected and immunoblotted as described in the legend for panels **(a)** and **(b)** (n = 3 biological replicates).

Next, the replication rate of both viruses was assessed in infected IFI16KO *vs* TC culture supernatants by ddPCR at different time points pi **Figure 18**. In the context of SARS-CoV-2, IFI16KO cells exhibited a significantly higher viral RNA load compared to TC cells at both 24 and 48 hpi **Figure 18a**. In contrast, at 144 hpi, NL63-infected IFI16KO cells showed a significant reduction of viral RNA levels compared to TC cells **Figure 18b**. Taken together, these findings illustrate that the absence of IFI16 has a different impact on the replication of highly *vs* low pathogenic HCoVs. Indeed, the replication of the highly pathogenic SARS-CoV-2 is enhanced in the absence of IFI16, while conversely the replication rate of the low pathogenic NL63is reduced.

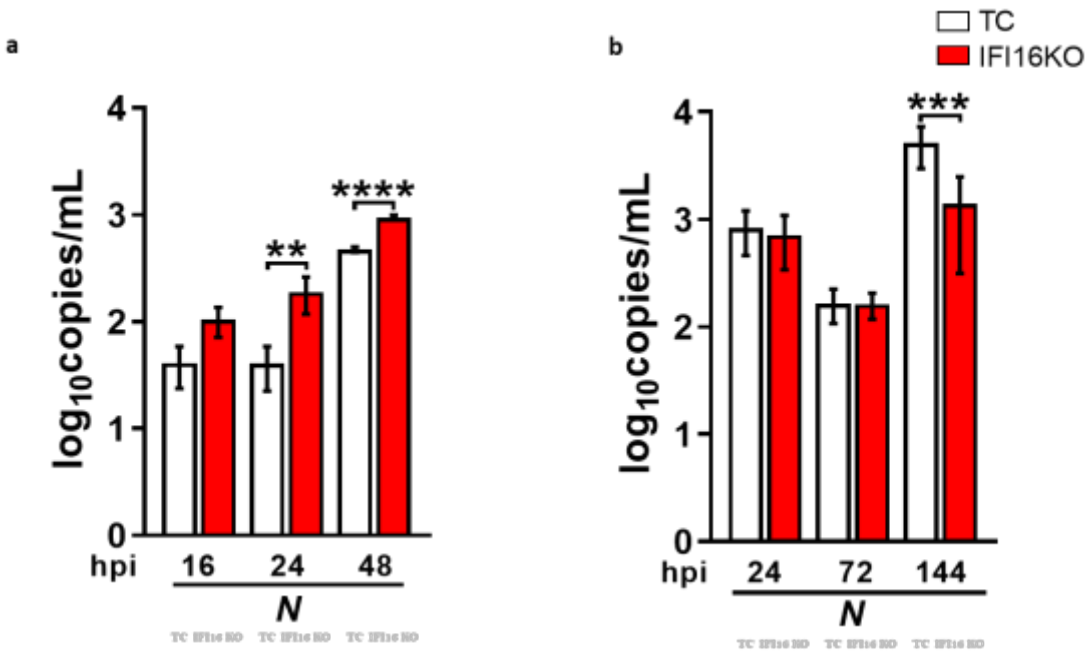

Figure 18 TC and IFI16KO cells were infected with SARS-CoV-2 and NL63
a, **b** TC and IFI16KO cells were infected with SARS-CoV-2 (**a**) or NL63 (**b**) at an MOI of 0.5 and 1, respectively. Cell-free supernatants were harvested at the indicated hpi, and the absolute quantification of viral RNA was determined by ddPCR. Data are presented as mean ± SD (two-way ANOVA, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n = 3 biological replicates).

