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The mechanism and consequences of SARS-CoV-2 spike-mediated fusion and syncytia formation

Maaran Michael Rajah^{1,2}, Annie Bernier³, Julian Buchrieser¹*, Olivier Schwartz^{1,2,4}*.

¹Virus & Immunity Unit, Department of Virology, Institut Pasteur; CNRS UMR 3569, Paris, France

²Université de Paris, Sorbonne Paris Cité, Paris, France

³Institut Curie, INSERM U932, Paris, France

⁴Vaccine Research Institute, Creteil, France

*Last co-authors

Corresponding authors: olivier.schwartz@pasteur.fr

Abstract

Syncytia are formed when individual cells fuse. SARS-CoV-2 induces syncytia when the viral spike (S) protein on the surface of an infected cell interacts with receptors on neighboring cells. Syncytia may potentially contribute to pathology by facilitating viral dissemination, cytopathicity, immune evasion, and inflammatory response. SARS-CoV-2 variants of concern possess several mutations within the S protein that enhance receptor interaction, fusogenicity and antibody binding. In this review, we discuss the molecular determinants of S mediated fusion and the antiviral innate immunity components that counteract syncytia formation. Several interferon-stimulated genes, including IFITMs and LY6E act as barriers to S protein-mediated fusion by altering the composition or biophysical properties of the target membrane. We also summarize the effect that the mutations associated with the variants of concern have on S protein fusogenicity. Altogether, this review contextualizes the current understanding of Spike fusogenicity and the role of syncytia during SARS-CoV-2 infection and pathology.

Introduction

Since their identification in the 1960s, coronaviruses have proven to be a persistent threat. Previous outbreaks of SARS-CoV (2001) and MERS-CoV (2012) as well as the commonly circulating HKU1, NL63, OC43, and 229E viruses have all collectively placed a significant burden on the global public health infrastructure [1]. The current global outbreak of SARS-CoV-2 has been truly staggering in its impact with over 220 million confirmed cases and 4.6 million deaths (as of September 20, 2021) [2]. Since the emergence of the ancestral Wuhan strain, several new variants have arisen and spread across the globe. The scale and impact of the pandemic and subsequent vaccination campaigns have piqued scientific interest in SARS-CoV-2; particularly into the virological and molecular determinants of SARS-CoV-2 pathology. The presence of syncytia in the lung tissues of infected patients represents one such pathological feature under scrutiny [3]. The contribution of syncytia to SARS-CoV-2 virology and pathology is currently unclear; however, this past year has seen an unprecedented flurry of research on the molecular and viral determinant of coronavirus induced syncytia formation as well as its potential impact on viral dissemination, pathology and the immune response.

Syncytia are large multinucleated cells produced by two or more cells fusing. During syncytia formation, the plasma membrane of different cells merge into a single lipid bilayer and their cytoplasmic contents merge [4, 5]. Many important physiological processes are dependent on cells undergoing syncytialization, including the formation of muscle fibers and the placental barrier as well as bone osteoclast differentiation [5, 6]. The fusion process is facilitated by a class of specialized proteins known as fusogens, which help overcome the natural repulsive forces and energetic barriers that act to keep cellular plasma membranes apart [7]. Mechanistically, cell-cell fusion is analogous to virus-cell fusion. Indeed, the membrane of enveloped viruses must fuse with cellular membranes in order for viral contents to be delivered into the cytoplasm, beginning the viral replication cycle. The parallels between the

mechanics of physiological cell-cell fusion and virus-cell fusion during infection is appropriately exemplified by the formation of the placenta. The vital placental barrier is formed by a layer of fused cytotrophoblasts called the syncytiotrophoblast [4, 5, 8]. Syncytins, the fusogens that facilitate cytotrophoblast fusion, are in fact derived from the fusogens of endogenous retroviruses (ERV); the genes of which were assimilated into mammalian genomes 10-85 million years ago [8].

Beyond this particular evolutionary association between viruses and cell-cell fusion exists a more immediate causal relationship, in which syncytia are induced as a consequence of viral infection. Such syncytia could potentially facilitate viral replication, dissemination, immune evasion as well as cause cytopathic effects and wider tissue damage [9]. The most direct example is the fusion-associated small transmembrane (FAST) proteins encoded by the non-enveloped reovirus [10]. These fusogens are uniquely specialized cell-cell fusion proteins and they increase pathogenicity and viral dissemination [10, 11]. In contrast, the fusion machinery of enveloped viruses primarily function to fuse the membrane of viral particles with plasma or endosomal membranes. In particular instances, they may induce infected cells to fuse with adjacent cells. Viruses from a broad range of families including Herpesviridae (varicella-zoster virus, herpes simplex virus 1/2, human cytomegalovirus), Paramyxoviridae (Sendai, Nipah, Hendra, measles virus, respiratory syncytial virus), Retroviridae (human immunodeficiency virus 1, human T-lymphotropic virus) have all been shown to form syncytia *in vivo* and/or *in vitro*. For a broader discussion on how, and potentially why, different viruses induce syncytia formation we refer the reader to the insightful review by Leroy and colleagues [9]). Syncytial cells have also been observed upon infection with some members of the Coronaviridae family including MERS-CoV, the currently pandemic SARS-CoV-2 and to a lesser degree SARS-CoV [9]. There have been many investigations into the highly antigenic SARS-CoV-2 Spike (S) protein, which is the viral fusogen. Recent characterizations have provided detailed insights into the properties and domains of the S fusogen and its interaction with receptors. There have also been

significant advances in the molecular understanding of the relationship between the SARS-CoV-2 S protein-mediated fusogenicity and the innate immune response.

In this article, we endeavor to summarize and contextualize the recent literature, including our own work, on the subject of SARS-CoV-2 induced syncytia formation. We summarize the clinical evidence for coronavirus-induced syncytia formation, examine the molecular determinants of S protein-mediated fusion, and discuss the antiviral innate immunity components that restrict cell-cell fusion. We also examine the molecular and virological implications of the S protein mutations carried by the novel variants of concern in terms of fusogenicity. Finally, we discuss possible consequences of virus-induced syncytia formation on viral infection, dissemination, immune response and pathogenicity.

Clinical Evidence for Coronavirus-Induced Syncytia formation

The clinical manifestations of SARS-CoV-2 infection can range from a mild febrile illness to acute respiratory distress to extensive and occasionally fatal lung damage [3]. The deterioration of lung tissue is characterized by diffuse alveolar damage, micro/macro-vascular thrombosis, and pneumocyte necrosis [3, 12, 13]. Many of these lung pathologies are also reminiscent of SARS-CoV and MERS-CoV infections [3, 14, 15]. The clinical features of COVID-19, especially the extensive lung thrombosis, are particularly severe in comparison to other causes of acute respiratory syndrome [3, 16]. A combination of hypoxemia, immune-mediated damage, and virus-induced cytopathic effect all contribute to lung epithelial destruction [3, 16, 17]. Amongst these factors, most of the described histopathology is likely related to the immune response. Indeed, infection of respiratory epithelial cells can lead to monocyte, macrophage and dendritic cell activation and the secretion of proinflammatory cytokines. Excessive cytokine production has been documented in the blood of patients presenting the most severe form of disease [18-21]. Virus-induced cytopathic effects may also directly damage the lung tissue while also triggering

further immune activation. A peculiar feature noticed in many clinical reports was the presence of multinucleated pneumocytes. One team reported the presence of infected syncytial pneumocytes in 36/41 patients who died of COVID-19 [3, 16]. This observation is supported by several COVID-19 autopsy reports from patients and non-human primate models which also describe the prevalence of syncytia in infected lung tissue [22-26]. It should be noted that these clinical observations are from patients presenting the most severe manifestation of COVID-19 disease and it is currently unclear if syncytia formation is also a feature of milder or asymptomatic SARS-CoV-2 infections. Autopsy reports conducted on patients who were infected with SARS-CoV and MERS-CoV have also reported the presence of giant multinucleated syncytial cells in the lungs, some of which are of epithelial origin [14, 15, 27-30].

