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SARS-CoV-2 and the Host Cell: A Tale of Interactions

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The ability of a virus to spread between individuals, its replication capacity and the clinical course of the infection are macroscopic consequences of a multifaceted molecular interaction of viral components with the host cell. The heavy impact of COVID-19 on the world population, economics and sanitary systems calls for therapeutic and prophylactic solutions that require a deep characterization of the interactions occurring between virus and host cells. Unveiling how SARS-CoV-2 engages with host factors throughout its life cycle is therefore fundamental to understand the pathogenic mechanisms underlying the viral infection and to design antiviral therapies and prophylactic strategies. Two years into the SARS-CoV-2 pandemic, this review provides an overview of the interplay between SARS-CoV-2 and the host cell, with focus on the machinery and compartments pivotal for virus replication and the antiviral cellular response. Starting with the interaction with the cell surface, following the virus replicative cycle through the characterization of the entry pathways, the survival and replication in the cytoplasm, to the mechanisms of egress from the infected cell, this review unravels the complex network of interactions between SARS-CoV-2 and the host cell, highlighting the knowledge that has the potential to set the basis for the development of innovative antiviral strategies.

Keywords: SARS-CoV-2, virus-cell interactions, innate immunity, receptors, virus replication

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INTRODUCTION

The Emergence of SARS-CoV-2

In December 2019 several cases of an atypical viral respiratory infection, later named COVID-19, emerged in Wuhan, China (1). A month later, in January 2020, a novel coronavirus was isolated from clinical specimens, phylogenetically related to betacoronaviruses that recently caused acute respiratory syndromes i.e., SARS-CoV and MERS-CoV (2). The novel coronavirus was named SARS-CoV-2 and, in March 2020, COVID-19 was declared pandemic by the World Health Organization (WHO). The origin of SARS-CoV-2 remains unclear, as reiterated by the WHO (3). Initially, a zoonotic origin was proposed, according to which "wet" markets in Wuhan would have been the first source for animal-human transmission (4), but the market samples that were collected did not allow to establish the exact zoonotic predecessor strain (5). Therefore, the exact place of origin and the possible intermediate hosts need to be further investigated. One of the viral strains closest to SARS-CoV-2, RaTG13, has been found in horseshoe bats (Rhinolophus affinis from Yunnan Province in China) and has a genome sequence identity of 96.2% (2). Interestingly, the variable loop region of the spike protein has a unique evolutionary history compared to the

rest of the SARS-CoV-2 genome overall, since it is similar to the same region observed in the coronavirus strain derived from the Malayan pangolin (pangolin-CoV-2020). It was therefore proposed that the Malayan pangolin may have been the intermediate host of SARS-CoV-2 (6). However, a direct origin from pangolin was challenged by the discovery of other viruses similarly close to SARS-CoV-2, found in bats from different locations of Southeast Asia (7).

An ancestral recombination event between the lineages leading to SARS-CoV-2 could therefore also have taken place in bats or in another intermediate host. Although pangolin-CoV-2020, RaTG13, and other bat CoVs are phylogenetically close to SARS-CoV-2, the viral genome underwent complex recombination events between divergent strains residing in different host species during its evolution, explaining the evolutionary histories of different genomic segments (8). Therefore, while bats are probably the reservoir hosts for this virus, it is likely that other mammalian species acted as intermediate hosts, as documented with civets and camels for SARS and MERS, respectively. Within these unknown intermediate hosts, SARS-CoV-2 acquired some or all the mutations needed for efficient transmission and replication into humans (9). In conclusion, although the molecular and phylogenetic analyses indicate the zoonotic hypothesis as the most likely, the origin and intermediate host species of SARS-CoV-2 remain uncertain and an unnatural origin of this coronavirus cannot be formally excluded [for a detailed review, see (10)].

Pathogenesis

According to the current best estimate of the Center for Disease Control and Prevention (CDC), the infection fatality ratio (estimated number of deaths per 1,000,000 infections) ranges from 20 in children and adolescents to 90,000 in adults older than 65, demonstrating high morbidity for the elderly population. Accordingly, it has been shown that the aging process predisposes older people to greater morbidity and mortality rates (11). It is estimated that 30% of the infections are asymptomatic (12) with more than 50% of transmission occurring in the pre-symptomatic phase (13), making the infection difficult to identify and contain. Similarly to SARS-CoV and MERS-CoV, SARS-CoV-2 may cause a severe respiratory syndrome, frequently associated with comorbidities (14, 15). The pathogenesis is characterized by diffuse alveolar damage occasionally accompanied by microthrombi and vascular damage with immune depletion [for a review see (16)]. Different transmission modes have been described for SARS-CoV-2, including aerosol, surface contamination, and the fecal–oral route (17–19) leading to severe flu-like symptoms that include fever, cough, and dyspnea. The incubation period ranges between 1 and 14 days and can progress to acute respiratory distress, pneumonia, renal failure and death [see (20)]. Respiratory failure in severe SARS-CoV-2 illness has been found to be associated with a hyper inflammation, which may be caused by a cytokine storm syndrome. Interleukin 6, interleukin 8, E-cadherin, MCP-1, VEGF, among other molecules, are involved in the cytokine release syndrome aggravated by trans signaling [reviewed in (21)], similarly to what has been observed in the case of SARS-CoV (22). These proinflammatory mediators can, in turn, perpetuate lung disease by elevating C-reactive protein from the liver through STAT3-IL-6 signaling (23), contributing to lung tissue damage.

SARS-CoV-2 Viral Particle and Genome Organization

SARS-CoV-2 is an enveloped, single-stranded positive-sense RNA virus with a diameter of 60–140 nm and spikes of 9–12 nm in length (**Figure 1**). It is part of the betacoronavirus genus, which includes MERS-CoV and SARS-CoV (24). The virus particle is made of structural viral proteins including spike (S), envelope (E), membrane (M), and nucleocapsid (N) protein (**Figure 1** and **Table 1**). The 419 amino acid-long N protein is the only structural protein inside the virion, associated with the viral genomic RNA via electrostatic interactions driven by positively charged amino acid residues and modulates RNA unwinding after entry into the cell (33). Other structural proteins are inserted into the lipidic viral envelope. The E protein forms an ion channel and participates in viral assembly, while the M protein is critical for incorporating essential viral components into new virions during morphogenesis. The S protein binds the receptor expressed by host cells and promotes fusion of the viral and cellular membrane [see (34) for a review]. The SARS-CoV-2 genome is ∼30 kb and encodes 14 ORFs (**Figure 2**). The genome is flanked by 5′ and 3′ untranslated regions (UTRs) that contain cis-acting secondary RNA structures essential for RNA synthesis. At the 5′ end, the genomic RNA features two large open reading frames (ORF1a and ORF1b) that occupy two-thirds of the capped and polyadenylated genome and encode 16 non-structural proteins (Nsps 1–16) that make up the replicase complex (**Table 2**). Nine accessory proteins—termed ORF3a, 3b, 6, 7a, 7b, 8, 9a, 9b, and 10—are encoded by homonymous orfs and, although deemed as non-essential for the virus replication *in vitro* (**Table 3**), are thought to exert important functions in modulating the host cell metabolism and antiviral immunity [see (78) for a review].

The Life Cycle

In the respiratory tract, SARS-CoV-2 invades preferentially mucus-producing goblet cells and the ciliated cells, as indicated indirectly by the topology of expression of host entry factors, by *in vitro* and *ex vivo* tropism studies and by post-mortem examinations (79–82). In addition, endothelial cells in the lung were also shown to be susceptible to infection *in vivo* (81, 83). The spike protein interacts with the host cell receptor hACE2 with the receptor-binding domain (RBD) (84, 85) followed by important conformational changes required to achieve infection (**Figures 3**, **4**). Accordingly, SARS-CoV-2 relies on cellular proteases for priming the spike protein (14, 84, 86). Conformational changes follow receptor binding and trigger the events leading to fusion with the cellular membrane followed by the penetration of the viral ribonucleoprotein complex into the cytoplasm [for a review on SARS-CoV-2 replication see (87)]. Once into the cytoplasm, the virus releases its RNA genome which is first translated to produce the viral replicase polyproteins pp1a and

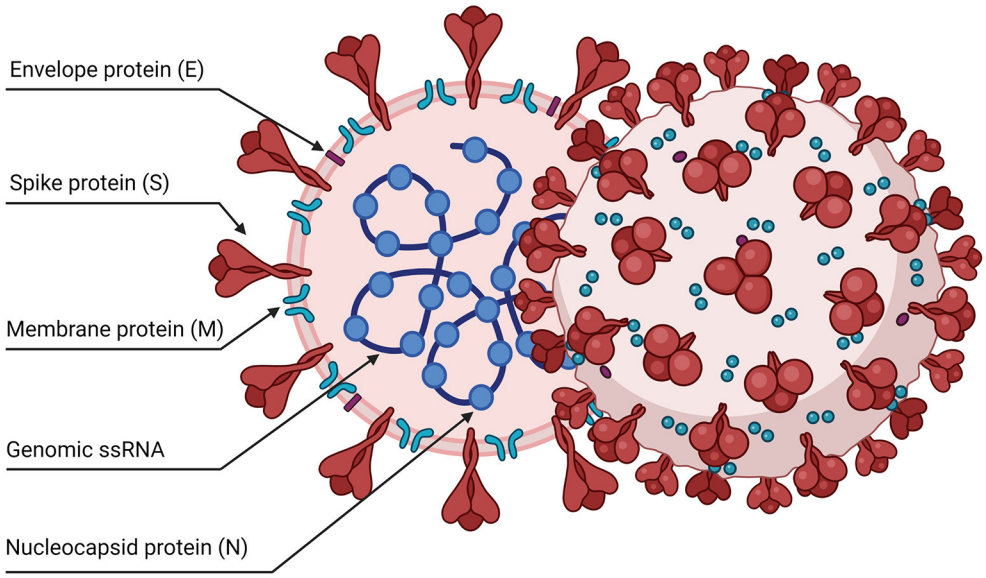

FIGURE 1 | Schematic representation of the SARS-CoV-2 viral particle. The virion contains a positive-sense, single-stranded RNA genome (+ssRNA) enclosed by a lipidic envelope and by structural viral proteins. The nucleocapsid protein (N) is associated with the RNA genome inside the virus particles. Other proteins are inserted in the lipid envelope: the spike trimers (S), the envelope (E), and membrane (M) proteins.

TABLE 1 | Structural proteins encoded by SARS-CoV-2 genome and their functions.

Structural protein	Function	References
N protein	Impairs IRF3 phosphorylation and nuclear translocation Prevents STAT1/STAT2 phosphorylation Prevents inhibition of viral mRNA translation Prevents GSDMD cleavage by caspase-1 Disassembles and prevents formation of stress granules	(25–28)
E protein	Forms an ion channel and participates in virion assembly	(29)
M protein	Essential for the incorporation of viral components during virion assembly Impairs MAVS self-association and association with SNX8	(30, 31)
S protein	Binds to the host receptor ACE2 and mediates fusion and entry	(32)

1ab and subsequently cleaved into smaller products by virus-encoded proteases. The viral polymerase transcribes a series of subgenomic mRNAs by discontinuous transcription, which are then translated into the viral structural proteins. The N protein forms a complex with the genomic RNA while the S, E and M proteins are inserted into the viral envelope at the ER and Golgi intermediate compartments. The newly assembled viral particles are then released from the infected cells by exocytosis (**Figure 3**).

The First Encounter With the Host Cell: Adsorption to the Cell Surface

As for many pathogens, glycoconjugates surrounding mammalian cells are also exploited by SARS-CoV-2 as attachment factors driven by non-specific electrostatic interactions which promote the primary virion-cell surface binding (88). Accordingly, different coronaviruses have been documented to bind host glycans, such as Heparan sulfate (HS) (89–91). The SARS-CoV-2 RBD contains a strongly electro-positive surface, which can accommodate 20 monosaccharides from heparin *via* hydrogen bonds and hydrophobic interactions. Notably, the glycan-binding surface on the RBD is adjacent to, but separate from, the ACE2-binding site, suggesting that ACE2 and HS interactions with the spike glycoprotein are not mutually exclusive (92–94). Despite 73% identity between SARS-CoV and SARS-CoV-2 RBDs, the electrostatic potential of SARS-CoV-2 is much higher, probably mediated by two amino acid substitutions (Thr to Lys 444, Glu to Asn 354) that enhance the predicted coordination with the 20 monosaccharide residues from heparin. HS is thought to enhance binding to ACE2 by promoting an RBD open conformation state (see below), therefore acting as a priming co-receptor that favors the interaction with ACE2 (92).

The important role of such primary interactions suggests pathogenic mechanisms as well as antiviral strategies. On one side it has been hypothesized that secretion of polycations from neutrophils may worsen SARS-CoV-2 infection by facilitating receptor-mediated entry following neutralization of electrostatic repulsive forces between the cell and the viral membranes (95). On the other side such preliminary interactions inspire strategies that could be exploited in therapies aimed at blocking the electrostatic binding (96): negatively charged polysulfates, such as

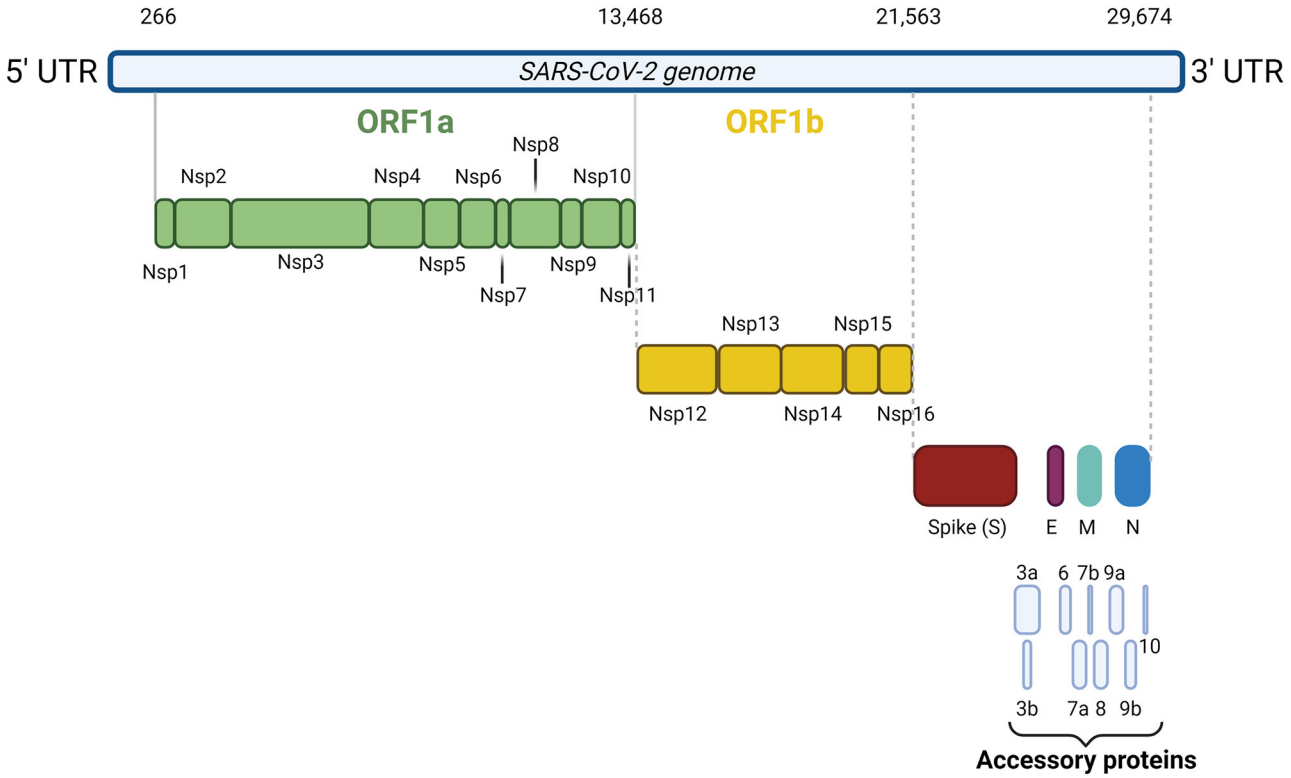

FIGURE 2 | SARS-CoV-2 genome organization. The 30 kb SARS-CoV-2 genome is flanked by 5′ and 3′ untranslated regions. At the 5′ end, the genomic RNA contains two large open reading frames (ORF1a and ORF1b) encoding 16 non-structural proteins (Nsps 1–16). At the 3′ end, the genome encodes the four structural proteins S, N, M, and E and nine accessory proteins, namely ORF3a, 3b, 6, 7a, 7b, 8, 9a, 9b, and 10.