5.3. Nuclear IFI16 translocates to the cytoplasm upon SARS-CoV-2 infection

To investigate the subcellular localization of IFI16 during SARS-CoV-2 infection, immunofluorescence analysis was performed using antibodies against IFI16 and the viral N protein. In mock-infected LLC-MK2 cells, IFI16 was predominantly localized in the nucleus, as expected. Upon infection with SARS-CoV-2, a notable redistribution of IFI16 from the nucleus to the cytoplasm was observed, particlulary at 16 hpi. This translocation coincided with with the accumulation of viral N protein, suggesting that cytoplasmic IFI16 may interact with viral components during infection **Figure 19a**. Line profile analysis of fluorescence intensity confirmed the partial overlap between IFI16 and N protein signals, indicating potential proximity or interaction in the cytoplasmic compartment. To further validate this potential interaction, a proximity ligation assay (PLA) was performed using anti-IFI16 and anti-N antibodies. In mock-infected cells, PLA signals were negligible, consistent with the absence of viral N protein and lack of close proximity to IFI16. In contrast, SARS-CoV-2 infected cells displayed distinct PLA puncta in the cytoplasm, with the number and intensity of these puncta increasing over time, reaching a

peak at 16 hpi. The discrete nature of PLA signals indicates that IFI16 and N protein are in close molecular proximity (<40 nm), supporting a potential direct or indirect interaction during infection **Figure 19b** . These observations complement the IF data, demonstrating not only the cytoplasmic relocalization of IFI16 but also its spatial proximity to vital components, suggesting a functional relevance in the host-virus interplay. Overalll, these findings demonstrate that SARS-CoV-2 infection triggers a dynamic relocalization of nuclear IFI16 to the cytoplasm, which may represent an early host response mechanism or a viral counterceration strategy **Figure 19**.