Several of the “common cold” coronaviruses including hCoV-HKU1, hCoV-NL63 and hCoV-229E form syncytia in cell culture models [31-33]. To our knowledge, there are no documented patient case of syncytia induced by these widely circulating coronaviruses. This is unsurprising as they are not known to be lethal, so autopsy reports are scarce. More *in vivo* evidence in animal models is required to assess the syncytogenicity of the common cold coronaviruses. It is currently unclear if or how SARS-CoV-2-mediated syncytia formation contributes to pathology; however, it may compound the detrimental effects of virus-induced cytopathicity and generalized immune-mediated damage and contribute to the deterioration of lung tissue seen in critical cases. Furthermore, syncytia may also contribute to viral dissemination and immune evasion by protecting the virus from immune cells and from neutralizing antibodies (discussed in later sections).

SARS-CoV-2 Viral Life Cycle and the S protein

The S protein is important for both virions and cell-cell fusion. As such, its genesis, action, processing, and translocation within the context of the viral life cycle is of importance to understand syncytia formation.

The surface of the SARS-CoV-2 virion contains the trimeric S protein, which protrudes from the surface of the virus giving the virion a “crown-like” appearance from which the family nomenclature of “corona” derives. The structure and features of the S protein are described in depth in a later section. The S protein is a class I fusogen (structurally comprised of α -helices) which facilitates membrane fusion after undergoing structural rearrangements which transition it from a metastable prefusion form to a fusion competent state [7, 34]. The fusion of the viral membrane with the cellular membranes and the deposition and uncoating of viral genomic RNA into the cellular cytoplasm represents the first stage of the viral life cycle (Fig. 1) [35]. This occurs when the S protein on the surface of the virions interacts with the human angiotensin converting enzyme 2 (ACE2) receptor at the surface of the target cell. Several auxiliary receptors such as L-SIGN, DC-SIGN, and neuropilin-1 (Nrp1), may facilitate attachment or entry [36, 37]. The virus can then fuse at the plasma membrane (early entry) or with endosomal membranes (late entry) during uptake (Fig. 1). When bound at the plasma membrane, the S protein may be primed by host factors like transmembrane serine protease 2 (TMPRSS2), which enhances SARS-CoV-2 entry [38-40]. After endocytosis, proteolytic activity by host factors such as cathepsin L/B (CTSL/B) and furin further assists in the priming the S protein (Fig. 1) [38, 41, 42].

Following fusion and uncoating, the first two-thirds (from the 5' end) of the viral RNA is translated from two open reading frames, ORF1a and ORF1b [35, 39]. The resulting polyproteins are co- and post-translationally processed into the non-structural proteins (nsp1-nsp16) that make up the viral replication and transcription complex [35]. Coronaviruses, like many other described viruses, co-opt intracellular membranes to form viral replication organelles that provide a protective microenvironment for the genomic viral RNA replication and protein transcription [35, 43]. The structural proteins S, envelope (E), nucleocapsid (N), and membrane (M) as well as several accessory proteins, are transcribed from ORFs from the final third of the viral genome from a nested set of sub-genomic mRNAs [35]. These accessory proteins are thought to play a role in viral pathogenicity and in modulating the host's immune response.

During the coronavirus assembly process, the E, M, and S proteins are translocated and inserted into the endoplasmic reticulum and are trafficked through the ER-Golgi intermediate compartment (ERGIC) to the Golgi apparatus (Fig. 1). The immature virions then bud into the ERGIC or Golgi compartments. Viral egress occurs through deacidified lysosome-dependent exocytosis [44]. During the course of S protein translocation and viral assembly, the S protein is subjected to processing by furin and other cellular proteases, generating the non-covalently associated S1 and S2 subunits which are primed for fusing with the membrane of naïve cells [45].

The role of S Protein in Syncytia Formation

Despite the majority of the genesis and processing of the S protein, as well as viral budding, occurring at intracellular membranes, we and others found that the S protein is present on the surface of infected cells [26, 46]. The interaction of the S protein on the infected cell surface with the ACE2 receptor on neighboring cells is the cause of syncytia formation (Fig. 1). This process is further augmented by the presence of the TMPRSS2 protease [46]. The exact role that auxiliary receptors may play in cell-cell fusion remains to be elucidated; however, Daly and colleagues note that knocking out Nrp1 in cells expressing ACE2 is detrimental to syncytia formation [47]. It has been suggested that Nrp1 facilitates earlier separation of the S2 domain, which contains the fusion machinery, by stabilizing the S1 domain [48]. However, prior to any receptor interaction, syncytia formation requires the S protein to be translocated to the plasma membrane.

Therein lies the first question regarding the mechanism of SARS-CoV-2 S protein-mediated syncytia formation. If throughout the viral life cycle, the S protein is associated with the intracellular membrane and the virus itself does not bud from the cell surface (Fig. 1), how then is the S protein able to localize there? Many factors act in conjunction to keep the S protein sequestered on the intracellular

membranes. In many coronaviruses, the M protein contains a Golgi localization signal and also associates with the S protein, effectively retaining it on intracellular membranes (Fig. 1) [49]. Furthermore, the S protein contains within its cytoplasmic tail a binding site for the cytoplasmic coat protein (COPI), which allows for retrograde transport from the Golgi to the ER and further exposure to M protein-mediated sequestration (Fig. 1) [49]. The E protein may also induce S retention by slowing down the cell's secretory pathway. The co-expression of M and E with the S protein reduces syncytia formation in cell culture models [49]. A recent investigation by Cattin-Ortolá and colleagues provides relevant mechanistic observation on how the S protein could escape its confines and localize on the cell surface [50]. They suggest that the COPI binding motif of the SARS-CoV-2 S protein tail might be sub-optimal. The presence of a histidine rather than a lysine at residue 1271 as well as threonine at residue 1273 in the S protein contributes to sub-optimal COPI binding and reduced intracellular accumulation [50]. Furthermore, the COPI binding site is in close proximity to the COPII (responsible for anterograde transport of protein from ER to Golgi) binding site and replacing the histidine with lysine at residue 1271 also reduced interaction with COPII [50]. This suggests that the S protein maybe more prone to exit from the ER (Fig. 1). The co-expression of M and E proteins reduced S surface expression but did not negate it [50]. These lower affinity binding motifs in the S protein cytoplasmic tail allow for the S protein to leak to the cell surface and promote syncytia formation (Fig. 1).

A study by Braga and colleagues further provides valuable insights into the mechanism of SARS-CoV-2 induced cell-cell fusion [16]. They screened for inhibitors of S protein-mediated syncytia formation and found that the most effective inhibitors shared the common characteristic of regulating intracellular calcium (Ca^{2+}) levels [16]. They noticed intracellular Ca^{2+} oscillations in individual S-expressing cells and in syncytial cells and they suggest that expression of the S protein amplifies spontaneous Ca^{2+} transients (Fig. 1) [16]. To better understand the relationship between Ca^{2+} fluctuations and syncytia formation, they focused on one of their strongest hits, the anthelmintic drug niclosamide which inhibits the Ca^{2+}