heparin or hyperbranched polyglycerol sulfate (HPGS), can bind to the spike protein, providing valid models to design polyanionic inhibitors of viral infection. Accordingly, heparin-derivatives such as unfractionated heparin, non-anticoagulant heparin, heparin lyases and low molecular weight heparin have been proposed for COVID-19 treatment, not only to treat thrombotic complications ranging from vascular micro-thromboses, venous thromboembolic disease, and stroke, but also to interfere with viral infection (97, 98). Preliminary attachment to cell surface glycoconjugates was shown for different viruses for which the inhibitory activity of heparan sulfate and similar polyanionic compounds has been observed *in vitro*. For SARS-CoV-2, as well as for other different viruses, the real therapeutic activity of these strategies awaits clinical demonstration (99).

The Interaction With Cellular Receptors

While the cell surface molecule ACE2 was soon identified to be the prominent receptor bound by the spike protein, other cell surface molecules have been later proposed to function as alternative receptors or co-receptors (**Figure 4**), including KIM1, AXL, L-SIGN, and DC-SIGN, and SR-B1 (100–104). For most of these molecules it remains unclear whether these are alternative receptors to ACE2, or whether their activity is limited to facilitating viral entry. In addition to describing the interaction with the main receptor ACE2, here we discuss the potential role of Neuropilin-1, independently reported by two research groups (105, 106).

ACE2

The human angiotensin-converting enzyme 2 (hACE2) was found to be the main host cellular receptor recognized by the S protein (84–86). ACE2 is a blood pressure and kidney function regulator of the renin-angiotensin-aldosterone system, expressed in most tissues of the body. It is essential for processing angiotensin 2 and therefore it is involved in vasoconstriction as well as in pro-fibrotic and pro-inflammatory processes (107). This receptor is organized as a homodimer, stabilized by B0AT1 (SLC6A19), a transporter which mediates uptake of neutral amino acids into intestinal cells (108). The spike glycoprotein recognizes the N-terminal peptidase domain of ACE2 (PD, 19–615 residues), which is also the catalytic domain of the protein. Hence, there is the possibility that viral infection could interfere with the angiotensin 2 pathway by contributing to pathogenesis. Given the widespread use of ACE2 inhibitors to treat hypertension and diabetes, a contribution of such medications to COVID-19 pathogenesis has also been investigated, but so far without finding clinical support (109).

The virion spike glycoprotein (S) is a class I viral fusion protein which forms a trimer and is processed by host proteases in two domains (S1 and S2) folding into a metastable pre-fusion

TABLE 2 | Non-structural proteins encoded by SARS-CoV-2 genome and their functions.

Non-structural protein (Nsp)	Function	References
Nsp1	Inhibits the translation machinery and production of immune defence factors Shuts off host mRNA translation	(35–37)
Nsp2	Interacts with prohibitin, maybe altering the host cell environment	(38)
Nsp3	Papain-like protease activity	(39)
Nsp4	Participates to the formation of sites for viral RNA synthesis and double membrane vesicles (DMVs)	(40)
Nsp5	Principal protease Impairs nuclear translocation of phosphorylated IRF3	(41, 42)
Nsp6	Participates to the formation of sites for viral RNA synthesis and DMVs Binds TBK1 preventing IRF3 phosphorylation Inhibits STAT1/STAT2 phosphorylation	(43–45)
Nsp7	Is involved in the primase complex	(46)
Nsp8	Is involved in the primase complex	(46)
Nsp9	RNA replicase activity	(47)
Nsp10	Cofactor for Nsp14 and Nsp16	(48)
Nsp11	No known function	
Nsp12	RNA-dependent RNA polymerase Suppresses nuclear translocation of phosphorylated IRF3	(49, 50)
Nsp13	Helicase/triphosphatase activity Binds and blocks TBK1 phosphorylation Disrupts TBK1 association with MAVS Hijacks USP13 Inhibits STAT1/STAT2 phosphorylation	(44, 51, 52)
Nsp14	Exoribonuclease activity Induces lysosomal degradation of IFNAR1 Blocks IRF3 nuclear translocation	(53–55)
Nsp15	RNA endonuclease Inhibits autophagy Blocks IRF3 nuclear translocation	(56, 56–58)
Nsp16	N7- and 2′O- methyltransferase activity Inhibits RIG-I and MDA5 Mimics host mRNA post-transcriptional modifications limiting IFIT restriction	(59, 60)

TABLE 3 | Accessory proteins encoded by SARS-CoV-2 genome and their functions.

Accessory protein	Function	References
ORF3a	Ion channel protein involved in cell cycle arrest and apoptosis Is involved in the activation of the inflammatory process Inhibits STAT1 phosphorylation	(44, 61–64)
ORF3b	Is involved in IFN (Type I) production and signaling inhibition Induces high levels of antibody production (immunodominant protein)	(65, 66)
ORF6	Is involved in IFN (Type I) production and signaling inhibition Has a potential highly pathogenic role in the cross-talk between SARS-CoV-2 and host signaling pathways Prevents STAT1/STAT2 nuclear translocation	(67–69)
ORF7a	May inhibit host translation Marginally inhibits STAT1 phosphorylation Inhibits STAT2 phosphorylation	(44, 70)
ORF7b	Inhibits STAT1/STAT2 phosphorylation	(44)
ORF8	Has a potential role in the immune evasion process to promote viral growth Prevents IRF3 nuclear translocation Induces MHC-I lysosomal degradation	(71–73)
ORF9a	No known function	
ORF9b	Prevents the interaction between TOM70 and Hsp90-bound TBK1 Interacts with RIG-I, MDA-5, MAVS, TRIF, STING, and TBK1 to impede the phosphorylation and nuclear translocation of IRF3 Suppresses formation of K63-linked polyubiquitination of NEMO	(74–76)
ORF10	No known function; not essential in humans	(77)

conformation. The S1 subunit binds ACE2 while the S2 subunit catalysez fusion with the target cell (110). The S1 C-terminal domain (CTD) contains the receptor binding domain (RBD, **Figure 5**), which is sufficient for binding to the ACE2-PD domain and is the main determinant of viral host range and tropism. The glycoprotein can be found in two distinct conformations, which make the RBD differently accessible to the receptor. While an "up" state readily exposes the RBD to receptor binding, a "down" state, which protects the RBD from crucial neutralizing antibodies, makes it less available for the interaction with ACE2 (**Figure 5B**). The SARS-CoV-2 spike predominantly acquires the latter conformation (32, 111) which makes receptor recognition less favorable while also contributing to decreasing the virus vulnerability to neutralization.

Our molecular understanding of the interaction between the spike protein and ACE2 builds from the knowledge gained from SARS-CoV studies (112), later confirmed by structural studies with SARS-CoV-2 (**Figure 5A**) (84, 85, 113). The RBD contains 2 subdomains: the "core loop" made of five-stranded anti-parallel β sheets (β1 to β4 and β7), with three short-connecting α helices (αA to αC) and the "extended loop" subdomain, which is a concave surface formed by two-stranded β sheets (β5 and β6) forming the interface with ACE2. Variations of ACE2 sequences in different animal species impact on the affinity of the binding with the S protein determining different degree of susceptibility to infection and virus transmission. Of note, within β6, residues Leu472, Asn479, Thr487 are critical for cross-species and human-to-human transmission of SARS-CoV. Met82 of human ACE2 interacts with Leu472, while the Asn82 in rat ACE2 introduces a glycan that produces steric hindrance, disrupting the binding. Indeed, some amino acidic variations in the recognition interfaces of SARS-CoV-2-RBD/ACE2 are likely to increase the binding affinity compared with SARS-CoV-RBD/ACE2 (85), possibly explaining the higher infection efficiency of SARS-CoV-2.

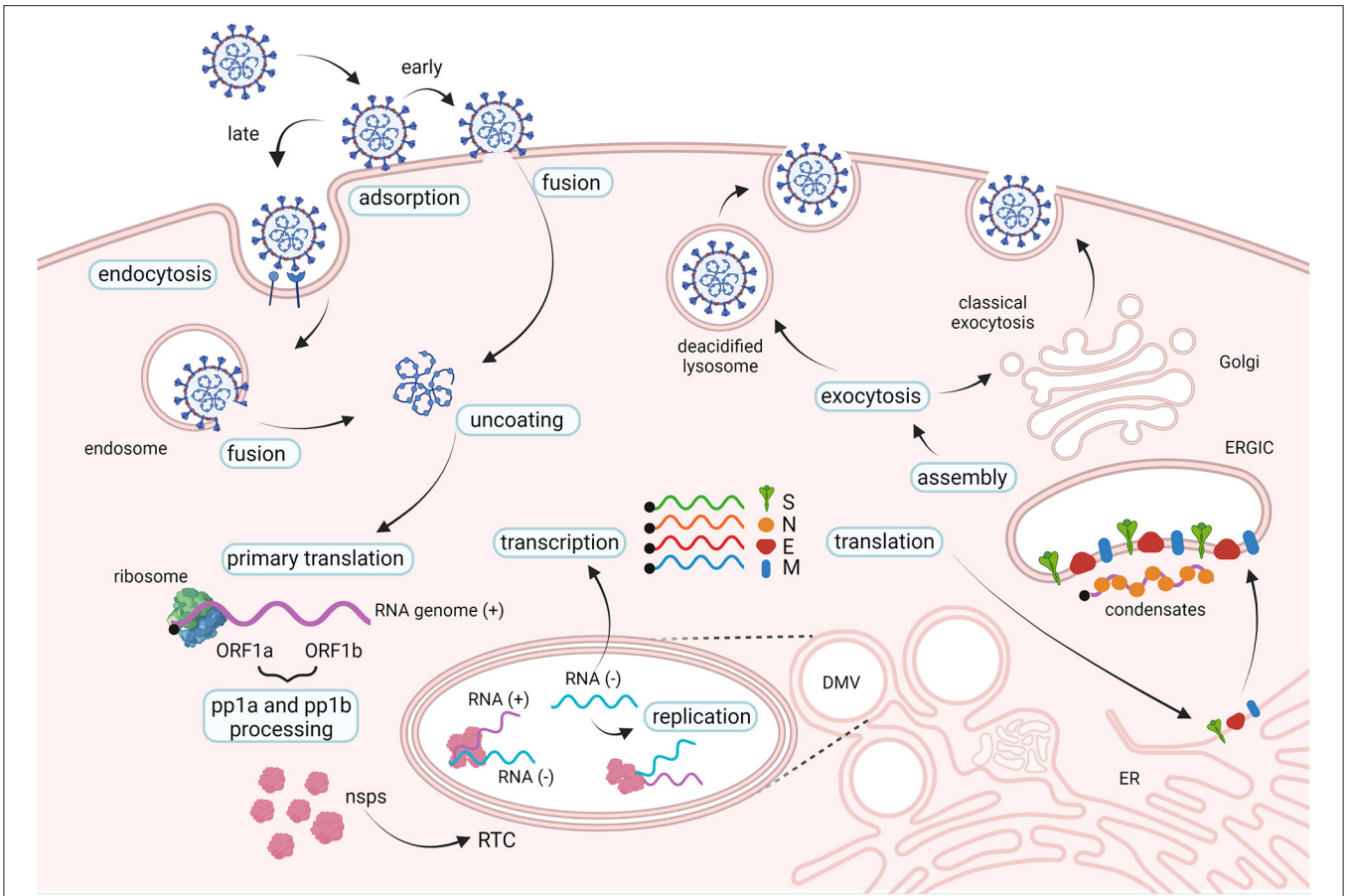

FIGURE 3 | The life cycle of SARS-CoV-2. SARS-CoV-2 interacts with cellular receptors such as human angiotensin-converting enzyme 2 (hACE2) and with host proteases which activate the spike proteins (not shown). Entry in the cell cytoplasm may occur in two ways: the viral particle is endocytosed before fusing with the endosomal membrane (late pathway), or the viral membrane fuses with the cell membrane at the cell surface (early pathway). Two large open reading frames, ORF1a and ORF1b, are immediately translated in polyproteins pp1a and pp1ab, that are processed into the individual non-structural proteins (nsps) which form the viral replication and transcription complex (RTC). Viral genomic RNA replication occurs in protective double-membrane vesicles (DMVs). Transcription and translation of the negative template result in the formation of structural proteins that are inserted into the endoplasmic reticulum (ER) membrane and transit through the ER-to-Golgi intermediate compartment (ERGIC). Here, condensates of newly produced genomic RNA and N proteins interact with E and M proteins resulting in assembly of viral particles, which bud into the lumen of secretory vesicular compartments. Virions are secreted from the infected cell by exocytosis in two ways: through the classical exocytosis pathway via the Golgi compartment or through the incorporation in deacidified lysosomes that fuse with the cellular surface membrane.