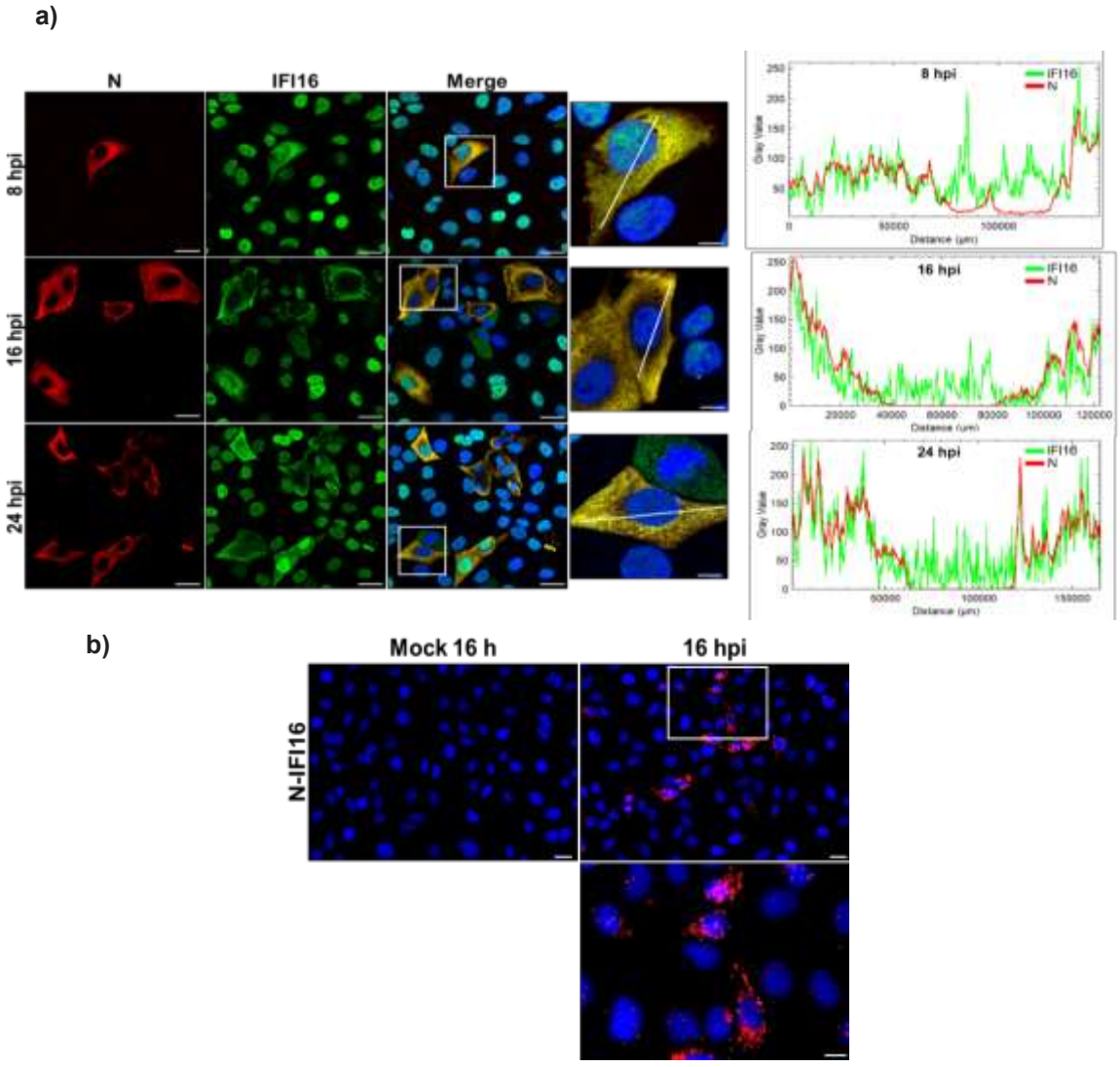

Figure 19 subcellular redistribution of IFI16 upon SARS-CoV-2 infection. LLC-MK2 cells were mock-infected or infected with SARS-CoV-2 for 16 h. Cells were fixed and stained with antibodies against IFI16 (green) and viral N protein (red), with nuclei visulaized using DAPI (blue). Magnified images show cytoplasmic translocation of IFI16 in

infected cells, partially colocalizing with N protein. Scale bars: 20 μm (overview), 10 μm (zoomed view). Proximity ligation assay (PLA) was performed using anti-IFI16 and anti-N antibodies to detect potential interactions.

5.4. IFI16 differentially modulates HCoVs replication

To gain more insight into the dual role of the IFI16 protein in the modulation of HCoVs infection we also assessed N protein and double-stranded RNA (dsRNA) expression levels, an intermediate of viral replication, by immunofluorescence analysis in both TC and IFI16KO cells infected with SARS-CoV-2 or NL63 **Figure 20**. Upon infection with SARS-CoV-2, IFI16KO cells exhibited a pronounced increase in viral markers, as evident by the elevated accumulation of both N protein and dsRNA at 16 and 24 hpi **Figure 20a, c**. In contrast, NL63 infection displayed an opposite trend, with markedly reduced viral signals in IFI16-deficient cells, particularly noticeable at the later time point of 144 hpi **Figure 20b, d**.