activated TMEM16 family of chloride channels and scramblases [16]. The expression of the ubiquitous TMEM16F was increased upon S protein expression (Fig. 1) [16]. The downregulation or overexpression of TMEM16F results in a corresponding decrease or increase in syncytia formation [16]. TMEM16F is a non-specific ion channel and a scramblase that translocates phospholipids bidirectionally between the plasma membrane leaflets. The translocation of specific phospholipids from internal to outer leaflets can serve as a fusion signal (Fig. 1) [51]. In resting cells, the phosphatidylserine (PS) phospholipid is found on the leaflet facing the cytoplasm. Its translocation to the exofacial leaflet serves as a signal for a variety of cellular processes, including cell-cell fusion (Fig. 1) [51]. The exposure of PS is associated with a variety of physiological and pathological cell-cell fusion events including skeletal muscle and myoblast formation and repair, macrophage fusion, placental trophoblast fusion, sperm and egg cell fusion during gamete formation, and cancer cell fusion [51]. It is also a marker of cell death through apoptosis. The presence of PS on the viral envelope is important for the entry of viruses including HIV and Ebola and some viruses like alphaherpesvirus induce PS exposure on the host membrane to facilitate infection [51-55]. The translocation of PS may influence the fusogenicity of the lipid membrane as well as assist in the recruitment and modification of fusion protein (the diverse functions of PS is thoroughly reviewed in [51]). Syncytium formed via S protein-mediated fusion also expose PS on their plasma membrane [16]. The activation of TMEM16F by S and the corresponding translocation of PS may represent one of the mechanisms by which SARS-CoV-2 induces syncytia. TMEM16F also plays a role in lipid scrambling in platelets during blood coagulation and its activation by S may contribute to the extensive thrombosis in severe cases of COVID-19 [16]. Additional studies will be needed to fully understand the contribution of PS exposition and other functions of TMEM16F to SARS-CoV-2 infection and syncytia formation [16, 51]. Braga et al. also note that the downregulation of TMEM16F does not affect MERS-CoV S protein-mediated syncytia formation [16]. Comparative investigations on this disparity between SARS-CoV-2 S and MERS-CoV S protein in relation to TMEM16F may further elucidate the mechanism of syncytia formation.

In brief, the SARS-CoV-2 S protein, which is normally sequestered on intracellular membranes, possesses suboptimal COPI binding sites that allow for leakage and translocation to the cell surface (Fig. 1). Once at the surface, the S protein interacts with ACE2 and host factors on adjacent cells to induce syncytia. In parallel, the expression of the S protein also affects intracellular Ca^{2+} fluctuations and results in the increased expression of the TMEM16F ion channel and scramblase. TMEM16F translocates PS from the cytofacial leaflet of the plasma membrane to the exofacial leaflet, which promotes the membrane's fusogenic properties (Fig. 1). Since the S protein plays a significant role in fusing together membrane during virus-cell fusion and in syncytia formation, a more in-depth molecular characterization of its interactions with host factors and with itself is warranted, which is the subject of the following section.

Molecular Mechanism of S protein-mediated fusion

The S protein is about 1271 amino acids long and is comprised of the S1 and S2 subunits separated by a polybasic cleavage site. Cellular proteases cleave between this S1/S2 site as well as at a S2' site in order to activate the S protein [38]. Hoffman and colleagues reported that optimization of the S1/S2 cleavage site facilitates cell-cell but not virus-cell fusion, further suggesting a prominent role in SARS-CoV-2 mediated syncytia formation [38]. The removal of the S1/S2 cleavage site reduces cell-cell fusion but does not inhibit viral entry [56]. Other studies suggest that removal of the furin cleavage site substantially reduces, but does not prevent, the production of infection particles and cell-cell fusion [57]. Viral replication was attenuated, but not abolished, in human respiratory cell lines and hamster and mouse models upon infection with SARS-CoV-2 lacking the furin cleavage site [58]. These studies highlight the significance of the S1/S2 furin cleavage site in SARS-CoV-2 infection particularly in case of syncytia formation. Indeed, natural occurring mutations in these sites augment syncytia formation and this is detailed in a later section.

The S1 subunit consists of the N-terminal domain (NTD) and the receptor binding domain (RBD). The function of the NTD is still under investigation, though work on other coronaviruses shows that the NTD may be associated with glycan recognition during initial attachment, receptor recognition and pre-to-post fusion transition of the S protein [59-61]. The C-type lectins DC-SIGN and L-SIGN may act as auxiliary receptors for SARS-CoV-2 by associating with the NTD [36]. SARS-CoV-2 S protein-mediated fusion may also be subject to antibody-dependent enhancement as the binding of antibodies to a specific site on the NTD induces an open RBD state which may enhance fusogenicity [62].

The RBD domain of the S1 interacts with the ACE2 receptor; the presence of which determines cellular tropism (Fig. 2) [63-65]. The RBDs of the S protein trimer exist in a state of dynamic equilibrium between “up” (open) and “down” (closed) conformations [66, 67]. In the “up” conformation, the RBD can bind to ACE2 and the binding of one of the RBDs monomers to ACE2 is followed by sequential opening up and binding of the remaining two RBD monomers to ACE2 [67]. As multiple receptors could bind to the S protein, considerable mechanical stress is exerted on the S1 [66]. The opening process disrupts contact between ACE2-bound S1 monomers, promoting their dissociation from the complex and revealing the trimeric S2 core, which houses the fusion machinery of the S protein (Fig. 2) [67]. The S2 domain is comprised of the fusion peptide (FP), heptapeptide repeat sequences 1 and 2 (HR1 and HR2), a transmembrane anchor (TA) and a C-terminal domain (CTD). Upon the release of the S1 domain, the unstructured HR1 in the S2 domain becomes helical, and thrusts the FP into the target cell membrane (Fig. 2) [66, 68]. The FP then disrupts the lipid bilayer of the target membrane and anchors it to the fusion machinery (Fig. 2) [69, 70]. On the other end of the S2 domain, the fusion machinery is anchored on either the virion membrane or the S-expressing cell membrane (in the case of syncytia formation), by the TA subdomain (Fig. 2). Upon insertion of the FP into the target membrane, three highly conserved hydrophobic grooves on HR1 are exposed, which allows for interaction with the HR2 subdomain [66]. The HR2 domain contains a rigid helix and a flexible loop [70, 71]. The interaction between HR1 and HR2

forms a six-helix bundle which brings the two anchored membranes into close proximity to one another, overcoming the natural repulsive force that keeps the membranes apart (Fig. 2) [66]. Once the energetic barrier for fusion is reduced, the outer leaflets of the membranes merge in a step referred to as hemifusion. This is followed by the formation of a pore which expands to create one continuous membrane, allowing mixing of the viral or cytoplasmic contents (Fig. 2) [66]. The CTD is associated with COPI/II binding and intracellular transport [50]. Furthermore, work by Sanders et al. found that the cystine-rich membrane proximal cytoplasmic region of the S protein CTD is necessary for syncytia formation, and that it associates with membrane cholesterol in a raft-independent manner (Fig. 1) [72].

The subdomains of the S protein play important roles in receptor recognition and binding, translocation, and fusogenicity. The S protein is also highly antigenic and has been subject to evolutionary pressure throughout the pandemic, leading to the rise of several variants of concern (VOC). These variants' S proteins contain several mutations that alter its propensity to carry out its aforementioned functions. The following section will examine how the mutations within VOC S proteins influence syncytia formation.

Variants of Concern S proteins and Fusogenicity

Since the start of the pandemic, the ancestral Wuhan strain has been replaced by variants containing several mutations throughout their genome. Variants Alpha (B.1.1.7) originating from the United Kingdom; Beta (B.1.351) from South Africa; Gamma (P1 and P2) from Brazil; and more recently Delta (B.1.617.2) from India are of concern both regionally and globally. While the variant-associated mutations are found throughout the viral genome, many mutations are associated with the S protein (Fig. 3). In addition to being the viral fusogen, the S protein is highly antigenic and therefore the target for neutralizing antibodies. As such, many of the mutations found in the variants' S proteins have arisen in

the context of antibody escape. The impact of the variants, with all of their accumulated mutations, on pathology is not clear, but some variants are more transmissible [73, 74]. Several recent studies have sought to characterize and compare the S proteins of the variants in terms of their fusogenicity.