NRP1

Neuropilin-1 (NRP1), is a multifunctional surface protein which binds secreted peptides such as VEGF and semaphoring (114). NRP1 plays an important role for the development of neurons and the cardiovascular system as well as for tumor growth and tumor vascularization (115). The protein recognizes furin-cleaved 192 substrates which, upon cleavage, expose a polybasic conserved C-end rule peptide (TQTNSPRRAROH). Accordingly, furin cleavage of the spike glycoprotein generates a C-end rule peptide, which was found to bind NRP1 and mediate cell entry through endocytosis (116). NRP1-driven virus entry is thought to facilitate infection of cells with low ACE2 expression level, such as olfactory endothelial cells (105), possibly by facilitating the separation of S1 and S2 (117). Indeed, entry promoted by NRP1 could have important neurological implications, especially in the olfactory-related region of the CNS, supporting the role of NRP1 in the neurological symptoms of SARS-CoV-2 (118). *In vitro* studies have shown that ACE2 expression in pulmonary and olfactory cells is low (119), while NRP1 appears to be abundantly expressed, consistent with an important role of the latter as a coreceptor to enhance cell tropism for the upper respiratory tract. Understanding the molecular details of the interaction between NRP1 and the furin-cleaved S1 domain may therefore help future studies for developing SARS-CoV-2 inhibitors (106). For over 20 years there has been strong interest in interfering with NRP1 binding to its ligands. For example, different strategies have been pursued to interfere with NRP1 binding to VEGF to block endothelial cell migration, including NRP1-based antibody therapy (120) and small molecules-based therapy to target the NRP1-b1 domain (121), the same found to interact with the SARS-CoV-2 spike. Monoclonal antibodies were therefore tested to establish the

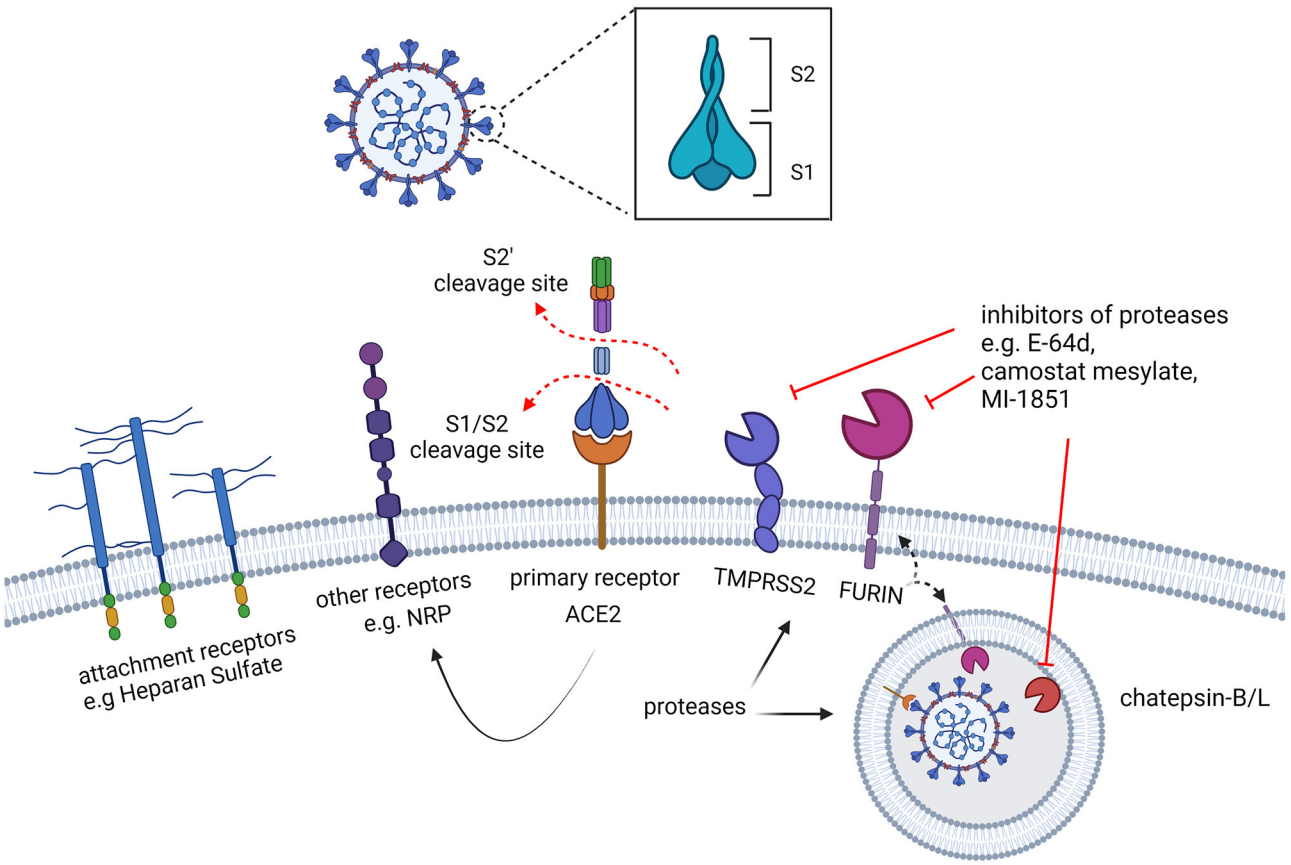

FIGURE 4 | Early interactions of SARS-CoV-2 with the target cells. The spike protein of SARS-CoV-2 recognizes surface receptors to bind and fuse with the cell. A preliminary interaction of the virus particle with the cell is mediated by attachment to cell surface glycans, such as heparan sulphate, followed by the interaction with specific receptors. While the human angiotensin-converting enzyme 2 (hACE2) is the main receptor recognized by the spike, other cell surface proteins, such as NRP, are also known to serve as receptors capable of triggering virus entry. The activation of the fusogenic activity of the spike protein also requires proteolytic processing by cellular proteases which are located on the cell surface or in the endosomal vesicles. Furin is required for the cleavage which separates the S1 and S2 subunits of the spike, predisposing the protein to the interaction with ACE2 and to the subsequent cleavage (S2') by the activating proteases: TMPRSS2, exclusively located at the cell surface, and cathepsins, in the endosomes.

functional relevance of the NRP1-b1-S1 molecular interaction. Therapies which block NRP1, such as antibodies-based therapy, small peptides-based therapy as well as small molecules-based therapy (122), may potentially be exploited to target also SARS-CoV-2. Accordingly, a selective NRP1 210 antagonist that interacts with the NRP1-b1 binding pocket was shown to reduce NRP1-b1/S1 binding and to inhibit viral entry (105).

Virus-Activating Interactions: The Cellular Proteases

In addition to receptor binding, efficient entry of coronaviruses also requires processing of the spike protein by cellular proteases. In the case of SARS-CoV-2, the proteolytic processing includes both a "priming" and an "activation" cleavage (86, 116). The first "primimg" cleavage reshapes the spike by cleaving S1 and S2, which remain non-covalently attached. The second cleavage (S2') occurs within S2, exposing the fusion peptide and activating its fusogenic potential (**Figure 6**). The priming processing predisposes the spike protein to the interaction with ACE2 and to the cleavage by the activating protease (86, 116). Proteolytic processing is therefore crucial for SARS-CoV-2 infectivity and has been intensively investigated to reveal which cellular factors are involved and in which cellular compartment they act. The spike of SARS-CoV-2 can be targeted by proteases during biogenesis in the Golgi of virus-producing cells and in target cells, either on the cell surface or inside an intracellular vesicle, depending on the entry pathway followed by the virus. After emerging from the producer cell, coronaviruses are known to infect target cells either by fusing with the host cell at the plasma membrane ("early pathway"), or within an intracellular vesicle following endosomal uptake ("late pathway"). SARS-CoV-2 is documented to exploit both pathways [**Figure 3**, see (123) for a review] and takes advantage of different host proteases encountered on its way including furin, transmembrane protease serine S1 member 2 (TMPRSS2) and endosomal cysteine protease Cathepsin B and L (CatB/L) (124). Such proteases play a crucial role during infection

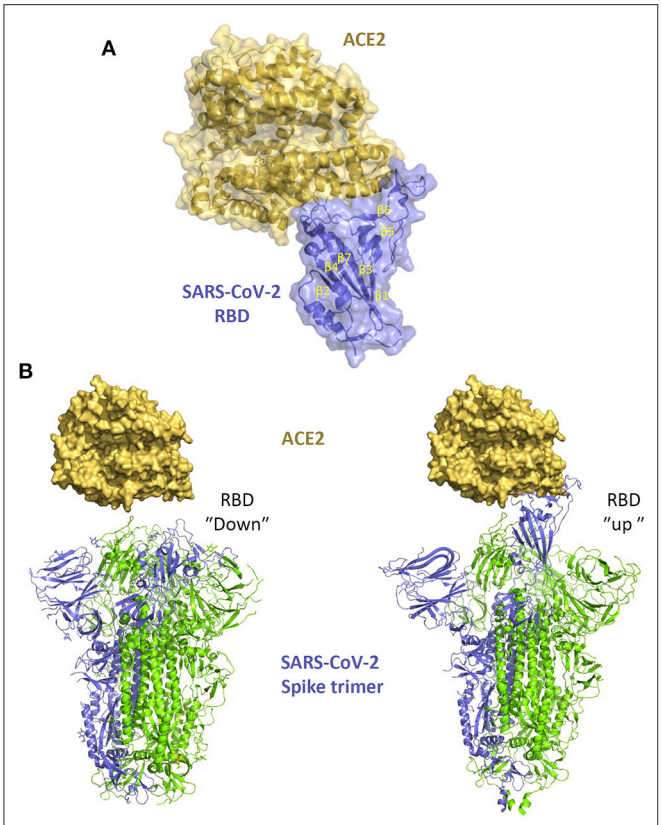

FIGURE 5 | The interaction of SARS-CoV-2 spike with ACE2. **(A)** structure of SARS-CoV-2 spike RBD in complex with ACE2 (PDB 6M0J). **(B)** different conformations of the spike trimer with RBD "down" (PDB 6ZB5) and RBD "up" (PDB 6zGG) which shows the exposure of the RBM within the RBB which facilitates the interaction with ACE2.

since they modulate viral infectivity, tropism, transmission and pathogenesis (125).

The Priming Proteases

The first protease cleavage ("priming") occurs at the boundary between the two functional subunits of the spike extracellular domain (S1 and S2, **Figure 6**) by recognition of a unique multibasic furin cleavage site (FCS) rich in Arginine (^{680}SPRRAR↓SV^{688}) which has been structurally well-characterized in complex with the protease (126). The presence of this sequence at the S1/S2 boundary is a crucial virulence determinant peculiar to SARS-CoV-2 since it is absent in most other coronaviruses, including SARS-CoV (127, 128). Only MERS-CoV contains a furin pseudo-binding site, though not as efficiently processed by the protease (128). In SARS-CoV-2, the FCS is a well-exposed loop within the spike structure, fully accessible to protease recognition. Indeed, from biophysical analysis (126) it was demonstrated that the cleavage loop (N657 to Q690) perfectly fits in the canyon-like crevice which identifies the substrate-binding pocket of furin. The higher proteolytic processing susceptibility of the spike from SARS-CoV-2 compared to that from others HCoV S, together with the ubiquitous expression of furin, may therefore facilitate viral entry into cells, leading to increased infectivity and transmissibility of the virus compared to SARS-CoV and MERS-CoV.

Furin is a membrane-bound protease and as such it is produced in the ER and transported through the secretory pathway. Like glycoproteins of other viruses (e.g., retroviruses), the spike glycoprotein can be processed by furin during biogenesis in producer cells. However, membrane-bound furin can also process the spike glycoprotein in target cells during the early steps of infection and, being also shed from the membranes, viruses can be primed by the protease in the extracellular space before attachment to target cells. Of note, the S1/S2 sequence boundary can be quickly lost because of lab adaptation to cell culture (129–131). Accordingly, virus replication *in vitro* results in deletion of the—RRAR—motif and increases the dependence of the virus on the endosomal pathway. This indicates that the emergence of the FCS could have been driven by *in vivo* selective pressure to fuse at the cell surface rather than in the endosomes, a pathway that allows the virus to avoid inhibition by IFITM proteins (132). Irrespective of the cellular site of cleavage, furin is expressed in different tissues and contributes to expanding the virus tropism to sites and organs usually refractory to infection by other coronaviruses (132). The increased tropism mediated by furin could also favor viral dissemination among individuals, since the virus can be shed in different body fluids and secretions (125).

In addition to increasing virus pathogenicity by altering virus particles infection, the ability of the spike protein to engage furin was also suggested to affect the normal physiology of the lungs because of molecular mimicry. An amino acid sequence similar to the FCS found within the SARS-CoV-2 spike is present in the human epithelial sodium channel α-subunit (ENaC-α), a protein involved in the homeostasis of airway surface liquid (133). This structural mimicry could therefore lead to a competition for furin in infected lung cells, with the virus interfering with fluid clearance from alveoli and contributing to edema and pulmonary pathology (134).

The Activating Proteases

The priming step gives rise to a spike structure made of S1–S2 non-covalently bound in a "pre-fusion" conformation which is also processed by other proteases, either on the target cell surface or within an endosome (127). This processing activates the fusogenicity of the spike protein, leading also to the formation of syncytia, which, as explain later, may contribute to pathogenesis and to spreading of the virus to neighboring cells.

Following protease-mediated "priming," the S1 subunit is released, exposing a S2' cleavage site (^{814}KR↓SF^{817}) located at the N terminal of the fusion peptide. Such "activation" cleavage can be performed by diverse proteases which are found in different cellular compartments. As already well-characterized for SARS-CoV and MERS-CoV, TMPRSS2 plays a major role in activating the spike glycoprotein at the cell surface of target cells [see (135) for a review]. In addition, Cathepsin L, a member of the lysosomal cysteine protease, activated at low pH, was also found to target the spike protein of incoming virions taken up into the endosomal pathway (136–138). Of