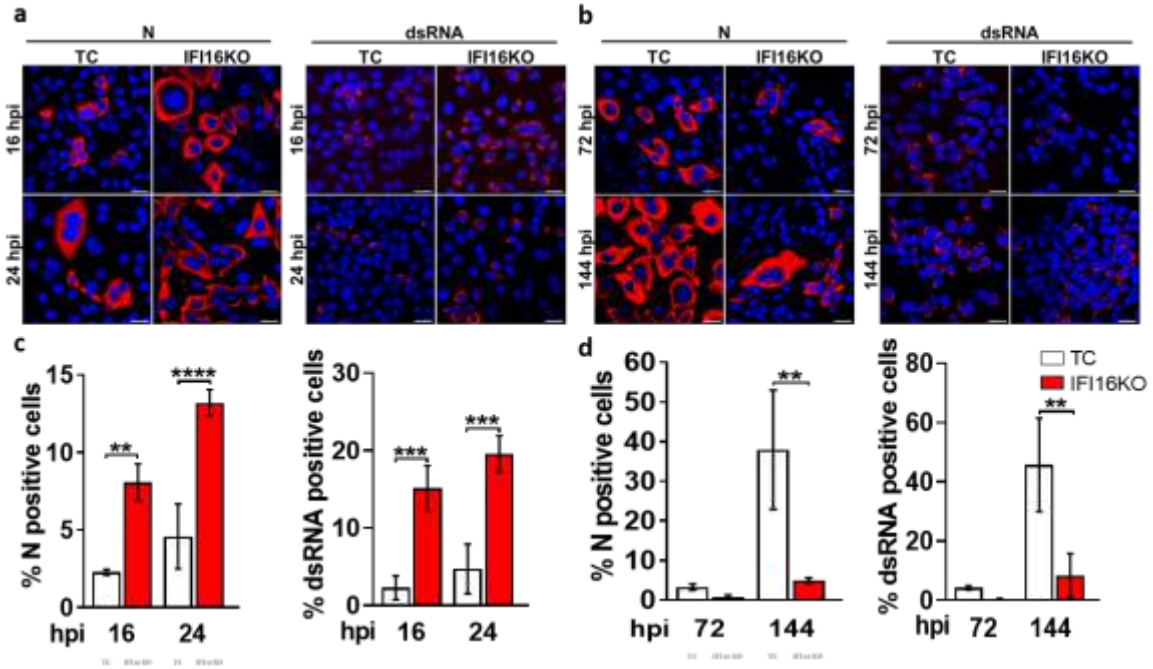

Figure 20 Immunofluorescence analysis for N or dsRNA, both labeled in red. Nuclei were visualized by DAPI (blue). **a, b** TC and IFI16KO cells were infected with SARS-CoV-2 **(a)** or NL63 **(b)** at an MOI of 0.5 and 1, respectively. At the indicated hpi cells were processed for immunofluorescence analysis for N or dsRNA, both labeled in red. Nuclei were visualized by DAPI (blue). Images were captured by confocal microscopy and are representative of six different fields from three independent experiments. Scale bars: 25 μm. **c,d** Images shown in panels **(a)** and **(b)** were quantified using THUNDER Imager 3D Live Cell (Leica Microsystems). Data are presented as mean ± SD of the percentage of cells positive for N or dsRNA per total cells. The analysis was performed in six different fields from three independent experiments (two-way ANOVA test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

The higher replication rate of SARS-CoV-2 in IFI16-depleted *vs* TC cells was confirmed by plaque assay titration of SARS-CoV-2 infectious viral particles in VeroE6 cells using cell culture supernatants from TC or IFI16KO infected cells **Figure 21**. At all tested time points, IFI16KO cells produced more visible plaques, indicating higher viral spread and cytopathic effect compared to TC cells **Figure 21a.**

Quantification of plaque-forming units (PFU) further supports this observation, indeed in **Figure 21b** we observed significantly elevated viral titers in IFI16KO cells compared to TC cells at 48hpi (569,000 *vs* 243,000 PFU/mL).

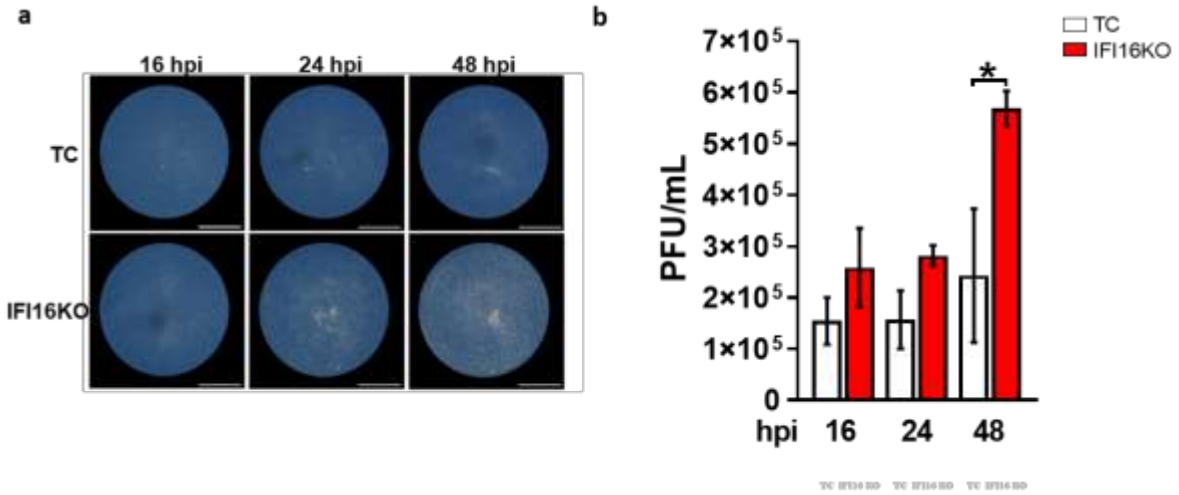

Figure 21 Plaque assay analysis on VeroE6 cells.
a TC and IFI16KO cells were infected with SARS-CoV-2 (MOI 0.5). Supernatants were collected at 16, 24, and 48 hpi and titrated by standard plaque assay analysis on VeroE6 cells. The cytopathic effect (CPE) on cultures was monitored by optical microscopy to demonstrate the extent of infection. Representative images of three independent experiments are shown. **b** Plaques were counted at 48 hpi using a microplate reader Cytation 5 (Biotech) and expressed as plaque forming unit (PFU)/mL. Data is presented as mean ± SD (unpaired two-sided Student's t-test. *$p < 0.05$, n = 3 biological replicates). Scale bar: 10.000 μm.