One of the earlier variants identified was the European variant, which contained the D614G mutation proximal to the S1/S2 cleavage site. This mutation shifts the conformation of the S protein to a state that is more competent for ACE2 binding, while not significantly altering antibody neutralization [65, 75]. The D614G replicates more efficiently in primary cell culture models [75]. Furthermore, greater levels of viral genetic material has been found in the upper respiratory tract of infected individuals which may be more associated with transmission rather than disease severity [73]. In terms of fusogenicity, pseudovirus assays have shown that the D614G substitution increases the efficiency of cellular entry [73, 76]. The D614G mutant produces more syncytia than the ancestral Wuhan strain in cell culture models [77, 78]. We and others have shown that relative to D614G, the Alpha, Beta, and Delta variants are more syncytogenic in cell culture [77, 79, 80]. The Alpha and Delta S proteins induce the most cell-cell fusion and they do so more rapidly than the other variants [77, 79]. The Beta variant S is also more fusogenic than D614G [77]. In the most general sense, the degree of syncytia formation seems to correlate with the affinity of the variant S proteins to ACE2 [77, 81]. A contradictory study found no difference (Alpha) or a slight reduction (Beta and Gamma) in variant S mediated cell-cell fusion; however, work by the same group found the Delta variant to be more syncytogenic [82, 83]. This discrepancy may be explained by variations in the experimental systems and the time-points in which syncytia formation was assessed. Highly fusogenic cells and late time-points may risk missing differences in fusogenicity. The D614G mutation alone elicited significant changes in the S protein that impacted its structure, receptor affinity and fusogenicity. As such, it is worth examining the changes elicited by other individual variant-associated mutations.

The S1 domain is part of the spike that is most exposed to neutralizing antibodies on the virion. As such, it is under persistent evolutionary pressure and prone to mutations. Some of the more recent variants (Alpha, Beta, Gamma) also share the N501Y mutation within the RBD. This mutation increases the affinity of S protein to ACE2 and viral transmission but does not affect antibody neutralization [77, 84-88]. The hydrophobic pocket formed by the Y41 and K353 regions of ACE2, interacts more efficiently with the Y501 mutant than with the wild type S [89]. Despite its higher affinity to ACE2, the N501Y mutation alone did not increase syncytia formation in cell culture [77]. E484K, another significant mutation within the RBD, present in Beta and Gamma variants, also later emerged in a subset of the Alpha lineage [90]. The RBD of Beta variant also includes the K417N (K417T in Gamma). The E484K and K417N mutations are associated with antibody escape, though they may alter ACE2 binding when in conjunction with N501Y [88, 90-92]. E484K may increase affinity to ACE2, whereas K417N seems to decrease it [85, 93]. Individually, both mutations reduced syncytia formation [77]. The E484K mutation has been suggested to allow for infection of H522 human lung cells independently of ACE2 [94]. This alternate entry pathway may rely on surface heparan sulfates and clathrin-mediated endocytosis [94].

Within the NTD domain of the variant S proteins are several mutations that are generally associated with antigenicity [92]. As previously mentioned, the NTD is also involved in receptor binding and structural changes, and some of the variant associated mutations within the domain have been shown to impact S protein-mediated fusogenicity. The Δ69/70 mutation that is associated with the Alpha variant increases infectivity through greater incorporation of cleaved S protein into virions [95]. Furthermore, Δ69/70 may compensate for the reduced infectivity brought about the antibody escape mutation in the RBD of the variants [95]. Interestingly, restoring the Δ69/70 mutation abrogates the relative increase in syncytia formation elicited by the Alpha S protein relative to the D614G S protein [95]. However, the deletion on its own within the D614G S protein is detrimental to syncytia formation [77]. The comparatively greater syncytia formation induced by the Alpha variant S protein may thus be a result of

the Δ69/70 mutation working in conjunction with other mutations. The deletion of 242-244 in the NTD of the Beta variant also restricts syncytia formation on its own [77]. It remains to be seen how it functions within the context of partner mutations. Interestingly, the D215G mutation of the Beta variant facilitates syncytia formation [77]. The mechanism of its action is unknown, and future investigations examining it within the context of L-SIGN, DC-Sign and neuropilin-1 binding and overall S structural changes would be of interest [36]. Such investigation could broaden the scope of target cells that may be able to form syncytia.

Of particular interest are the P681H and P681R mutations found in the S1/S2 cleavage site of Alpha and Delta variants, respectively. The P681 site is positioned in a surface-exposed loop of the S protein and is accessible to proteases. A recent investigation in the Alpha variant with the P681H mutation showed that there is a greater exposure of the furin cleavage site which naturally increases affinity to furin [96]. The presence of more cleaved Spike in the virion or at the cell surface can increase fusogenicity. Indeed, we showed that the P681H mutation drastically increased syncytia formation [77]. It has also been suggested that O-glycosylation modulates furin cleavage of the S protein, and the presence of the P681 region facilitates the activity of glycosylation enzyme GALNT1 [97]. How the variant-associated mutations affect glycosylation and subsequent furin cleavage would be of future interest. Work by Saito and colleagues suggest that the P681R mutation of Delta variants increases S protein cleavage and subsequent viral fusion [80]. The P681R mutation was also responsible for larger syncytia forming in experiments conducted with artificial virus reversed engineered to contain the mutation [80]. Infection with virus housing the P681R resulted in higher pathogenicity in a hamster model: animals infected with the mutant virus experienced greater weight loss and reduced pulmonary function than with the D614G virus [80].

The A570D and S982A mutations, also within proximity of the cleavage site of the Alpha variant, did not significantly affect syncytia formation individually; however, structural studies suggest that they may reduce contact between individual chains of the trimeric spike [77, 98]. In conjunction with other

mutations like P681H, they may augment cleavage of the S protein [98]. The D1118H mutation in the S2 of the Alpha variant, between the HR1 and HR2 domain potently increases syncytia formation [77]. Characterization of this mutation in the literature is scarce. From its location, it would not be unreasonable to speculate that it may impact the fusion machinery and HR1/HR2 association. Further investigations examining the effects of D1118H on the structure and function of the S protein, and the overall infectivity of the S protein would be of interest. A more thorough understanding of the variants and their respective mutation, especially in *in vivo* models, would be of great value in understanding the transmission and pathology of the novel variants.

Modulation of S-mediated fusion by host factors

Restriction and enhancement factors play a complex role in regulating viral infection. Several of these are membrane-bound and operate at the entry and fusion step of the viral life cycle. This section will examine the role of key membrane-bound restriction and enhancement factors on S protein-mediated fusion and syncytia formation. Upon infection, cytosolic sensors detect viral genetic material and trigger the expression of type I, II and III interferons (IFN), which subsequently set off signaling pathways that promote the expression of a wide array of interferon-stimulated genes (ISGs) to impede viral replication. One such family of ISGs are the interferon-induced transmembrane (IFITM) proteins. Functional IFITMs can be further categorized into three clades and despite the nomenclature only members of clade 1 – IFITM1, 2 and 3 as well as the mouse specific IFITM6 and 7 – are truly IFN induced, ubiquitously expressed and possess bona fide innate immune activity [99]. IFITMs restrict a broad spectrum of enveloped viruses including influenza, HIV-1, Ebola, Marburg, dengue, Zika, West Nile, Japanese encephalitis, Rift Valley Fever as well as members of the coronavirus family [100-102].

IFITM1 localizes on the plasma membrane, while IFITM2 and 3 both transit through the plasma membrane and then localize within the endo-lysosomal compartments. IFITMs prevent viruses from crossing the cellular membrane and accessing the cytoplasm, possibly by altering the rigidity and the curvature or the composition of the membrane [100, 102-104]. This serves to increase the energetic barrier for fusion, hindering virus-cell fusion at hemi fusion or pore formation stage [105, 106]. In the case of coronaviruses, IFITMs block the entry of MERS-CoV, SARS-CoV, hCoV-NL63 and hCoV-229E [107-109]. However, the interaction between IFITMs and coronavirus S protein-mediated fusion is complex. Work by Zhao and colleagues demonstrated that the hCoV-OC43 uses IFITM2 and 3 as entry factors to facilitate infection [110]. Furthermore, they also identified mutations within the IFITM1 and 3 that can convert them from inhibitors of MERS-CoV and SARS-CoV S mediated entry, into enhancers [109]. The contradictory effects of IFITMs and their mutations on different coronaviruses may be a result of how and where they interact with the different cellular receptors [109].