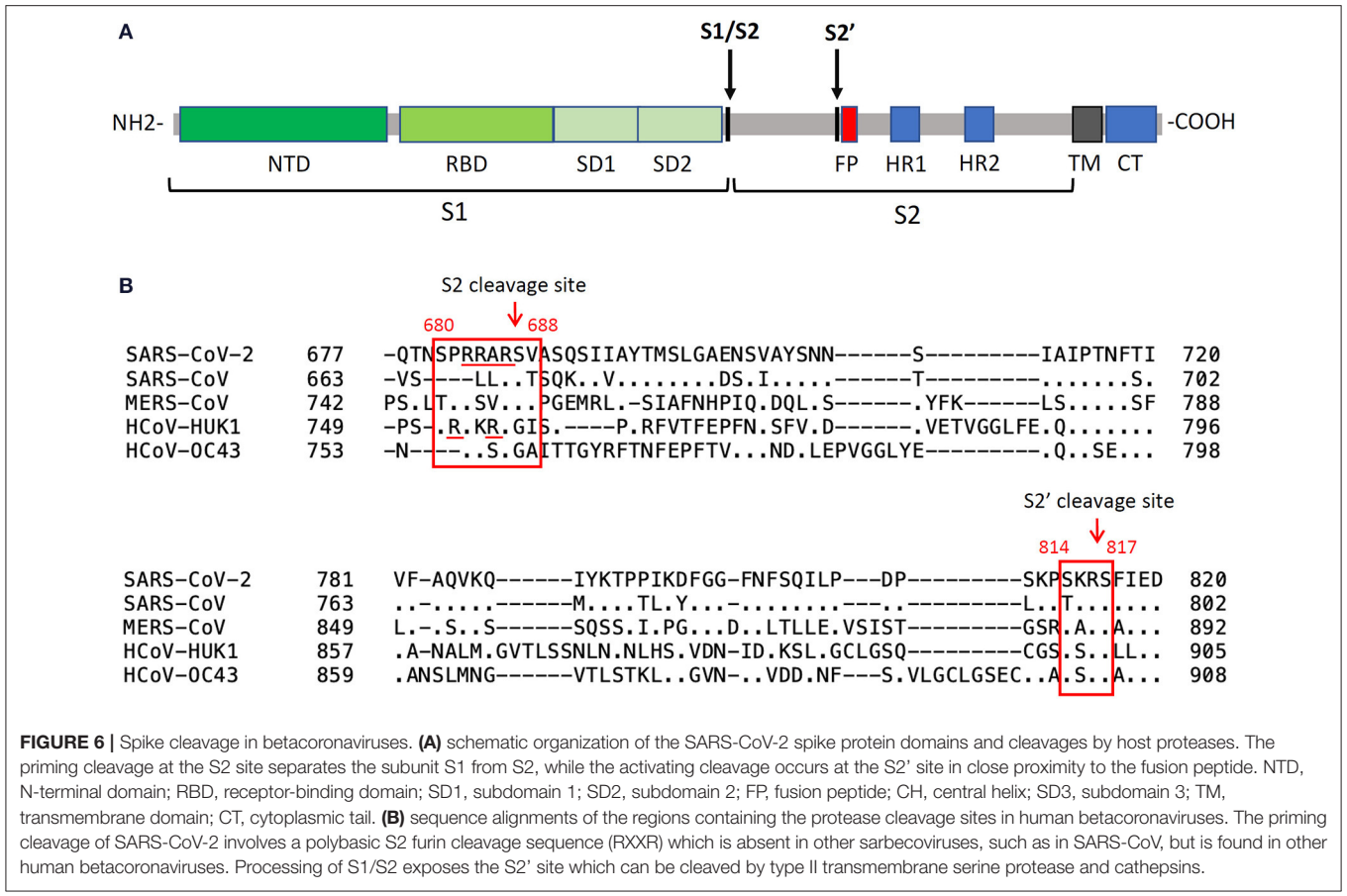

FIGURE 6 | Spike cleavage in betacoronaviruses. **(A)** schematic organization of the SARS-CoV-2 spike protein domains and cleavages by host proteases. The priming cleavage at the S2 site separates the subunit S1 from S2, while the activating cleavage occurs at the S2' site in close proximity to the fusion peptide. NTD, N-terminal domain; RBD, receptor-binding domain; SD1, subdomain 1; SD2, subdomain 2; FP, fusion peptide; CH, central helix; SD3, subdomain 3; TM, transmembrane domain; CT, cytoplasmic tail. **(B)** sequence alignments of the regions containing the protease cleavage sites in human betacoronaviruses. The priming cleavage of SARS-CoV-2 involves a polybasic S2 furin cleavage sequence (RXXR) which is absent in other sarbecoviruses, such as in SARS-CoV, but is found in other human betacoronaviruses. Processing of S1/S2 exposes the S2' site which can be cleaved by type II transmembrane serine protease and cathepsins.

note, while activation by TMPRSS2 requires the prior furin priming cleavage, the spike can be activated independently on priming by cathepsins within an endosomal vesicle (139), giving SARS-CoV-2 the possibility to exploit different entry pathways. Accordingly, it has been observed that in Calu-3 cells the virus exploits a pH-independent early pathway by exploiting proteases such as TMPRSS2, enriched in airway epithelial cells, while in simian epithelial Vero cells SARS-CoV-2 is suggested to follow a low pH endosomal entry pathway supported by proteases such as Cathepsin L (132, 140). The observation that TMPRSS2 is enriched in nasal and bronchial tissues may therefore explain the high efficiency of SARS-CoV-2 infection of these tissues via respiratory droplets (80).

Could Inhibitors of Cellular Proteases Be Used as Antivirals?

Given the importance of proteases during the early phases of the infection process, inhibition of furin, TMPRSS2 or cathepsins has been proposed in order to interfere with "priming" and "activation" cleavages of the trimeric S glycoprotein (141–143). Proving this concept, Hoffmann and coworkers (127) were the first to report the ability of a TMPRSS2 inhibitor, camostat mesylate, in preventing viral entry in lung Calu-3 cells, possibly by interfering with the activating cleavage.

Furin-specific inhibitors, such as decanoyl-RVKR-chloromethylketone (CKM) and naphthofluorescein, were also identified as potential antiviral leads able to prevent both priming of S glycoprotein and syncytia formation (144). In contrast, other studies highlighted how inhibitors of furin may be not sufficient to block virus infection, given the redundancy with other proteases (145). Along the same line, E-64d (a Cathepsin B and L inhibitor) was found to act efficiently against the virus only when used simultaneously with a TMPRSS2 inhibitor, further corroborating the concept that SARS-CoV-2 can exploit more than one protease to proteolytically activate the S glycoprotein. This is consistent with the evidence that simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors *in vitro* prevents SARS-CoV entry into cells expressing ACE2 and TMPRSS2 (146). The cooperation of different protease inhibitors could therefore be a promising strategy to treat COVID-19 (144, 145, 147), as shown with a combination of camostat mesylate and MI-1851 (a synthetic furin inhibitor) which promoted a strong and synergic reduction of viral replication in human airway epithelial cells. Of note, the experimental system used could lead to discordant results given that SARS-CoV-2 can follow different entry routes in different cells and therefore be processed by different proteases. However, even though TMPRSS2 and lysosomal cathepsins have been both demonstrated to have cumulative effects with furin

on activating SARS-CoV-2 entry, inhibition of TMPRSS2 was found to be sufficient to prevent SARS-CoV-2 entry in lung cell lines and primary lung cells (148), raising hopes for their future use as antivirals *in vivo*. Accordingly, promising preclinical results obtained with Nafamostat, another TMPRSS2 inhibitor (149, 150), are currently awaiting clinical confirmation (151).

Syncytia Formation by SARS-CoV-2 Spike: A Fusogenic Interaction of Infected and Non-infected Cells

As observed with many enveloped viruses, such as HIV-1, RSV, and herpesviruses, cell surface expression of viral glycoproteins may promote fusion between infected cells and non-infected cells that express a functional virus receptor [see (152) for a review]. This interaction causes the formation of multinucleated syncytia which may contribute to virus propagation and to tissue damage. While syncytia were never observed with SARS-CoV-1, infection with SARS-CoV-2 is reported to induce the formation of multinucleated cells *in vitro* (153–156) as well as *in vivo* (154, 157–159), leading to the fusion of pneumocytes (157) with the contribution of lymphocytes (159) in the lungs. The propensity of the SARS-CoV-2 spike to induce the formation of multinucleated cells could be related to the processing by cellular proteases, such as furin and TMPRSS2 (156, 159), which activates and unleash the fusogenic potential of the viral glycoprotein not only on the viral particle but also on the cell surface. Of note, as well as requiring recognition of the cognate receptor and the activation by proteolytic processing, fusion depends also on the engagement of the calcium-activated ion channel TMEM16 in cells expressing the spike protein (158). On this basis, approved drugs inhibiting TMEM16 were found not only to block syncytia formation but also to prevent cytopathic effects and inhibit virus replication *in vitro*, suggesting a novel antiviral target.

The Importance of Lipids for SARS-CoV-2 Entry

The success of the fusion process also depends upon the composition of the viral and host membranes, given that the biophysical properties of different lipid species determines membrane fluidity and curvature [see (160) for a review]. If on one side phosphatidylethanolamine and cholesterol are found to enhance membrane fluidity and promote a negative curvature critical for viral fusion, lysophospholipids (LPLs) promote positive curvature and inhibit fusion (161). Accordingly, compounds interfering with lipid metabolism are proposed as drugs for the treatment of COVID-19, capable of inhibiting virus replication and syncytia formation. In particular, 25-hydrocholesterol (25HC) was shown to block membrane fusion by depleting cholesterol from the plasma membrane following activation of acyl-CoA:cholesterol acyltransferase (154, 162). The importance of the cholesterol metabolism for SARS-CoV-2 infection was also demonstrated by the inhibition of virus replication *in vitro* upon treatment of 27-hydroxycholesterol, an endogenous oxysterol metabolite of cholesterol targeting lipid rafts and the late endosomal compartment (163) with no cytotoxic effects *in vitro*. The potential of targeting viral fusion by altering the lipid composition of biological membranes is therefore an antiviral strategy that should be further explored to test its applicability *in vivo*.

The Interaction With Fc Receptors: A Potential Alternative Entry Pathway

Soon after SARS-CoV-2 was isolated, serious concerns were raised about the possibility that the virus could take advantage of antibody dependent enhancement of infection (ADE, **Figure 7**), posing a problem for vaccine development and therapeutic approaches in patients infected with different SARS-CoV-2 variants (164). The molecular bases of the ADE rely on poorly neutralizing or non-neutralizing antibodies binding to surface viral antigens and promoting viral particle uptake into cells that express an Fc receptor, thus enhancing the infection of the host (165). Of note, ADE could be potentially caused by the presence of suboptimal concentrations of neutralizing antibodies or antibodies with decreased affinity produced in the host either following an earlier infection, vaccination, or passive transfer (166). The ADE phenomenon has been reported for some human viruses, such as dengue, Zika and respiratory syncytial virus (RSV), and also in some veterinary pathogens such as foot-and-mouth disease virus (FMDV), porcine reproductive and respiratory syndrome virus (PRRSV) [for a review see (167)].

Myeloid cells, including monocytes, macrophages and dendritic cells (DCs), are considered likely targets of ADE infection, since they express the FcγR which could confer the virus the ability to infect cells even in the absence of the spike cognate receptor (167). For non-macrophage-tropic respiratory viruses (such as RSV), the formation of immune complexes (i.e., virus-antibody complexes) in lung and airway tissues induce a local activation of the complement system and release of cytokines resulting in heavy inflammation and airway obstruction that eventually can lead to acute respiratory distress syndrome (168) closely similar to COVID-19 clinical manifestations, which could involve the complement system activation (169).

Concerns about the possibility of ADE for SARS-CoV-2 were raised looking at its close relative SARS-CoV. The two viruses have a genome sequence similarity of 79.5% and they also share the canonical receptor for viral entry (ACE2), which is bound by SARS-CoV-2 with 10–20 times increased affinity compared to SARS-CoV. ADE could result from poorly neutralizing antibodies raised by previous infections with other coronaviruses, since the S protein sequence similarity between different coronaviruses suggests the presence of cross-reactive epitopes (169). Indeed, in the case of SARS-CoV, anti-spike antibodies were reported to bind Fcγ receptors on the surface of M2 macrophages normally devoted to anti-inflammatory functions (170). Accordingly, anti-spike antibodies were shown to increase the infection of monocytes and lymphocytes by SARS-CoV *in vitro* in the absence of the canonical viral receptor, indicating an ACE2 independent entry mechanism (171). Understanding whether ADE can occur during SARS-CoV-2 infection has been fundamental for the evaluation of vaccine safety and the development of immunomodulatory

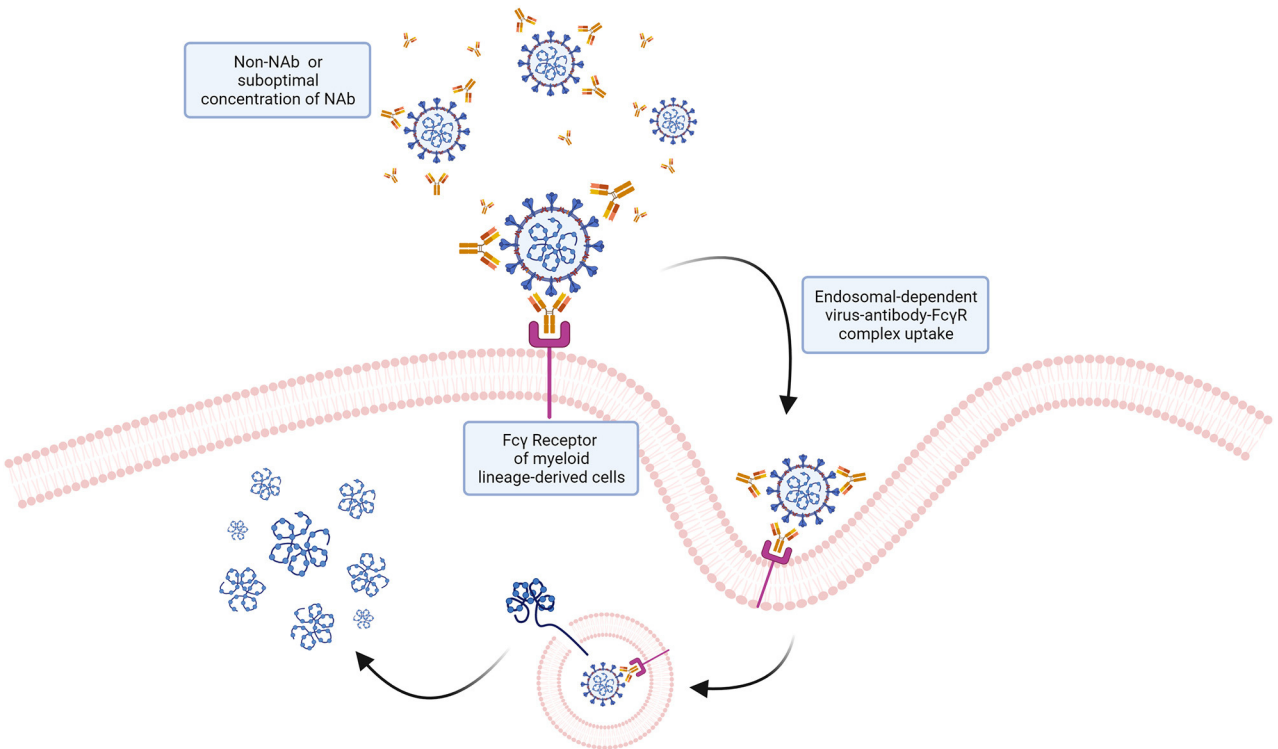

FIGURE 7 | ADE: a potential mechanism of ADE for SARS-CoV-2? Antibodies interacting with virions without neutralizing the ability of the virus to infect target cells could potentially mediate viral particles uptake by cells expressing Fcγ receptors via endocytosis. Internalized virions could then fuse with the endosomal membrane and release the genome into the cytoplasm. While this mechanism, known to favor infection of other viruses, has been hypothesized, no evidence so far has demonstrated that ADE occurs with SARS-CoV-2.

therapies. Fortunately, up to date, there is no proof that ADE occurs in SARS-CoV-2 infections and there is no evidence that the available vaccines for COVID-19 are eliciting ADE in subjects that are reinfected. Indeed, it appears that vaccinated subjects are less severely affected by the disease. However, it will be important to keep monitoring for signs of ADE to optimize and update current prophylactic and therapeutic interventions.