5.4. IFI16 regulates HCoV replication independently of innate immunity

To rule out that the absence of IFI16 affected the innate immune response to HCoV infections, potentially influencing viral replication, we evaluated *IFN-β* mRNA expression levels by RT-qPCR in TC and IFI16KO cells infected with either SARS-CoV-2 **Figure 22a** or NL63 **Figure 22b**, along with the two-IFN-inducible genes *Mx1* and *IFIT1*. Neither infection resulted in a significant induction of *IFN-β* expression or downstream genes. The slight induction of the *IFN-β* gene transcription in infected TC cells observed at the later time points was significantly reduced in IFI16KO cells and it could be probably due to the response of the cells to the virus-induced damage.

These results suggest that the activation of the innate immune response is minimal in IFI16-proficient cells, at least in terms of IFN production, and only slightly affected in IFI16-deficient cells, suggesting that the role of IFI16 in viral replication is IFN-independent.

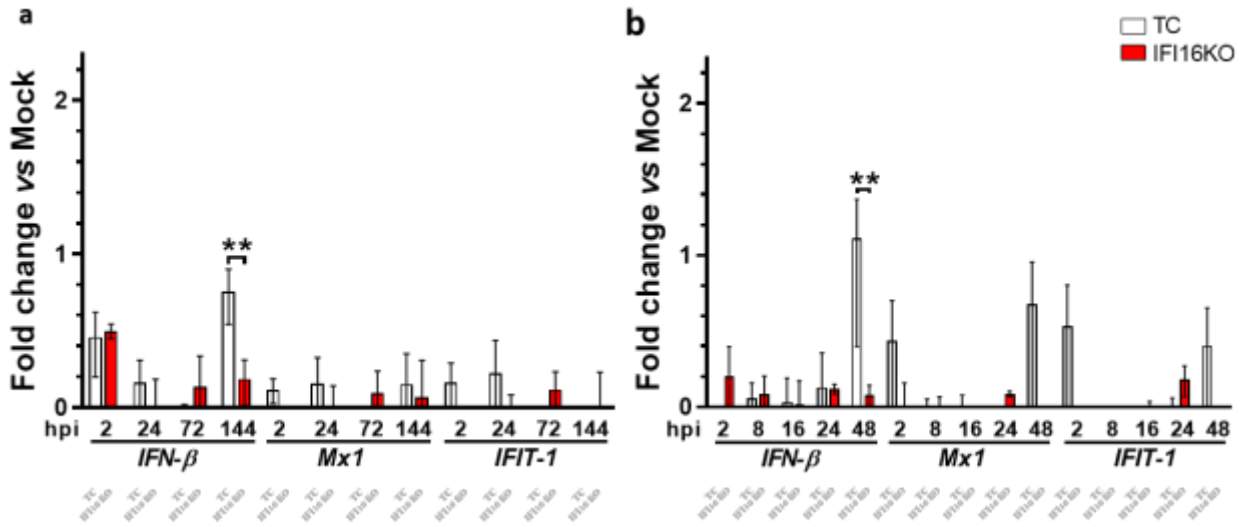

Figure 22 qRT-PCR Analysis.
a, b TC and IFI16KO cells were infected with SARS-CoV-2 **(a)** or NL63 **(b)** at an MOI of 0.5 and 1, respectively. Cell monolayers were harvested at the indicated hpi, and total RNA was extracted. Transcripts of the indicated viral genes were assessed by qRT-PCR. The data were normalized against GAPDH levels and presented as fold induction relative to mock-infected cells. Data are presented as means ± SD (two-way ANOVA; $^{**}p < 0.01$, n = 3 biological replicates).