There have been several reports examining the role of IFITMs on SARS-CoV-2 S mediated fusion. Work by Shi and colleagues show that IFITM1, 2 and 3 are restriction factors of SARS-CoV-2 infection, with IFITM1 being particularly potent [102]. Another study using pseudotyped lentiviral vectors expressing SARS-CoV-2 S protein found that IFITM2 was the most potent inhibitor of entry and that the polybasic S1/S2 cleavage site makes S protein particularly sensitive to IFITM2 [111]. Shi et al. also suggest that restriction by IFITM3 is independent of its palmitoylation, a post-translation modification thought to affect hydrophobicity and location of IFITM3 to membrane compartments [102, 112]. Since the palmitoylation site is necessary for the antiviral activity of IFITM3 against IAV, hCoV-229E and hCoV-NL63, this may suggest that the restriction of SARS-CoV-2 infection at the endosome may occur in a different manner [102, 109, 112]. Shi et al. confirm that the amphipathic helix domain within the hydrophobic region of IFITM3, is necessary for restricting S mediated fusion. The amphipathic helix is believed to insert into membrane bilayers and mechanically induce a curvature and rearrange lipids in a manner that

antagonizes fusion [105, 113-115]. One investigation suggests that endogenous IFITMs in human lung cells may on the contrary, facilitate SARS-CoV-2 infection [116]. The authors proposed that IFITMs interact with the S protein and that reduction of endogenous IFITMs drastically impedes viral replication [116]. They also found that IFN- β enhanced SARS-CoV-2 infection in small airway epithelial cells [116]. The discrepant observations might be a result of the different IFITM expression systems or cell culture models. Further investigations examining the relationship between IFITMs and SARS-CoV-2 are warranted.

SARS-CoV-2 mediated syncytia formation is effectively restricted by IFITMs, with IFITM1 displaying the most potent activity. We reported that restriction of syncytia formation by IFITMs is subject to reversion by TMPRSS2 [46]. TMPRSS2 drastically augmented S protein-mediated syncytia formation, whether IFITMs were present or absent [46]. This protease also increases infection by SARS-CoV-2 and reverts the inhibitory effect of IFITMs on viral infection [102]. Studies on other coronaviruses have also found that TMPRSS2 thwarts the antiviral effects of IFITMs [117, 118]. It would be of future interest to characterize the relationship between TMPRSS2, ACE2, and IFITMs to better understand how TMPRSS2 subverts the potential biophysical constraints imposed by IFITMs. Understanding the mobility and interaction dynamics of these three interactors during infection or syncytia formation may be informative of the precise mechanism in which syncytia formation is restricted. Of note, we also observed that syncytia induced by the S proteins of D614G, Alpha, and Beta variants are effectively restricted by IFITMs [77].

Other membrane-bound restriction factors impact S-protein mediated syncytia formation. The IFN inducible Lymphocyte antigen 6 complex locus E (LY6E) is a glycosylphosphatidylinositol (GPI)-anchored protein that associates with membrane lipid rafts [119, 120]. LY6E is a pro-viral factor that promotes the uptake of Yellow Fever virus, the uncoating of influenza A, and HIV-1 infection of high CD4-expressing cell [120-122]. It is also a receptor for syncytin-A, a mouse endogenous retroviral envelope [123]. However, LY6E is an inhibitor of hCoV-OC43, hCoV-229E, SARS-CoV, SARS-CoV-2, and MERS-CoV infection and associated syncytia [124, 125]. The precise mechanism by which LY6E inhibits membrane

fusion is not clear [124]. It does not affect S protein maturation or expression of surface receptors [124]. The GPI-anchor which associates LY6E to lipid rafts is crucial to its activity. Lipid rafts are cholesterol and glycosphingophospholipid rich regions in the plasma membrane that compartmentalize important processes including ligand receptor engagements. The mechanism by which LY6E exerts its restriction is likely different than that of IFITMs. The antifungal drug amphotericin B (AmphoB), which binds cholesterol in the plasma membrane and increases its fluidity, can rescue restriction by IFITMs but not LY6E. Understanding the precise mechanism by which LY6E restricts fusion would be of interest.

Recently, the ISG Cholesterol 25-hydroxylase (CH25H) has been described as an inhibitor of SARS-CoV-2 membrane fusion [126, 127]. CH25H is induced by SARS-CoV-2 infection and converts cholesterol into 25-hydrocholesterol (25CH) [126]. 25CH inhibits viral-cell fusion by depleting fusion accessible plasma membrane cholesterol through the activation of acyl-CoA: cholesterol acyltransferase (ACAT) localized on the ER membrane [126]. CH25H expression also significantly reduces SARS-CoV-2 S protein-mediated syncytia formation [127]. These observations further emphasize the significance of cholesterol access to S protein-mediated fusion.

In summary, a few important ISGs act to antagonize SARS-CoV-2 S mediated cell-cell fusion by altering the target membranes. IFITMs likely restrict syncytia formation by altering the biomechanical properties of the plasma membrane, whereas LY6E does so through an unknown mechanism. CH25H facilitates the depletion of membrane cholesterol and the compositional change is also unfavorable to cell-cell fusion. Molecular mechanisms by which ISGs inhibit virus-cell and cell-cell fusion remain a pertinent field in understanding SARS-CoV-2 induced pathology.

The potential roles of syncytia formation in SARS-CoV-2 infection

SARS-CoV-2 mediated syncytia formation has been proposed to be a potential contributor to overall pathology. However, its relative significance and its general contribution to the viral infection remains unknown. Formation of abnormal multinucleated pneumocytes represents a form of cytopathic effect. There are significant differences between physiological and pathological syncytia. During physiological syncytogenesis, such as in placental trophoblast formation, the cell cycle is arrested at the G0 phase [128]. The expression of the Syncytin-2 (SYN2) fusogen is also restricted to the G0 phase, which allows for strict regulation of the physiological cell-cell fusion process [128]. The transient expression of SYN2 in cells in any other phase (S,G2,M) of the cycle results in the formation of unstable and functionally compromised syncytia [128]. Furthermore, physiological fusion processes like placental formation is controlled through polarized expression of the receptor and fusogen [8]. SYN2 expression is limited to a few cytotrophoblasts, whereas its receptor (major facilitator superfamily domain-containing protein 2) is expressed on syncytiotrophoblasts [8]. In stark contrast, unregulated pathogen-induced syncytia formation, in cells which normally do not fuse, is likely to be pathological.

Unnaturally occurring syncytia cells are susceptible to rapid cytopathicity. This phenomenon of “syncytial apoptosis” has been well documented in HIV-1 infections *in vitro*, *in vivo* and in infected patient tissues [129]. Upon cell-cell fusion, the HIV-1 envelope protein triggers a pro-apoptotic signaling pathway that triggers karyogamy (nuclear fusion) and DNA damage [129]. SARS-CoV-2 S protein-mediated syncytia also collapse and die during the later stages of the process, and of particular interest is the observation that the nuclei within S protein-mediated syncytium cluster together (Fig. 4 top left)[46, 130]. Future investigations characterizing the cell death pathways triggered within SARS-CoV-2 induced syncytium, and the roles of other viral proteins would be of interest. There is also a possibility that the death of infected cells may release virions into the surrounding environment, spreading the infection (Fig. 4 top left). Syncytial pneumocytes in COVID-19 patient tissues are scarce and the degree to which cytopathic effects contribute to pathology is unclear. There is also a paucity of information regarding presence, clearance or

turnover of syncytia during early infection. There is also possibility that if syncytia are prone to premature death, they may be detrimental to viral replication. The relationship between syncytial death and viral replication remains to be explored more thoroughly.