The Interaction of SARS-CoV-2 With the Endocytic Pathway and the Antiviral Potential of Inhibiting Endosomal pH

Following receptor binding and the possible uptake into a vesicle, the fusion mechanism is triggered by S2 [see (172) for a review]. The S2 sequence contains regions conserved among CoVs required for the fusion machinery, notably a fusion peptide and two conserved heptad repeats (HR) instrumental for bringing viral and cellular membranes in close proximity for fusion. After activation of the spike glycoprotein, a conformational change occurs leading to the release of the fusion peptide and the interaction with the cellular membrane (172). The collapse of S2, which bridges the virus and cellular membranes, pulls the two membranes together with HR1 and HR2 forming the canonical 6-helix bundle first reported for CoVs in mouse hepatitis virus (MHV) (173) and then for other coronaviruses (141). As mentioned earlier, SARS-CoV-2 can fuse either at the cell membrane or after having been taken up by endocytosis. Understanding which entry pathway plays a crucial role *in vivo* remains an important issue which also indicates whether endo-lysosomotropic compounds can be used as possible therapeutic agents. In the meantime, the possibility of inhibiting virus infection by interfering with the maturation of endosomes has been investigated intensely. As mentioned earlier, CatB and CatL are the main proteases required for SARS-CoV-2 activation in the endosomes and both are active only at an acidic pH. Accordingly, as the endosomal vesicle is formed and clathrin breaks off, the pH of the vesicle decreases and the endosome matures to late endosome (pH of 5.5–6), which eventually fuses with the lysosome reaching a pH of ∼4.6 (174). Understanding which cellular factors govern endosome acidification has become crucial to identify cellular functions required for the virus to infect cells using this pathway. Viral envelope fusion with the endolysosomal membrane cells depends, among other factors, on the lysosomal two-pore channel 2 (TPC2) (175). TPCs are dimeric ion channels composed of a duplicated domain architecture and are considered an evolutionary bridge to four-domain voltage-gated Ca^{2+} and Na^{+} channels [for a review see (176)]. The opening of TPCs is known to induce a strong sodium-driven depolarization in the endo-lysosomal membrane, which has been demonstrated to facilitate membrane fusion (177). Even though the specific role

of TPC2 for virus escape into the cytoplasm is not completely clear, inhibition of TPCs should both impair the fusogenic potential of the endo-lysosomal system and disrupt the correct intracellular vesicle trafficking, resulting in inhibition of viral replication (178, 179). Accordingly, it has been demonstrated that knockdown and pharmacological inhibition of both TPC2, mainly expressed in late endosomes/lysosomes, and TPC1, which mainly localizes to early endosomes, attenuate intracellular trafficking of MERS-CoV through the endolysosomal system (180). Accordingly, Naringenin (Nar), which is the predominant flavanone in grapefruit and was found to impair TPC2 (181), has been investigated for its effects against SARS-CoV-2, after its activity had previously been observed against viruses that enter via a pH-dependent pathway such as Hepatitis C virus (182), influenza A virus (183), dengue virus (184), and Zika virus (185). Interestingly, Nar suppresses also inflammatory cytokine production through both transcriptional and post-transcriptional mechanisms, by regulating lysosome function, resulting in the inhibition of TNF-α and IL-6 secretion by macrophages and T cells (186, 187), which could contribute to prevent the excess of inflammation in COVID-19 patients. Like Nar, the TPC-specific blocker tetrandrine was also found to prevent cellular entry, viral-endosome membrane fusion and capsid disassembly required for successful virus entry (176, 188, 189). In addition, the endo-lysosomal lipid phosphatidylinositol-3,5-bisphosphate [PI(3, 5)P2] has also emerged as a direct channel activator that binds to TPCs (190) suggesting that inhibiting PI(3, 5)P2 could have antiviral effects. Accordingly, inhibition of the phosphoinositide kinase PIKfyve has been demonstrated to prevent infection by Zaire ebolavirus and SARS-CoV-2 (191). While these results obtained *in vitro* suggest the strong antiviral power of inhibiting the correct endolysosomal functionality, the toxicity and efficacy of these treatments *in vivo* remain to be tested, especially considering the ability of SARS-CoV-2 to exploit both pH-dependent and -independent pathways. Such adaptability of SARS-CoV-2 could be at the basis of the controversy surrounding the use of hydroxychloroquine for treating COVD-19. Quinine-derivative drugs, and hydroxychloroquine in particular, are well-known compounds with lysosomotropic activity which inhibit the replication of coronaviruses *in vitro* (192, 193) and were soon proposed as possible treatments for SARS-CoV-2 (194). However, the antiviral efficacy of the drug in cell culture (195–197) is in stark contrast with its inefficacy as a prophylactic and therapeutic drug in clinical settings (198–200). Accordingly, the ability of SARS-CoV-2 to exploit TMPRSS2, avoid the endosomal compartment and fuse at the cell surface could explain such discrepancy and limit the efficacy of drugs targeting the pH acidification (201).

The Virus in the Cytoplasm

Interaction of Nsp1 With the Translation Machinery and the Host RNA

After the virus accesses the cytoplasm, the replicative cycle begins with the translation of single-stranded positive-sense genomic RNA (ssRNA+). The translation of ORF1a and ORF1b from the genomic RNA produces two polyproteins, pp1a and pp1ab. Like all viruses, SARS-CoV-2 depends on the host cell translation machinery (**Figure 8**). Accordingly, RNA-interactome studies revealed the association of the viral RNA with components of the eukaryotic translation machinery, such as initiation factors, the cap- and poly(A)-binding proteins and ribosomal proteins (202–205). Furthermore, coronaviruses have evolved specialized mechanisms to hijack the host gene expression machinery and employ cellular resources to regulate viral protein production. Such mechanisms include inhibition of host protein synthesis and endonucleolytic cleavage of host mRNAs (206). A pivotal role in regulating the translation machinery of the host cell is played by the viral protein Nsp1, which is among the first proteins to be expressed after cell entry and causes repression of host translation (207). For SARS-CoV, Nsp1 has also been demonstrated to induce an endonucleolytic cleavage of the host RNA (208), while sparing from degradation viral mRNA by a yet unknown mechanism, likely mediated by the 5' UTR of the virus transcript (209). By combining cryo-electron microscopy and biochemistry, SARS-CoV-2 Nsp1, the host shutoff factor, has been recently demonstrated to bind the ribosomal mRNA channel (210). The protein was shown to interact with a range of different ribosomal conformations, as it co-migrates with both the 40S and the 80S complexes, competing with mRNA for ribosome binding. Nsp1 associates with the 40S ribosomal subunit with its C terminus interfering with the level of mRNA entry into the ribosome channel, thereby inhibiting host translation (210). As observed in the high-resolution structure of the 40S–Nsp1 complex, the C-terminal part of Nsp1 in the mRNA entrance channel folds into two helices. The first helix, composed of C-terminal residues 153–160, interacts with uS5 and uS3 [as reviewed (211)] through multiple hydrophobic side chains. The two helices are connected by a short loop containing the KH motif that establishes stacking interactions with helix h18 of the 18S rRNA through U607 and U630, as well as backbone binding. The second helix (residues 166–178), interacts with the phosphate backbone of h18 via two conserved arginines, R171 and R175 (210). Overall, these interactions tightly bind Nsp1 to the 40S subunit to cause translation inhibition by sterically occluding the entrance region of the mRNA channel. Interestingly, this inhibition mechanism may be unique to SARS-CoV-2 and other closely related beta-coronaviruses because the C-terminal region of Nsp1 is shorter in alpha-coronaviruses and is not highly conserved among other beta-coronaviruses, including MERS-CoV [as reviewed (212)]. Nevertheless, the viral mRNA has been demonstrated to be more efficiently translated than host mRNAs in the presence of this mechanism. Further details were emerged by studying the eukaryotic ribosome during initiation with the cricket paralysis virus (CrPV) internal ribosome entry site (IRES), which can directly recruit and assemble with the 40S or 80S ribosome without requiring any eIFs (213). Cryo-EM analyses with CrPV IRES have suggested that Nsp1 may act by changing the conformation of ribosomal subunit resulting in incorrect positioning of the mRNA 3' region (211). The conformation of the 40S ribosomal subunit in the Nsp1-40S complex appears to be in a "closed state," suggesting that Nsp1 not only plugs the mRNA channel but also changes the 40S subunit conformation making it incompatible with loading of mRNA. Nsp1 is proposed

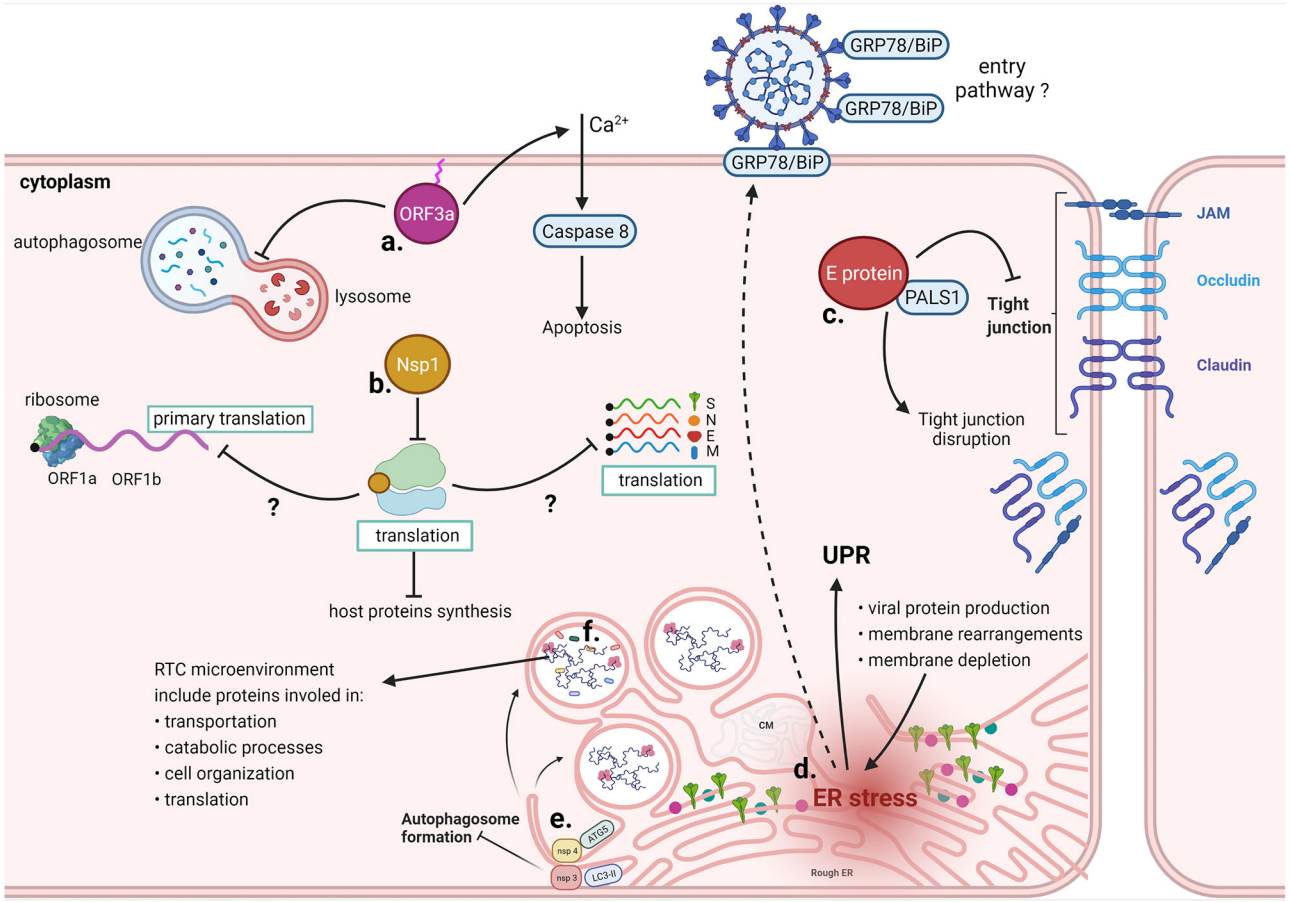

FIGURE 8 | Cytoplasmic interactions of SARS-CoV-2. a. ORF3a requires binding to the plasma membrane to promote Ca^{2+}-dependent apoptosis and prevents autophagosome-lysosome fusion. b. Nsp1 sterically occludes the mRNA entrance channel of the ribosome, prevents the physiological conformation of 48S PIC, and reduces mRNA transport through the NPC. Molecular mechanisms whereby Nsp1 specifically blocks only host translation have yet to be clarified. c. Computational predictions indicate that SARS-CoV-2 E protein binds to PALS1, normally involved in tight junctions maintenance, leading to their disruption. d. Excessive viral protein production, membrane rearrangements leading to the formation of DMVs and membrane depletion due to virus budding from ERGIC contribute to ER stress, which results in the UPR. Prolonged ER stress induces missorting of GRP78/BiP to the cell membrane, likely increasing infectivity upon binding of the chaperone with the SARS-CoV-2 spike protein. e. Nsp3 and Nsp4 prevent the formation of autophagosomes by inducing the formation of DMVs; ATG5, LC3 and other cellar factors involved in the autophagosome formation colocalize with the non-structural proteins. f. Different host proteins have been identified in the RTC microenvironment, including proteins involved in transportation, catabolic processes, cell organization and translation.

to dislodge the canonical tRNA-mRNA interaction from the 40S subunit, interfering with the joining of the 60S subunit to form the 80S initiation complex (211). Overall, these results indicate that the C-terminal domain of Nsp1 is necessary and sufficient for inhibition of translation. Instead, its N terminus may play a role in suppressing host gene expression of Type I interferon in infected cells, as described later (214), and regulating cellular mRNA stability (215). Accordingly, an attenuating mutation of Nsp1 was found to decrease the ability of SARS-CoV to replicate in cells with an intact interferon response (216). However, how SARS-CoV-2 RNA escapes inhibition of translation remains unclear, even though it has been postulated that interactions involving the viral 5' UTR may result in the "unplugging" of Nsp1 from the 40S ribosome during initiation of viral translation (211). The SL1 hairpin structure found at the 5' leader sequence is required for the interaction of the RNA molecule with the Nsp1 C-terminal domain, allowing viral RNA to be translated despite Nsp1 binding to the ribosome (37).

In addition to altering the initiation of translation, the observation that the overall transcriptome profile is perturbed by Nsp1 in infected cells has led to the hypothesis that this viral protein functions autonomously as an mRNA export inhibitor (217). Nsp1 directly binds the mRNA export factor NXF1 and reduces its interaction with the nuclear pore complex (NPC), thereby reducing expression of host mRNAs, including those which encode antiviral factors. Nsp1 disrupts NXF1 interaction with adaptor proteins which regulate mRNA interaction, and with the NPC (217). Accordingly, Nsp1 has been observed in the vicinity of the NPCs, raising the possibility that Nsp1 may shuttle between the nucleus and the cytoplasm, with its steady-state distribution being predominantly cytoplasmic. The effect on mRNA nuclear export results in an increased availability of the

host translational machinery for viral mRNAs, thus favoring virus replication. SARS-CoV-2 Nsp1 therefore suppresses host gene expression by both inhibiting translation and blocking mRNA nuclear export, two functions performed by distinct domains within the C- and N-terminal regions of the protein (217).