6. DISCUSSION

Bats are natural reservoirs for many viruses, including SARS-like coronaviruses, in part due to adaptations of their immune system, such as the loss of the entire PYHIN gene family, which includes the DNA sensor IFI16 (Fan et al. 2022). IFI16 is mostly known for its antiviral activity against DNA viruses, indeed, upon binding to viral DNA, IFI16 undergoes oligomerization and recruits host factors necessary to build up an antiviral response (Madden et al. 2022). However, recently the role of IFI16 in the sensing and control of RNA virus replication has emerged as well (Tang et al. 2022). Notably, several studies have attributed to IFI16 the capacity to limit infection of some RNA viruses, including IAV (Jiang et al. 2021), CHIKV (Kim et al. 2020), and PRRSV-2 (Chang et al. 2019b) . Yet, the role of IFI16 in RNA virus regulation, particularly HCoVs, remains poorly understood. This study addresses this gap by revealing a previously underappreciated role of IFI16 in modulating HCoV replication, with distinct effects on two genetically and pathogenically different coronaviruses: the highly pathogenic β-HCoV SARS-CoV-2 and the low pathogenic α-HCoVNL63, using the monkey kidney epithelial cells LLC-MK2, which are permissive to both viruses. We demonstrate that IFI16 restricts SARS-CoV-2 replication, while promoting NL63 replication.

Firstly, we define the kinetic of SARS-CoV-2 and NL63 in our cellular model. Results show that LLC-MK2 cells are permissive to both viruses, representing a valuable *in vitro* model for studying HCoV infections.

Using *in vitro* approaches, we observed a significantly increased viral replication of SARS-CoV-2 in the absence of IFI16-protein, indeed, in IFI16KO cells we observed higher levels of viral RNA, viral structural proteins (N and S), and infectious viral particles release, compared to control cells. In addition, immunofluorescence analysis confirmed these results, since the expression of N protein and of dsRNA, an intermediate of viral replication, were significantly higher in IFI16-depleted cells. All together, these results suggest that IFI16 displays an antiviral effect against SARS-CoV-2 replication. Intriguingly, in contrast to this inhibitory effect, our results indicate that IFI16 seems to support NL63 infection, as evidenced by decreased levels of viral RNA, N protein and dsRNA in IFI16-depleted cells. Importantly, in both cases, IFI16 activity appears independent of type I interferon signaling, as neither IFN-β nor its downstream effectors Mx1 or IFITI were significantly induced upon infection in either TC or IFI16KO cells. This suggests that IFI16 exerts its role on SARS-CoV-2 and NL63 through interferon-independent mechanisms.

In the case of SARS-CoV-2 it is possible that IFI16 interacts with viral components or modulates cellular pathways critical for viral replication. This is supported by emerging evidence that IFI16 can recognize RNA viruses and influence their replication (Li et al. 2024) although the exact mechanism such as direct sensing of viral RNA intermediates like dsRNA remains to be determined. In addition, IFI16 has been shown to interfere with viral transcription and translation machinery in some RNA virus infections (Mishra et al. 2022), further supporting its potential antiviral function during SARS-CoV-2 infection. Instead, the mechanism behind IFI16 pro-viral role on NL63 replication could involve indirect interactions between IFI16 and host factors that promote viral replication, such as RNA-binding proteins or lipid metabolism regulators.

The contrasting roles of IFI16 in these two coronaviruses highlight the complexity of host-virus interactions and suggests that IFI16 may act as a molecular switch in antiviral immunity, with outcomes dependent on viral strain and host contexts.

While our data provides compelling evidence of IFI16's involvement in modulating SARS-CoV-2 and NL63 replication, several key questions remain and warrant further investigation. The precise mechanism by which IFI16 influences SARS-CoV-2 or NL63 replication remains unclear and thus further studies are required to elucidate these mechanisms. Future studies should investigate the subcellular localization of IFI16 during HCoV infections, since a cytoplasmic translocation of IFI16 could allow its interaction with viral components. In addition, techniques such as co-immunoprecipitation (co-IP), RNA immunoprecipitation (RIP), surface plasmon resonance (SPR), and proximity ligation assay (PLA) could help determine whether IFI16 directly binds viral proteins or HCoV RNA. Also, it could be interesting to expand the knowledge of IFI16 activity on other RNA viruses, such as Zika virus (ZIKV), Dengue virus (DENV), and respiratory syncytial virus (RSV).

In conclusion, the unexpected role of IFI16 in RNA virus infection opens a promising area of research in host-virus interactions. Further exploration into its molecular functions, interactions, and regulatory mechanisms will be essential to fully exploit as a potential therapeutic target or biomarker in the fight against current and future viral pandemics.

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