The formation of syncytia may also have immunological consequences. Zhang and colleagues found the presence of lymphocytes contained within syncytia as a cell-in-cell structure among COVID-19 patient lung tissue [26]. Using cell culture models, they demonstrated that syncytia formed through S protein-mediated fusion can internalize various T and B cell lines, monocytes and peripheral blood mononuclear cells (Fig. 4 bottom left) [26]. The internalized cells readily underwent shriveling, deterioration of the plasma membrane and death (Fig. 4 bottom left) [26]. These studies provide a possible mechanism by which syncytia may contribute to lymphopenia among patients with COVID-19 [26]. There is also an outstanding question whether S-mediated syncytia trigger elements of the innate immunity. Cell-cell fusion events induced by other pathogens have been documented to induce interferon expression. Syncytia induced by measles virus amplifies IFN- β production *in vitro* [131]. Similarly, cell-cell fusion induced by the bacterial pathogen *Burkholderia pseudomallei* elicits type I IFN expression through the DNA sensing cGAS-STING pathway [132]. This reportedly occurs independently of bacterial ligands [132]. How S protein-mediated pathological syncytia formation is related to immune activation or recognition remains an outstanding question.

Recent work suggests that S protein-mediated syncytia formation results in the activation of Caspase-9, followed downstream by the activation of Caspase-3/7, and later by gasdermin E (GSDME)-mediated pyroptosis [133]. This inflammatory form of cell death may have significant implication in triggering the immune response and warrants further investigation (Fig. 4 top left). The adaptive immune response also has profound implications for SAR-CoV-2 mediated cell-cell fusion. Antiviral antibodies may have contradictory roles in cell fusion [134]. Some neutralizing antibodies restrict cell-cell fusion by locking the spike in a pre-fusion state, whereas some weakly neutralizing antibodies behave as allosteric effectors

that promote S2 unsheathing and increase syncytia formation [134]. The opposing activities of neutralizing antibodies will need to be elucidated further in order to be understood in terms of pathology.

Finally, syncytia formation can have direct virological consequences rather than just trigger a generalized cytopathic effect or immune response. Syncytia may allow viruses to spread directly from cell-to-cell without having to enter the extracellular environment. This strategy shields the virus from neutralizing antibodies, physical barriers like the mucociliary blanket, as well as components of the immune system (Fig. 4 bottom right) [135]. Many respiratory viruses such as measles, influenza, respiratory syncytia virus, parainfluenza virus, and human metapneumonia virus exploit this mechanism of dissemination [135]. As already discussed above, nonenveloped reoviruses employ evolutionarily conserved fusogens, dedicated to the induction of cell-cell fusion, as a mechanism of cell-to-cell transmission [10, 11]. Recent cell culture studies suggest that SARS-CoV-2 cell-to-cell dissemination is a possible route of spread (Fig. 4 bottom right). Cell-free virus was effectively neutralized by monoclonal antibodies and convalescent plasma; however, cell-to-cell viral spread was either not or less sensitive to neutralization (Fig. 4 bottom right) [130, 136]. The direct spread of HIV from cell-to-cell has also been demonstrated to be less sensitive to the restrictive effects of antiretrovirals and neutralizing antibodies [137-139]. A more thorough understanding of the significance of SARS-CoV-2 cell-to-cell spread to the pathology and antibody response is warranted. Beyond direct cell-to-cell spread, syncytia can contribute to the overall infectious dose and viral dissemination upon being dislodged (Fig. 4 top right). In reconstituted primary bronchial epithelia, multi-ciliated cells and basal cells form syncytia which are then released into the apical lumen [140]. Vesicular inclusions containing virus particles were detected within the released syncytia, potentially suggesting continued viral replication [140]. Infected single and syncytial cells that are released may thus spread infection (Fig. 4 top right). Like several of the aforementioned processes, syncytial release is not unique to SARS-CoV-2 [140]. Infection of epithelial cell cultures from

macaque respiratory tissue with measles virus also results in the formation and shedding of infectious syncytia [141].

From a methodological standpoint, syncytia formation in cell culture can also be utilized as an effective, albeit imperfect, surrogate to characterize the early stages of virion entry via S protein fusogenicity. It has the added benefit for being suitable for biosafety level 2 research environments, when the S protein is expressed alone, in the absence of infectious virus. We also developed a rapid, sensitive and semi-automated assay we termed “S-Fuse”, which employed virus-induced syncytia formation as a marker to detect and measure infectious virus in various human samples [46, 142]. The S-Fuse assay is now routinely used in our laboratory to assess the activity of anti-SARS-CoV-2 monoclonal antibodies or other antiviral molecules, and to measure the neutralizing antibody response in sera from vaccinated and convalescent individuals [81, 143]. Quantitative syncytia formation has also been employed in high-throughput drug screens in order to identify inhibitors of S mediated fusion [16, 72]. There are several reporter systems that can be used to quantify syncytia formation and they are compared by Sanders et al [72].

Conclusion and Perspectives

Several histopathological reports have identified the presence of infected multinucleated syncytial cells in COVID-19 patient lung tissues. The degree to which syncytia affect pathology is not fully understood. Recent studies have provided valuable insights into the role of syncytia in SARS-CoV-2 infection as well as into mechanism of their formation. Syncytia might facilitate infection by disseminating the virus through cell-to-cell contacts or upon cell death, targeting immune cells, and protecting the virus against neutralizing antibodies. On the contrary, rapid syncytial collapse may be detrimental to viral replication

and could trigger an inflammatory immune response. In either case, SARS-CoV-2 syncytia most likely contribute to viral pathogenicity.

Syncytia formation is sensitive to the antiviral innate immune response. Some ISGs modify the membrane in ways that are unfavorable to fusion. SARS-CoV-2 is a rapidly evolving virus, with many mutations within the S protein. The individual mutations harbored by Alpha, Beta, Gamma, and Delta variants have significant impact on S function and syncytia formation, both individually and cumulatively. The impact of syncytia in the pathology of emerging variants remains an outstanding question.

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Figure Legends

Figure 1: Schematic representation of the diverse activities of the SARS-CoV-2 spike (S) protein during infection and syncytia formation. Viral infection begins when the S protein on the surface of the virion interacts with the ACE2 receptor. During early entry, the S protein is processed by the TMPRSS2 protease and fusion occurs on the plasma membrane (PM). The S protein can also fuse with endosomal membranes during late entry, upon being primed by cathepsins and furin. The positive-sense single stranded (+ss) viral genomic RNA is deposited in the cytoplasm and translated. Viral RNA replication and transcription occur on membranes. Upon being transcribed, the S protein is translocated and inserted into the endoplasmic reticulum (ER) and is generally sequestered within intracellular membranes by the membrane structural protein (M). The expression of the S protein leads to intracellular calcium fluctuations and the increased expression of the TMEM16F scramblase. TMEM16F exposes the phosphatidylserine (PS) from the cytosolic leaflet of the PM to the exofacial leaflet. The transcribed S protein is processed by furin and transported throughout the ER-Golgi network. During COPI (retrograde) and COPII (anterograde) transport, leakage of the S protein can occur from vesicles (not shown in simplified schematic). The S protein is then translocated to the PM, where it associates with cholesterol, and induces syncytia formation by interacting with receptors on neighbouring uninfected cells. The sequestered S protein is packaged into virions that bud into the Golgi or ER-Golgi intermediate compartment (ERGIC), and virions exit the cell via deacidified lysosome-dependent exocytosis (not shown). The ER, ERGIC, and Golgi membranes are bilayers which are represented as single lines in this schematic.

Figure 2: Molecular mechanism of Spike (S) protein-mediated virus-cell or cell-cell fusion. **Top:** the functional subdomains of the S protein. N-terminal domain (NTD), receptor binding domain (RBD), fusion peptide (FP), heptapeptide repeat sequences 1 and 2 (HR1 & HR2), transmembrane anchor (TA), C-terminal domain (CTD). **Bottom: 1)** The S protein associates with the ACE2 receptor via its RBD. It is also

processed by cellular proteases at the S1/S2 or S2' sites (not shown). **2)** The S1 domain is released which allows for HR1 to extend and thrust the FP into the target cell plasma membrane. The FP anchors the target membrane while the TA anchors the fusion machinery to the viral or infected cell membrane. **3)** Interactions between HR1 and HR2 results in a hairpin-like foldback that overcomes the energetic barrier to fusion and brings the membranes close together. A hemi-fusion step occurs where the outer layers of the fusing membranes merge (not shown). **4)** The association of HR1 and HR2 and the corresponding membrane fusion result in the formation of a pore that will gradually expand. The viral or cytoplasmic contents are then merged.