Following translation, a Replication and Transcription Complex (RTC) is assembled, involving several non-structural proteins including Nsp12, the RNA-dependent RNA polymerase (RdRp), and its two accessory factors Nsp7 and Nsp8, that all together form the RdRp core complex. It is thought that additional subunits bind this complex, including Nsp9, Nsp10, Nsp13, Nsp14, and Nsp16 (218). The RTC is involved in two processes of the coronavirus replicative cycle: genome replication and transcription of subgenomic mRNAs, which are then translated into *structural* and accessory proteins (219). In analogy with our knowledge from other coronaviruses, we can deduce that several host factors associate with the RTC of SARS-CoV-2 to modulate transcription. The involvement of host factors in the functionality of coronaviruses RTC was investigated recently using proximity labeling with the mouse hepatitis virus (MHV) model (220). More than 500 host proteins were found associated with the RTC microenvironment, including factors involved in transportation, catabolic processes, cell organization and translation. The latter category was enriched by factors involved in translation initiation, such as the ribosomal proteins Rpl13a and Rls24d1 and several subunits of the eIF3 complex, which were confirmed to be crucial for MHV replication. The association of factors orchestrating translation initiation was confirmed for SARS-CoV-2 by a recent study which found Nsp9 interacting with eIF4H, a partner of the initiation factor eIF4A (221). Accordingly, the eiF4 inhibitor zotatifin was observed to exert a strong antiviral activity and on these bases was approved for a phase-I clinical trial for the treatment of COVID-19 (222), leading the way to exploit the interface between the viral replication machinery and the host factors as target for therapeutic intervention.

The Virus Interaction With Intracellular Membranes

The replication of coronaviruses and many other positive-sense RNA viruses occurs in the cytoplasm of infected cells, in association with modified membranes that are transformed into distinctive structures called viral replication organelles (ROs). ROs provide platforms that facilitate viral RNA synthesis by concentrating relevant factors, while preventing the exposure of viral replication intermediates to cytosolic innate immune sensors (223). The formation of invaginations can occur at different membranes of various organelles, including the endoplasmic reticulum (ER), the endolysosomes, and mitochondria (224, 225). Such membranous structures may take the shape of convoluted membranes (CVs) and double-membrane vesicles (DMVs), originating from the endoplasmic reticulum, and the recently discovered double-membrane spherules (226). Viral RNA synthesis was shown to predominantly occur within such DMVs, with a pore connecting the interior of the vesicles to the cytosol for transport of viral RNAs and metabolites required for RNA synthesis.

The membrane-spanning Nsp3, Nsp4, and Nsp6 have been proven to be implicated in diverting cellular endomembranes into replication organelles, with other non-structural proteins and unknown cellular factors possibly involved in the process (224, 227). Such proliferation and reorganization of intracellular membranes by viruses is reminiscent of the formation of autophagosomes, which nucleate in the cytoplasm to originate a double membrane organelle. It is therefore plausible that SARS-CoV-2 exploits cellular factors required for autophagy alongside viral proteins for the biogenesis of DMV. Accordingly, coronaviral Nsp3 and Nsp4, which are co-translationally inserted in the ER, are sufficient to induce the formation of convoluted membranous structures in human cells and by inducing the formation of DMVs they inhibit the formation of canonical autophagosomes (45, 228). Cellular factors involved in autophagosome formation, such as ATG5 (229), LC3 (230), and ptdIns3P (231), were observed to colocalize with non-structural viral proteins on DMVs, and to be required for coronavirus replication, though perhaps in a cell-type specific fashion.

The ability of coronaviruses to induce formation of DMVs associates also with the modulation of lipid biosynthesis, resulting in a selective lipid composition with alteration of glycerophospholipids and cholesterol levels. To this end, coronaviruses hijack host factors to manipulate lipid mobilization and synthesis. Accordingly, sterol regulatory element-binding proteins (SREBPs), which regulate the biosynthesis of cellular cholesterol and fatty acids, were found to play an essential role for DMV formation by MERS-CoV (232). In addition, TMEM41B, an ER resident protein involved in induction of autophagy (233) and thought to be required for coronavirus replication, was identified as a crucial factor for the replication of diverse coronaviruses possibly by mobilizing cholesterol and other lipids for DMV formation (234). The antiviral effect of drugs targeting lipid biosynthesis, such as AM580 (232), demonstrate that interfering with lipid homeostasis could become an effective antiviral strategy. Further elucidation of the mechanisms and factors required for DMV formation will however be crucial to understand to what extent this process can be targeted to interfere with virus replication.

Virus Assembly and the Interaction With the Secretory Pathway

As recently proposed in a cryo-ET study (235), nucleocapsid assembly of SARS-CoV-2 is driven by the interaction between the N protein and the viral gRNA, giving rise to viral ribonucleoparticles (vRNPs) in a beads-on-a-string like conformation, resembling the helical filaments already observed for other coronaviruses (236). This conformation allows maintenance of high steric flexibility between the vRNPs, ideal for the incorporation of the SARS-CoV-2 large genome. It has been estimated that vRNPs are made of ∼12 N monomers and 800 nt RNA and that each virion contains ∼35–40 vRNPs (237). The pivotal role of the N protein in nucleocapsid assembly is further supported by its ability to undergo phase separation with RNA, as reported by several independent studies (238–243). Specific gRNA elements were proposed to differentially interact with the N protein, either promoting liquid-liquid phase

separation (LLPS) or impeding it. A mixture of LLPS promoting elements, such as the 5'end and the nucleocapsid encoding region found at the 3'end, characterize the full lenght gRNA and favor a selective LLPS resulting in the exclusion of host mRNA from packaging (239). An L/Q rich region found in the central intrinsically disordered region (IDR) of N is supposed to be responsible for these interactions (243). As suggested by others and despite previous indications of phase separation being implicated in other viral contexts (242), it is currently impossible to exclude that phase separation is just an epiphenomenon due to the high concentration and multivalency of N proteins. For this reason, further studies are needed to elucidate the implication of LLPS in the viral replication cycle, eventually addressing its physiological relevance in SARS-CoV-2 assembly (239), as already proposed (242). Interestingly, recent analyses using fluorescent microscopy and FRAP indicate accumulation of RdRp at the level of N/RNA condensates (241). Even though this seems in contrast with the concept that replication occurs in ER DMVs, a dual involvement of N protein in both assembly and replication was recently demonstrated. It appears that phosphorylation of the N proteins in a particular S/R rich region, found in proximity of the L/Q-rich region of the central IDR, allows it to recruit host factors (e.g., RNA helicases), to eventually promote viral RNA template switching and subgenomic mRNA transcription. On the other hand, hypophosphorylation of the N protein in the same domain is responsible for its incorporation into virions (238, 243).

Altogether, these findings are pointing at the central role of the condensates involving the N protein, indicating this as a potential therapeutic target for future antiviral therapies against SARS-CoV-2. Possible compounds could either induce condensate dissolution (e.g., 1,6-hexanediol) and size modifications (e.g., kanamycin, lipoic acid) and/or alter protein/RNA ratio (e.g., kanamycin) (239). It is reasonable to assume that condensate formation could be advantageous to concentrate viral components in an isolated compartment in the cytosol, possibly improving nucleocapsid assembly. However, the mechanism underlying the recruitment of these condensates to the membranes, where subsequent steps in virion assembly take place, remains unclear (242).

The final assembly of SARS-CoV-2 virions requires M, E, S and N proteins and takes place at the level of intracellular membranes belonging to the ER-Golgi intermediate compartment (ERGIC), as previously shown for SARS-CoV-1 and other coronaviruses (236, 244). Virus budding occurs mainly at the level of the ERGIC membrane arrangements where vRNPs were observed to accumulate on the cytosolic side (235), while S trimers were found to be facing the luminal side. S trimers and M protein alone were shown to be unable to induce membrane curvature, consistent with previous evidence from SARS-CoV (244). Nonetheless, it appears that the M protein interacts with vRNPs (243) possibly driving their accumulation at the ERGIC membrane and together causing its curvature, which might also be dependent on the E protein, as suggested by studies with other coronaviruses (236). The evidence that some individual vRNPs are oriented on the viral envelope in an "eggs-in-a-nest" shaped assembly supports the role of specific M-N interactions driving packaging of new virions (237). Moreover, additional evidence shows that the N protein undergoes phase separation with soluble fragments of the membrane associated M protein, highlighting an important interaction that reinforces the pivotal role of M protein in recruiting the assembled nucleocapsid to the ERGIC compartment (243). Furthermore, the simultaneous expression of M, N, S, and E was shown to be pivotal for efficient viral assembly and it was also demonstrated that both E and M play a central role in inducing the retention of S in the ERGIC compartment. A recent study shows that M is able to directly interact with S through a C terminal retention signal, while E indirectly induces S retention by altering the cell secretory pathway, possibly by acting as a viroporin (245). The accumulation of M and E at the level of ERGIC/Golgi membranes could alter the activity of glycosylases, hence disrupting the N glycosylation profile of the S protein. The advantage given by the modulation of S maturation might be related to the ability of the virion to attach to lectins found at the surface of permissive cells, as proposed for SARS-CoV (246). Interestingly, a recent publication (247) suggests that the glycosylation profile of the Spike protein can be affected by inhibitors of protein glycosylation such as Miglustat and Celgosivir, resulting in misfolded S protein accumulation and reduction of infectious viruses release, highlighting the antiviral potential of these drugs.

The completion of the first step along the egress pathway of SARS-CoV-2 is established by the virus budding in the lumen of the ERGIC compartment. From this point on, it was originally believed that SARS-CoV-2 would follow the biosynthetic secretory pathway engaged by other enveloped viruses. However, recent findings suggest that SARS-CoV-2 and other betacoronaviruses exploit a lysosomal exocytic pathway that results in non-lytic release of the virions (248) confirmed also by recent cryo-imaging studies which indicate the formation of tunnels connecting multi virus-containing vesicles to the cell membrane, possibly supporting the lysosomal mediated exocytosis (249). These tunnels were not observed in another independent study, which instead indicates that virus egress follows a secretory pathway involving fusion of small vesicles generally containing only one viral particle (250). Nevertheless, by following such unconventional non-lytic egress pathway, viruses are co-trafficked together with the ER chaperone GRP78/BIP which could therefore assist the correct folding of newly synthesized viral protein. Interestingly, given the low pH, the lysosomal egress pathway could cause a premature activation of the viral particles. However, late endosomes/lysosomes of infected cells were shown to be deacidified and carry lysosomal enzymes with reduced activity. The exact deacidification mechanism has not yet been elucidated, with one possibility being that this is actively promoted by the infected cells to relieve viral-induced stress (248, 251). However, one deacidification pathway involves ORF3a which may act as a viroporin, consistent with previous knowledge involving SARS-CoV (63, 252, 253).

Interestingly, perturbation of lysosomal pH could also disrupt the antigen cross-presentation pathway and the endolysosomal Toll-like receptor signaling (248), benefiting virus survival. Since the lysosomal exocytic pathway depends on small GTPases, such

as Arl8b and Rab7, which regulate intracellular trafficking, these have also become possible targets to inhibit the latest stage of the virus life cycle (248, 254).

The Interaction of ORF3a With the Apoptosis and Autophagy Pathways

It has been well-established that the SARS-CoV accessory protein ORF3a induces apoptosis, Golgi fragmentation, and accumulation of intracellular vesicles (255). Even though data with SARS-CoV-2 remain scarce, the pro-apoptotic activity of SARS-CoV-2 ORF3a has been confirmed in different cell lines, where the viral protein was shown to activate mainly the extrinsic apoptotic pathway by promoting cleavage of caspase-8 (64). Features that regulate SARS-CoV ORF3a intracellular transport include a tyrosine-based sorting motif (YXXΦ) and a diacidic EXD domain (256), a cysteine-rich sequence and a region with potassium channel activity (257). The protein ORF3a from SARS-CoV-2 shares 73% amino acid homology with its counterpart in SARS-CoV, maintaining the cysteine-rich sequence, the YXXΦ motif and the potassium channel region (64) and exerting a relatively weaker apoptotic activity than that associated with SARS-CoV ORF3a, correlating with the lower virulence of the pandemic virus. Interestingly, some of the mutations naturally occurring in the SARS-CoV-2 ORF3a coding sequence have been recently demonstrated to significantly affect protein stability and secondary structure (258), possibly affecting the virus pro-apoptotic activity. *In-silico* studies suggest that these mutations may help the virus to evade the host immune system because of the loss of B-cell putative epitopes (258), indicating a possible trade-off between the loss of apoptotic activity in favor of immune evasion. Nevertheless, residues of SARS-CoV-2 ORF3a predicted to favor stability of the protein central pore remain unaltered conserved (259) indicating that, though lower than in SARS-CoV, the ability to induce apoptosis remain important. Since the ion channel activity of ORF3a is required for its pro-apoptotic activity, inhibiting its function may still provide a direction toward interfering with coronaviruses cytopathology (257).

As a catabolic pathway of mammalian cells, autophagy controls viral infections at multiple levels by causing destruction of viruses, regulating inflammatory responses, and promoting antigen presentation. Moreover, viruses manipulate autophagy for their immune evasion, replication, and release from infected cells [for a review see (260)]. SARS-CoV-2 ORF3a has been shown to inhibit autophagosome-lysosome fusion by disrupting the assembly of the RAB7-HOPS fusion machinery, evidence obtained with ectopic expression of ORF3a (261). The HOPS tethering complex regulates the activity of SNARE proteins which govern autophagosome fusion with lysosomes (262). The C-terminal region of SARS-CoV-2 ORF3a was reported to interact with VPS39, a component of the HOPS complex, and to disrupt the assembly with the SNARE proteins (263). Interestingly, given the conformation similarity between DMVs and autophagosomes, ORF3a disruption of RAB7-HOPS interaction may also be beneficial for the virus to prevent DMVs from fusing with lysosomes, thereby promoting the conditions for optimal viral replication (261).

The Interaction of SARS-CoV-2 With the ER

Infections caused by coronaviruses, such as SARS-CoV, MHV, IBV, and HCoV-HKU1, are known to induce ER stress, resulting in the activation of the unfolded protein response (UPR). This is regulated by ER transmembrane sensors PKR-like ER protein kinase (PERK), inositol-requiring protein 1 (IRE1) and activating transcriptional factor 6 (ATF6), leading to increased expression of ER chaperons, to global repression of protein synthesis or even to apoptosis when the ER stress becomes persistent [reviewed in (264, 265)]. ER stress is demonstrated by the induction of Immunoglobulin heavy chain-binding protein (BiP, also known as glucose-regulated protein 78, or GRP78) or glucose-regulated protein 94 (GRP94) in cells infected with SARS-CoV (265). From our knowledge based on SARS-CoV, ER stress is induced by different mechanisms which are consequences of the viral replicative cycle in the host cell. First, the UPR can be triggered by ER saturation following the extensive production of viral proteins, particularly the spike and the accessory proteins ORF3a, ORF6, ORF7a, and ORF8ab (266–268). In addition, contributions to ER stress originate from the formation of DMVs and other membrane rearrangements as well as from the ER membrane depletion following virus budding from the ERGIC and autophagy (265). More directly, the central region of the S1 subunit (amino acids 201–400) of SARS-CoV was found responsible for activating the transcriptional activity of GRP78 and GRP94 promoters, eventually leading to UPR (269). ORF8b was instead shown to contribute to ER stress by forming aggregates dependent on a C-terminal VLVVL motif (253). The contribution of ORF8 to ER stress was additionally confirmed by studying two genotypes of SARS-CoV-2, namely ORF8L and ORF8S, which carry a Leucine or a Serine at position 84 and were identified during the early stages of the pandemic in China. Despite lacking the aggregation motif found in SARS-CoV (270), both ORF8L and ORF8S are able to trigger the activation of ATF6 and IRE1 (71) and PERK (271). SARS-CoV-2 ORF8 is encoded by a hypervariable gene and several different polymorphisms have already been identified, as reported by a recent review (73). A large deletion (Δ382) which abolishes ORF8 expression associates with a favorable clinical outcome despite no appreciable effects on viral replication (272) demonstrating the contribution of ORF8 to SARS-CoV-2 virulence. Given all these indications that point toward a crucial role in pathogenesis, inhibiting ORF8 effector functions could be used to improve the clinical course of the infection (273).

Another contribution to the induction of UPR comes from the region IV of the SARS-CoV-2 spike protein which was observed to interact with the substrate-binding domain (SBD) of BiP (254). Conditions of prolonged ER stress result in an altered trafficking of BiP to the cell surface, causing its accumulation on the cell membrane or in extracellular secretions. Indeed, increased levels of BiP in lung pneumocytes and macrophages was observed in patients with COVID-19 (274). Interestingly, cell surface accumulated BiP has been shown to function as attachment factors for MERS-CoV, promoting viral infection (275). SARS-CoV-2 spike protein might also bind BiP, presumably facilitating viral entry and boosting infection (254). In addition to characterizing several pathological conditions,

such as diabetes, obesity, and neurodegeneration, persistence of ER stress resulting from the accumulation of misfolded proteins can be elicited during aging by a decline of ER chaperone activity. Age and comorbidities therefore may predispose to ER stress caused by coronavirus infection, as well as lead to an increased susceptibility to infection due to accumulation of BiP on the cell surface (276, 277), therefore contributing to the severity of the coronavirus diseases in elderly or chronically diseased individuals.

If from one side it appears that the interaction between SARS-CoV-2 proteins and UPR effectors leads to the activation of this stress response, another recent article suggests that, even though CoVs infection initiate ER stress signaling and induces UPR components at the mRNA level, these are inhibited at the protein level (278). This could indicate that coronaviruses, including SARS-CoV-2, might have evolved strategies acting at posttranscriptional or translational level to escape antiviral response placed by BiP, IRE1α, and HERPUD. The evolution of a viral mechanism to counteract the effects of ER-stress is consistent with the emergent notion that UPR provides a crucial contribution to the activation of innate antiviral signaling, as seen with flaviviruses (279). Strikingly, treatment of cells with thapsigargin, a guaianolide which induces ER-stress by inhibiting the ER Calcium ATPase, represses replication of SARS-CoV-2 and other CoVs, counteracts virus mediated BiP downregulation, activates IRE1α and outweighs coronavirus mediated inhibition of global protein synthesis, thus becoming an attractive antiviral drug candidate (278, 280).

The Interactions of SARS-CoV-2 With Adhesion Molecules

The enigmatic envelope (E) protein is the smallest among SARS-CoV-2 structural components acting as a viroporin and a virulence factor proposed to affect assembly, budding and envelope formation (29). Other than assisting virus replication, the E protein was also observed to interfere with the control of cell polarity and cell-cell junction integrity in epithelial cells. For both SARS-CoV and SARS-CoV-2, the E protein was shown to bind PALS1 PDZ domain, via the C-terminal DLLV motif (281–283). PALS1 is part of the CRUMBS3-PALS1-PATJ polarity complex, which is crucial for the establishment and maintenance of epithelial polarity. Accordingly, in cells infected with SARS-CoV, PALS1 was shown to relocalize to virus assembly sites resulting in the disruption of tight junctions. Disruption of tight junctions could also be the result of an interaction between the C-terminal domain of SARS-CoV-2 E protein and the PDZ domain of Zona Occludens-1 (ZO1), known for its pivotal role in tight junction formation (283). At the same time, the E protein was shown to also cause disruption of adherens junctions by impairing E-cadherin cell surface delivery (281, 282, 284). The impairment of both types of junctions could therefore explain the alveolar desquamation observed in SARS-CoV infection and the disruption of the epithelial barrier eventually favoring viral dissemination through systemic circulation and the amplification of the inflammatory response (281). Based on the genomic differences with SARS-CoV, a stronger interaction with PALS1 was predicted *in silico* based on two polymorphic residues at the C-terminus domain, postulating a crucial pathogenic role of the E protein of SARS-CoV-2 (282). Accordingly, the complex between SARS-CoV-2 E protein and PALS1 was characterized by cryo-EM indicating a possible target for novel peptide and small molecule inhibitors (285).

Virus Interaction With Cellular Innate Immunity

Upon infection, SARS-CoV-2 pathogen associated molecular patterns (PAMPs) can be recognized by several host pathogen recognition receptors (PRR), predominantly including Toll-like receptors (TLRs), retinoic-acid inducible gene I (RIG-I) and melanoma differentiation-associated 5 (MDA-5). Receptors intercepting SARS-CoV-2 can be found either in the endosomal compartment, where TLR-7 and TLR-8 can recognize the ssRNA viral genome, or in the cytoplasm, where RIG-I and MDA-5 initiate a signaling cascade in response to the ssRNA genome and the replication-intermediate dsRNA, respectively (**Figure 9**). RIG-I and MDA-5 signal through the same cascade: upon target RNA recognition, the CARD domain of these sensors recruit E3 ligases (TRIM25 and Riplet) that form a K63-linked polyubiquitin scaffold, allowing RIG-I and MDA-5 to interact with mitochondrial antiviral signaling protein (MAVS) on the outer mitochondrial membrane and form MAVS aggregates. These aggregates will in turn recruit TRAF family E3 ubiquitin ligases, which form new K63-linked polyubiquitin that activate TANK binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), eventually leading to the production of type 1 interferons (IFNα/β) and activation of NF-κB [for a review see (286)].

Interferon production is pivotal for the induction of an antiviral state and the activation of adaptive immunity in both the infected cell and the surrounding cells, as it has both autocrine and paracrine effects. Interferon molecules, upon binding to their corresponding receptors IFNAR1/IFNAR2 on cells surfaces, activate a signaling cascade mediated by the Janus kinase 1 (JAK1), Tyrosine kinase 2 (TYK2) and signal transducer and activator of transcription (STAT1 and STAT2) (213). Upon phosphorylation, these mediators undergo dimerization and nuclear translocation, ultimately leading to the activation of several IFN-stimulated genes (ISGs) and contributing to the establishment of the antiviral state (287).

SARS-CoV-2 Interaction With the RIG-I Signaling Pathway

Several SARS-CoV-2 proteins have been shown to inhibit IFN-β promoter activation (54). Screening evidence reports that 11 viral proteins, both structural and non-structural, can antagonize the RNA-dependent IFN-β induction by targeting different components of the intracellular antiviral response triggered by RIG-I.

SARS-CoV-2 ORF9b was shown to localize on mitochondrial membrane and elicit the suppression of type I IFN response through association with TOM70, one of RIG-I mitochondrial interacting proteins besides MAVS, TRAF3 and TRAF6 (288) (**Figure 9**). In particular, upon viral RNA recognition, TOM70 mediates the interaction between MAVS and Hsp90-bound

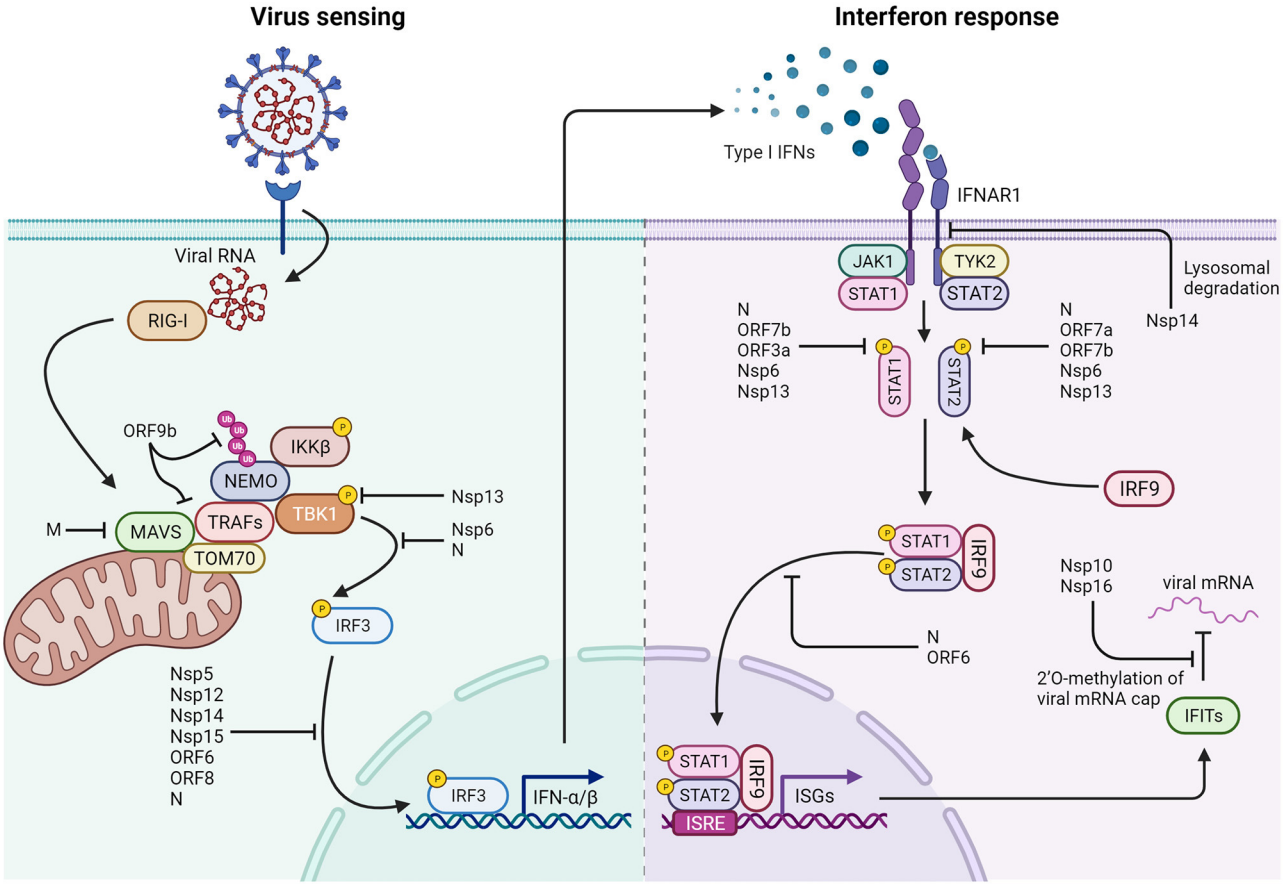

FIGURE 9 | Interactions of SARS-CoV-2 with cellular components of the immune system. **Left**: SARS-CoV-2 triggers several mechanisms that hinder type 1 interferon production by preventing both viral RNA detection and activity of the IRF3 transcription factor. ORF9b interacts with TOM70 on the mitochondrial surface and prevents K63-polyubiquitination of NEMO. The M protein prevents MAVS aggregation and interaction with downstream proteins. Nsp13 blocks TBK1 phosphorylation by inhibiting its interaction with MAVS, while Nsp6 binds TBK1, and N protein prevents IRF3 phosphorylation. Nsp5, Nsp12, Nsp14, Nsp15, ORF6, ORF8, and N are reported to impair nuclear localization of phosphorylated IRF3. **Right**: Mechanisms that impair the response to type I interferons: Nsp14 induces lysosomal degradation of type 1 IFN receptor. Nsp6, Nsp13, ORF3a, ORF7a, ORF7b, and N prevent STAT1 and STAT2 phosphorylation. ORF6 and N protein hamper phosphorylated STATs nuclear translocation. In addition, Nsp10 and Nsp16 are responsible for 2'O-methylation of viral mRNA cap, limiting IFITs activation and translation.

TBK1 and IRF3 (289). ORF9b can prevent the interaction of TOM70 with Hsp90, thus impairing this signaling pathway. The residues found to be crucial for the interaction between TOM70 and ORF9b are TOM70 E477 and ORF9b S53, and the interaction is facilitated by the binding between TOM70 R192 and the ORF9b EEVD motif (74). Since SARS-CoV-2 is detected mainly by RIG-I (76), the inactivation of this pathway strongly affects the host immune response. Consequently, the development of molecules inhibiting the interaction between ORF9b and TOM70 represents a possible therapeutic approach. Besides interacting with TOM70, ORF9b interrupts the K63-linked polyubiquitination of NEMO, hence preventing IKK activation and IKKβ phosphorylation, ultimately blocking NF-κB activation (76). In addition, ORF9b has been suggested to act at the convergence point among the RIG-I/MDA-5-MAVS, TLR3-TRIF, cGAS-STING pathways, that is TBK1, and inhibit them all. This protein also interacts with STING preventing TBK1 phosphorylation and TRIF interaction with TBK1. The presence of anti-cGAS-STING pathway proteins in SARS-CoV-2, despite it being an RNA virus, suggests that this pathway is involved in SARS-CoV-2 clearance (75).

Recent results also suggested that the M protein inhibits the RIG-I mediated antiviral response by interacting with the MAVS modulator. Mechanistically, the M protein impairs the MAVS aggregation in particular by disrupting MAVS self-association and its association with SNX8, thus altering the recruitment of downstream TRAF3, TBK1, and IRF3 which are required for an efficient innate antiviral response (31).

Additional proteins interfere with IFN-β induction by interacting with TBK1, which is essential for the phosphorylation and subsequent activation of IRF3. Indeed, Nsp6 has been shown to reduce the activity of genes driven by the IFN-β promoter *in vitro* (44) by binding TBK1 and reducing phosphorylation of IRF3. Interestingly, the naturally occurring L37F mutation of this

protein is associated with a milder outcome of the infection (290), although the impact of this mutation on RIG-I signaling has not yet been directly documented.

Nsp13, the SARS-CoV-2 helicase, is another inhibitor of the type I IFN response (54). Nsp13 binds and blocks TBK1 phosphorylation, thus resulting in decreased IRF3 activation and IFN-β production. Recent results suggest that Nsp13 suppresses type I IFN production by disrupting the association of TBK1 with MAVS (52). Furthermore, Nsp13 hijacks the deubiquitinase USP13, which deubiquitinates and therefore stabilizes Nsp13. Interestingly, knockout of USP13 or treatment with USP13 inhibitor mitigates the Nsp13 inhibition of type I IFN response and suppresses viral replication. Therefore, USP13 inhibitors could be used to suppress virus replication by targeting Nsp13 for degradation (52).