Figure 3: Spike protein mutations associated with Alpha, Beta, Delta and Gamma SARS-CoV-2 variants. N-terminal domain (NTD), receptor binding domain (RBD), fusion peptide (FP), heptapeptide repeat sequences 1 and 2 (HR1 & HR2), transmembrane anchor (TA), C-terminal domain (CTD).

Figure 4: The potential consequences of syncytia formation on SARS-CoV-2 pathology. **Center:** Cells infected with SARS-CoV-2 express the spike protein at the surface and form large multinucleated syncytia. **Top left:** The process of syncytial death via apoptosis or pyroptosis can release virus to infect neighbouring cells and/or trigger an inflammatory response. **Top right:** Infected syncytia can lift off to contribute to viral dissemination and the overall infectious dose. **Bottom left:** Syncytial cells can target lymphocytes for cell-in-cell mediated death. **Bottom right:** Syncytia can facilitate cell-to-cell spread of the virus and shield the virus from neutralizing antibodies.

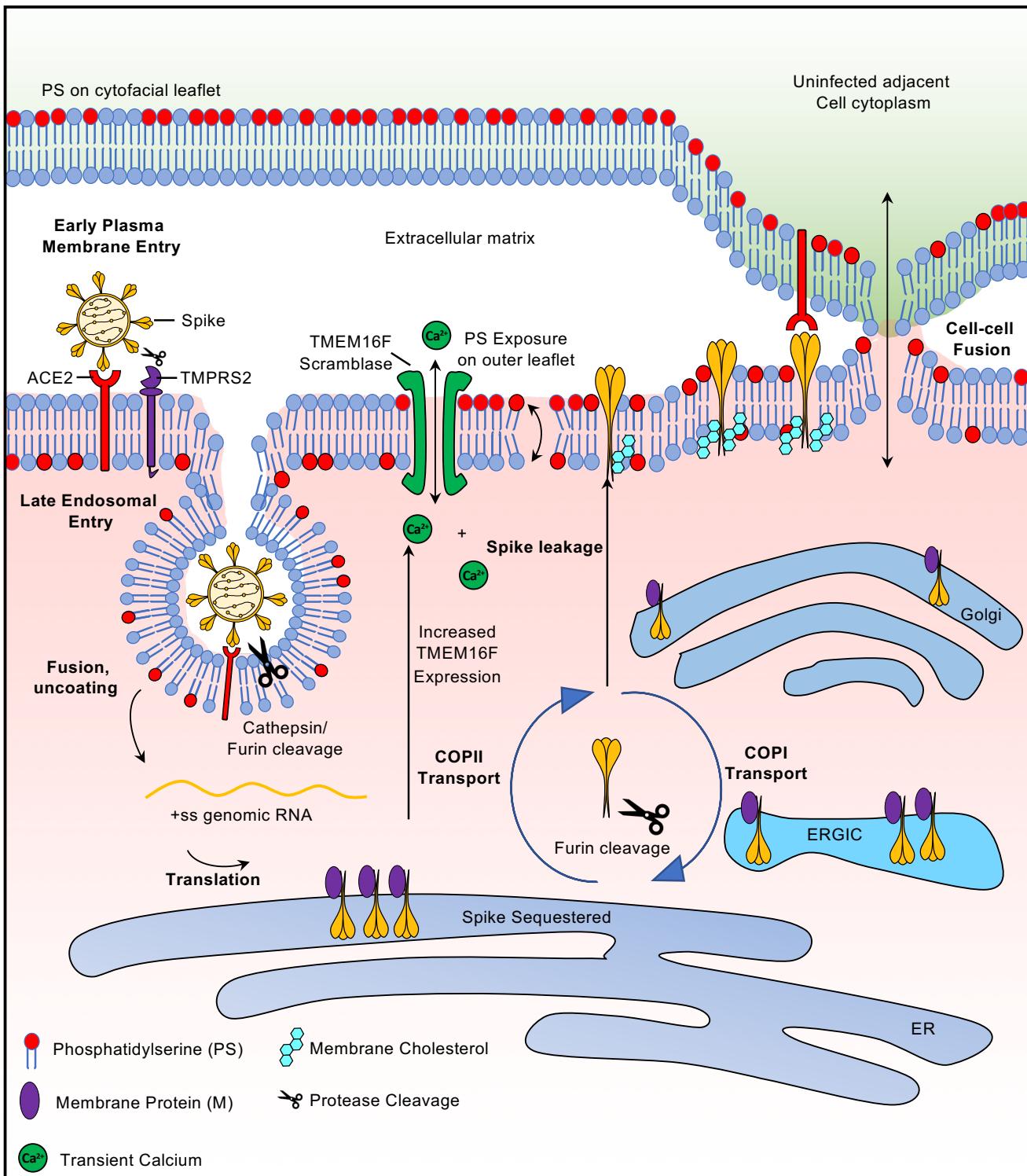


Figure 1

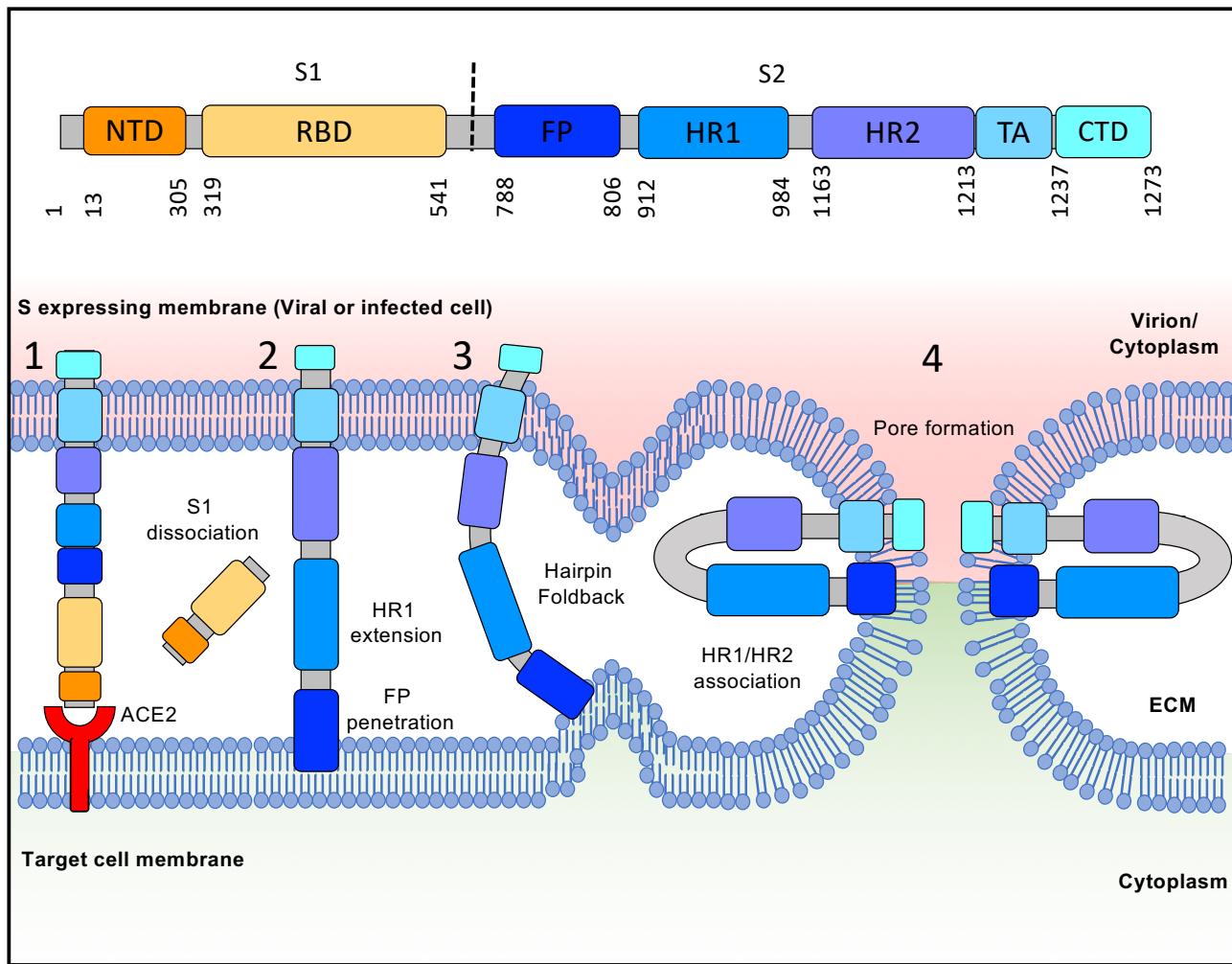


Figure 2

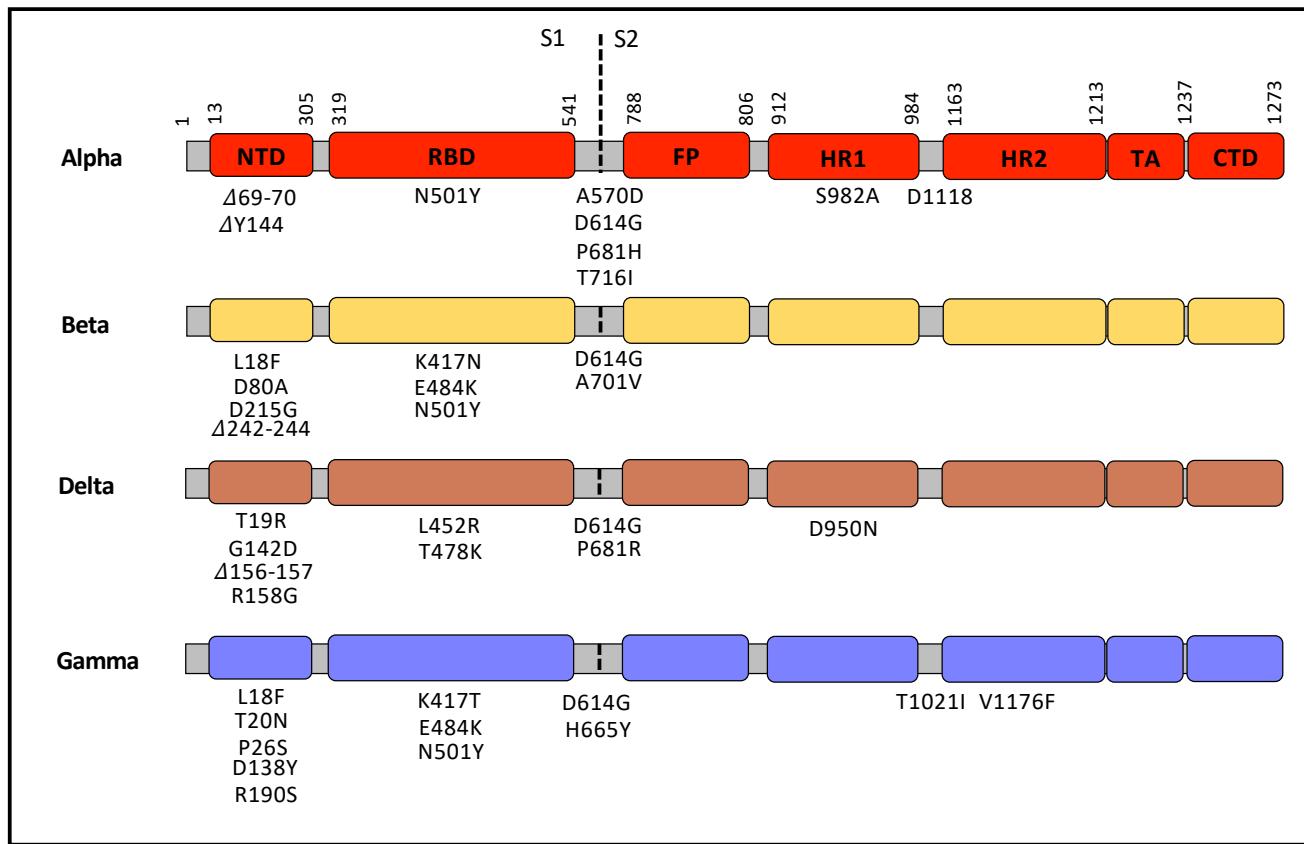


Figure 3

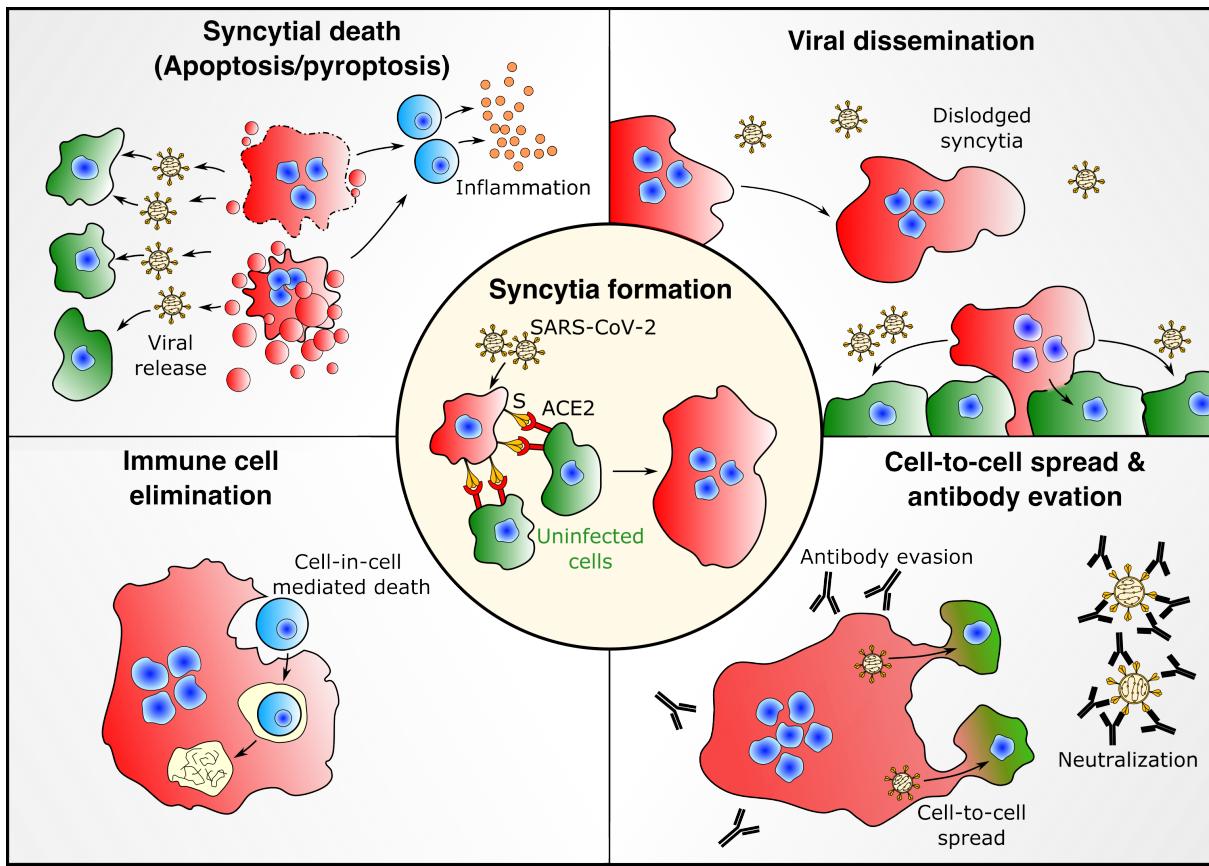


Figure 4