Several proteins have been shown to impair either phosphorylation or nuclear translocation of IRF3. Nsp5 counteracts RNA-dependent IFN-β induction by acting downstream of IRF3 cytoplasmic phosphorylation mediated by TBK1 and IKKε. Indeed, Nsp5 was shown to impair the nuclear localization of phosphorylated IRF3 rather than its stabilization or phosphorylation (42). Nsp12, an RNA dependent RNA polymerase, was also reported to inhibit the IFN signaling (54), by suppressing the nuclear translocation of IRF3 which is essential for the promotion of production of IFN-α/β. The mechanism was shown to be independent from its polymerase activity (50), though results from a recent report confuted this activity (291). Nsp14 and Nsp15 have also been reported to block IRF3 nuclear translocation (57), SARS-CoV-2 Nsp15 being less potent in counteracting both IFN-β induction and signaling than the SARS-CoV analog, perhaps contributing to the more favorable clinical outcome of SARS-CoV-2 (292).

ORF6 and ORF8 are also reported to inhibit the nuclear translocation of IRF3. Two of the genotypes of ORF8, ORF8L, and ORF8S, were shown to be capable of down-regulating IFN-β, interferon-stimulated genes (ISGs) ISG15 and ISG56 production, and impaired IRF3 nuclear translocation in response to poly (I:C). This down-regulating effect was found at the level of both protein abundance and mRNA levels, but it remains unclear whether SARS-CoV-2 ORF8 physically interacts with transcription factors involved in IFN-β regulation (71). The ability of ORF6 to inhibit IRF3 nuclear translocation is conserved between SARS-CoV-1 and SARS-CoV-2, depending on the sequence DEEQPMEID found at its C-terminus, which could indicate another possible therapeutic target for anti-COVID-19 therapy (54).

Finally, the N protein co-localizes with RIG-I by interacting with its DExD/H domain and, with still undefined mechanisms, impairs both IRF3 phosphorylation and nuclear translocation rather than IRF3 production, thus impacting IFN-β production (293).

SARS-CoV-2 Interaction With Interferon Signaling

Interferon signaling is inhibited in host cells by a number of SARS-CoV-2 proteins that generally modulate STAT1 or STAT2 phosphorylation, thus preventing their nuclear translocation (**Figure 9**). So far, the viral proteins that have been reported to have such activity include N, ORF3a, ORF6, ORF7 (including both ORF7a and ORF7b), Nsp6, Nsp13, and Nsp14. The nucleocapsid (N) protein antagonizes IFN-I signaling by preventing STAT1 and STAT2 phosphorylation and interfering with the interaction of STAT1 with JAK1 and of STAT2 with TYK2, suggesting a competitive binding to STAT molecules with the respective downstream kinases. In addition, the interaction between SARS-CoV-2 N and STAT1/STAT2 prevents their IFN-induced nuclear translocation. The N-terminal 361 amino acids of N were found to be sufficient for its activity, including cytoplasmic localization, suppression of phosphorylation and interaction with STAT1/STAT2 (25).

ORF6 exerts its immune escape activity by inhibiting both IRF3 activation and STAT1 nuclear translocation. Nuclear translocation of STAT proteins is known to occur upon their phosphorylation and dimerization and to depend on the activity of the KPNA1-KPNB1 complex, which docks the import complex to the nuclear pore complex (NPC) (294). The formation of an ORF6-Nup98-Rae1 complex leads to the inability of the cargo-KPNA1-KPNB1 complex to dock at the NPC, and thus promotes cytoplasmic accumulation of phosphorylated STATs (68). ORF7a marginally inhibits STAT1 phosphorylation, while the effect of ORF7a and ORF7b on STAT2 is more pronounced and ORF7b is active also against STAT1 (44). Further analysis found the phosphorylation antagonizing effect of ORF7a to be mediated and enhanced by K63-linked polyubiquitin chains at lysine 119 (52). ORF3a contributes to the inhibition of STAT1 phosphorylation and marginally suppresses STAT2 phosphorylation (44). Nsp6 and Nsp13 act similarly on interferon signaling by suppressing STAT1 and STAT2 phosphorylation. Instead, Nsp14 acts upstream of STAT1 and STAT2 phosphorylation by inducing the lysosomal degradation of the type I IFN receptor IFNAR1, thus preventing the activation of STAT1 and STAT2 transcription factors (295).

SARS-CoV-2 Suppression of the Inflammasome and Pyroptosis

Pyroptosis has been determined to play a critical role in establishing an early antiviral response. The process is dependent on the activation of the inflammasome, a multiprotein complex consisting of pro-caspase-1, ASC and inflammasome nucleators such as NOD-like receptors (NLRs), AIM2 and pyrin. Of the many NLRs described so far, NLRP3 is strongly induced by viral infection, thus leading to inflammasome formation (296). NLRP3 inflammasome activation leads to pro-caspase-1 auto-cleavage and generation of active caspase-1 that, in turn, will process pro-IL-1β and pro-IL-18 into IL-1β, and IL-18, respectively. The secretion of these cytokines is pivotal for the recruitment of effector immune cells to the site of infection and for the establishment of an adaptive immune response. In addition to the processing of pro-IL-1β and pro-IL-18, active caspase-1 also cleaves Gasdermin D (GSDMD) (296), releasing a N-terminus fragment which interacts with membrane lipids for the formation of transmembrane pores, that cause the release of cytokines and danger signals and ultimately lead to cell death (297). SARS-CoV-2 nucleocapsid protein can interact with GSDMD

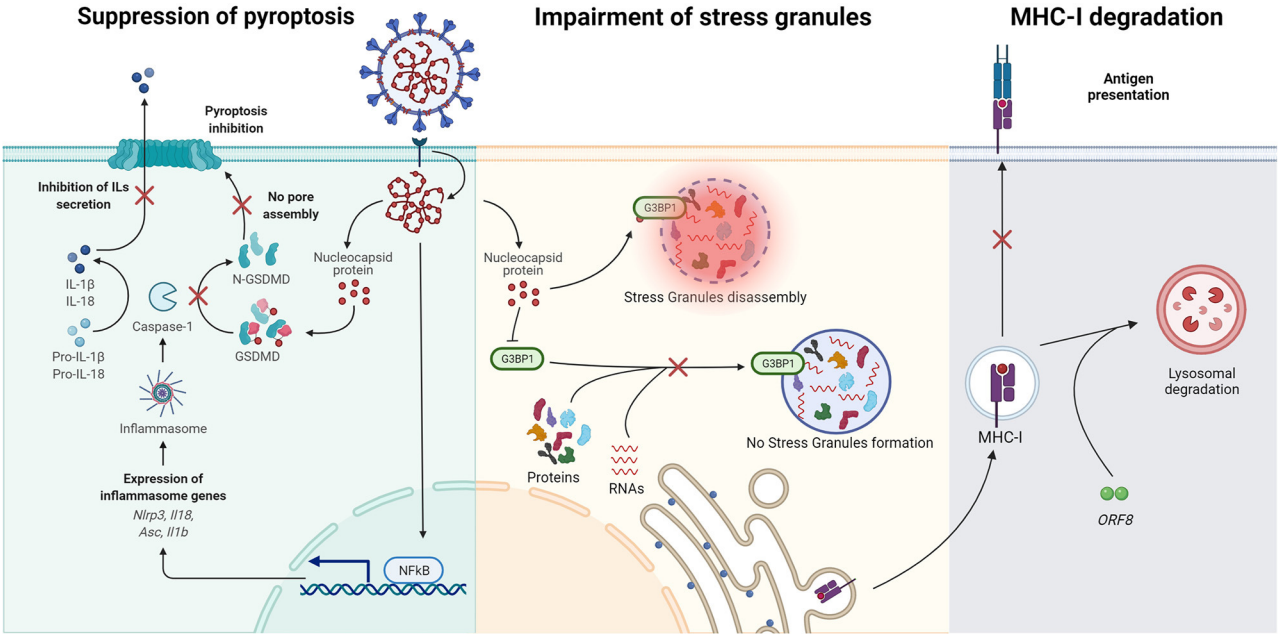

FIGURE 10 | Additional mechanisms for immune evasion. **Left**: impairment of pyroptosis. The interaction of the N protein with the linker peptide of GSDMD prevents cleavage of GSDMD by inflammasome-activated caspase-1, thus preventing N-GSDMD from assembling pores in the cell membrane and triggering pyroptosis. **Center**: impairment of Stress Granules. Binding of N protein to G3BP1 prevents G3BP1 interaction with other stress granules proteins, hampering formation of new stress granules and disassembling those already formed. **Right**: MHC-I degradation. SARS-CoV-2 ORF8 induces MHC-I lysosomal degradation, thus reducing antigen presentation on host cells.

and prevent caspase-1-mediated cleavage (**Figure 10**). Because NLRP3 inflammasome formation is unaffected by SARS-CoV-2 nucleocapsid protein, the natural consequence of GSDMD protection is the intracellular accumulation of IL-1β and IL-18 that cannot be released. This mechanism could therefore delay the initial antiviral response and contribute to the asymptomatic phase of COVID-19 infection (298). Opposing this evidence, multiple studies have in contrast shown that the inflammasome is activated in COVID-19 patients, linking this activation to a more severe disease (299–301). It was proposed that this can trigger a feedback loop of IL-1β signaling that also induces interleukin-6 (IL-6) production [reviewed in (302)]. Since high levels of IL-6 are found in severe COVID-19 cases, this step may provide a crucial contribution to the cytokine storm observed in patients (303).

Other Interactions Contributing to Immune Evasion by SARS-CoV-2

Besides the mechanisms employed by SARS-CoV-2 to evade the action of the innate immune system, SARS-CoV-2 adopts additional strategies to escape the multifaceted immunity of infected cells (**Figure 10**). As we have described earlier, SARS-CoV-2 non-structural protein 1 (Nsp1) binds to the 40S subunit of the ribosome and shuts off host RNA translation (304), a mechanism that was also observed in other coronaviruses including SARS-CoV [reviewed in (214, 305)], and which limits the ability of the host cells to express antiviral activities.

An additional mechanism is provided by Nsp16, which has been identified to be responsible for viral mRNA cap 2'O-methylation in other coronaviruses (306). 2'O-methylation of mRNA cap allows viruses to avoid restriction by IFN-induced proteins with tetratricopeptide repeats (IFIT) by mimicking the structure and post-transcriptional modifications of endogenous, eukaryotic mRNA (307). Nsp10 has been observed to associate with Nsp16 and to be required to achieve mRNA cap methylation in SARS-CoV (308). The same complex with the same methyltransferase activity is found in SARS-CoV-2 (309).

Nsp15 was shown to inhibit autophagy induction (295), while the N protein prevents the inhibition of viral mRNA translation. Accordingly, one of the innate mechanisms employed by eukaryotic cells to hamper viral infection is based on the formation of stress granules (SGs), cytoplasmic mRNA-protein aggregates. These ribonucleoprotein complexes are capable of trapping viral mRNA [reviewed in (310)], thus limiting their translation. Most viruses have evolved mechanisms to escape this inhibition by prevention of SG formation [reviewed in (305)], and SARS-CoV-2 makes no exception. SARS-COV-2 N protein was found to bind the core protein of SGs Ras-GTPase activating SH3-domain-binding-protein 1/2 (G3BP1/2) (262, 306). By specifically binding G3BP1/2, the N protein prevents the formation of stress granules and disassembles already formed granules by blocking the interaction between G3BP1/2 and other SG-related proteins, thus facilitating viral assembly. The N protein exerts this activity via formation of liquid-liquid phase separation (LLPS) condensates. In particular, N protein

intrinsically disordered region 1 (IDR1) is responsible for phase separation with G3BP1 (307). G3BP1/2 additionally functions as a positive regulator of RIG-I and significantly improves RIG-I-mediated IFN-β synthesis (308, 309) and of cGAS (308). This may be a potential mechanism by which the SARS-CoV-2 N protein affects IFN-β via the RIG-I signaling pathway (50).

MHC-I expression seems also to be dysregulated by SARS-CoV-2, a unique feature not shared with other SARS-CoV strains (72). HEK cells infected with SARS-CoV-2 were observed to decrease their MHC-I expression after infection. A similar effect was observed in mice, where MHC-I expression in lung epithelial cells was lower in animals with ongoing virus replication compared to animals that had fully recovered. ORF8 was found the most likely culprit for this process by selectively targeting MHC-I for lysosomal degradation via autophagy, protecting cells from cytotoxic T lymphocytes-mediated killing. Some *in vitro* evidence suggests that ORF8 may have more roles in immune evasion as previously described. Its ectopic expression has been correlated with a reduced expression of several IFNγ-induced antiviral molecules with different biochemical activities in lung epithelial cells (311). Interestingly, the same group observed that ectopic expression of ORF8 in HEK293 cells reduced the expression of different antiviral proteins, suggesting that the effects of ORF8 on ISGs may be cell type specific.

CONCLUSIONS

After SARS-CoV-2 emerged in 2019, the scientific community has engaged in an unprecedented effort to elucidate the virus replication mechanism, decipher the host immune response and understand the immunopathogenesis caused by the infection. Accordingly, a clearer picture of the complex pattern of molecular interactions of SARS-CoV-2 with host cells is now helping to prepare novel strategies for prevention and treatment. Here we have shown how in <2 years researchers have produced 3D structures revealing the fine details of the interaction between viral proteins and host factors, obtained exhaustive proteomics, transcriptomics, and interactomics profiles which have mapped the intricate interplay of the virus with the cells, and identified novel host factors crucial for the outcome of the infection. All this would not have been possible in such a short period of time without the prior knowledge built after years of coronavirus research triggered by previous outbreaks and epidemics, especially those involving SARS-CoV and MERS-CoV. However, some aspects of the interaction between the virus and the host cell require an improved understanding to unveil the mechanisms supporting virus replication and identify novel viral inhibitors.

The precise understanding of SARS-CoV-2 entry pathways remain essential to identify new antiviral targets, which together with neutralizing antibodies could prevent infection of the host cell. Moreover, a key early event that deserves further investigation is the specific host translation inhibition by Nsp1, an activity which could reveal a crucial Achille's heel of the virus. In general, much mystery still surrounds some ORFs and Nsps of SARS-CoV-2, which are likely to play important roles in regulating cellular pathways and in tuning the cellular environment for optimal virus replication while avoiding immunity. This is for example the case of ORF3a and its effect on the apoptotic machinery. Indeed, blocking this viral protein could re-establish the defective autophagosome-lysosome fusion of infected cells, thereby preventing formation of DMVs and favoring recognition by the immune system. At the same time, the mechanism of viral exit from the infected cell remains uncertain and insights on this step of the infectious cycle are pivotal to understand how spreading of the virus in the host can be inhibited, possibly leading to the development of new drugs.

In conclusion, while the pandemic is slowing down thanks to the global administration of vaccines, the research should continue in order to fully understand the mechanisms of SARS-CoV-2 interaction with the cell, providing results that can be translated into treatments and preparing the world for future viral outbreaks.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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