

SARS-CoV-2 Infection and Autophagy

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The coronavirus disease pandemic, which profoundly reshaped the world in 2019 (COVID-19), has affected over 200 countries, caused over 500 million cumulative cases, and claimed the lives of over 6.4 million people worldwide as of August 2022. The causative agent is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Depicting this virus' life cycle and pathogenic mechanisms, as well as the cellular host factors and pathways involved during infection, has great relevance for the development of therapeutic strategies. Autophagy is a catabolic process that sequesters damaged cell organelles, proteins, and external invading microbes, and delivers them to the lysosomes for degradation. Autophagy would be involved in the entry, endo, and release, as well as the transcription and translation, of the viral particles in the host cell.

COVID-19

macroautophagy

ATG proteins

mitophagy

1. Introduction

The coronavirus disease pandemic, which profoundly reshaped the world in 2019 (COVID-19), and is currently ongoing, has affected over 200 countries, caused over 500 million cumulative cases, and claimed the lives of over 6.4 million people worldwide as of August 2022 [1]. The causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a member of the Coronaviridae family of viruses, a group of positive-sense, single-stranded RNA genome viruses of approximately 30 kb in length [2]. Its genome consists of 11 genes with 11 reading frames that produce 16 non-structural proteins (NSP1 to NSP16) and 4 structural proteins, including the fusion trimeric spike (S), the envelope protein (E), the nucleocapsid protein (N), and the membrane glycoprotein (M) [3]. These reading frames generate eight accessory proteins: ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8a, ORF8b, and ORF9b [3][4]. NSPs are key for viral RNA replication and immune avoidance, and the accessory proteins play diverse roles, aiding in viral infection, replication, and transmission [5]. **Figure 1** contains a diagram of the SARS-CoV-2 virus genome, highlighting the two open reading frames: ORF1a and ORF1b [4].

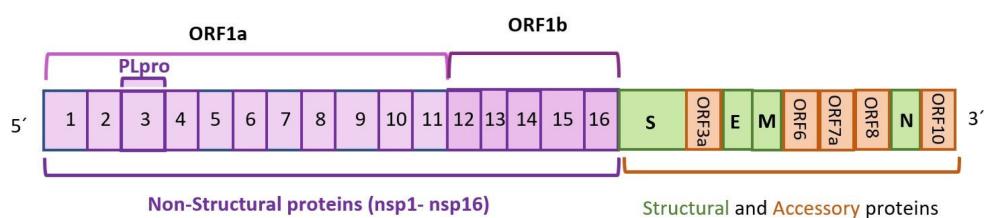


Figure 1. Genome of SARS-CoV-2 virus showing ORF1a and ORF1b open reading frames.

Autophagy is a catabolic process that sequesters damaged cell organelles, proteins, and external invading microbes and delivers them to the lysosomes for degradation [6][7][8][9]. It is a fundamental and evolutionarily conserved eukaryotic cellular process that has multiple effects on cell survival, homeostasis, and immunity [10]. Non-canonical autophagy describes other processes that use the autophagy molecular machinery, such as phagocytosis, inflammatory signaling, and secretion [11].

Autophagy plays an essential role in promoting RNA virus replication by inhibiting innate antiviral immune responses [12] or promoting infectivity via the autophagy-related secretion of vesicles loaded with virus [13][14][15][16].

Coronaviruses can cause respiratory infections of varying severity. Seasonal human CoVs (HCoVs) can cause mild to moderate upper respiratory tract infections with cold-like symptoms in humans [17]. On the other hand, highly pathogenic beta-CoVs have been responsible for multiple deadly outbreaks in the 21st century, including SARS-CoV (2003), the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, and the current SARS-CoV-2 (2019) [17][18]. For the ongoing pandemic, self-isolation quarantine and vaccination are still the mainstream strategies in responding to the virus, and therapeutic strategies remain challenging. For knowledge and therapeutic purposes, depicting the virus' life cycle and pathogenic mechanisms, as well as the cellular host factors and pathways involved during SARS-CoV-2 infection has great relevance [19]. The viral replication process comprises mainly six steps: binding and attachment, where the virus and the host cell receptors interact—this interaction is performed by the viral protein spike, (S) and the cell host receptors, angiotensin-converting enzyme-2 (ACE2), TMPRSS2 and integrins [19]; viral entry, using the two entry pathways of membrane fusion and endocytosis—where the virus fuses with the host cell membrane via the cleavage of protein S by the serine proteases of the host cell endocytic pathway; transcription and translation, where viral proteins are translated from viral RNA; viral replication; nucleocapsid packaging, where new virion packaging at the endoplasmic reticulum (ER) and Golgi apparatus occurs; budding and egress, i.e., the release of viral particles by exocytosis, as these RNA viruses traverse the Golgi apparatus and trans-Golgi network (TGN), where envelope proteins receive additional post-translational modifications and exit the cell via lysosomal exocytosis [20].

2. Overview of Autophagy

Canonical autophagy includes three different major types: (1) microautophagy, which implies the direct sequestering of small molecules by the invagination or protrusion of the membranes of lysosomes [21]; (2) chaperone-mediated autophagy (CMA), which transfers proteins or other targeted molecules through a chaperone to the lysosome, specifically to its LAMP2A receptor; regarding this, Cuervo and colleagues led to the identification of the lysosome-associated membrane protein type 2A (LAMP-2A) as a CMA receptor [22]; LAMP2A is a single-span membrane protein with a very heavily glycosylated luminal region and a short (12 amino acids) C-terminus tail exposed on the surfaces of the lysosomes, where substrate proteins bind [23]; (3) macroautophagy, which is the third and most prevalent form, in which double-membrane vesicles called auto-phagosomes form and engulf the substrates to be transported to the lysosome, in which they then fuse and release their cargo for degradation [24][25].

The molecular machinery involved in macroautophagy is composed of evolutionarily conserved autophagy-related proteins (ATG) [7][26][27].

ATG protein complexes are sequentially recruited for autophagosome biogenesis, and, after successive steps, the autophagosome fuses with a lysosome, forming the degradation compartment called the autolysosome (**Figure 2**).

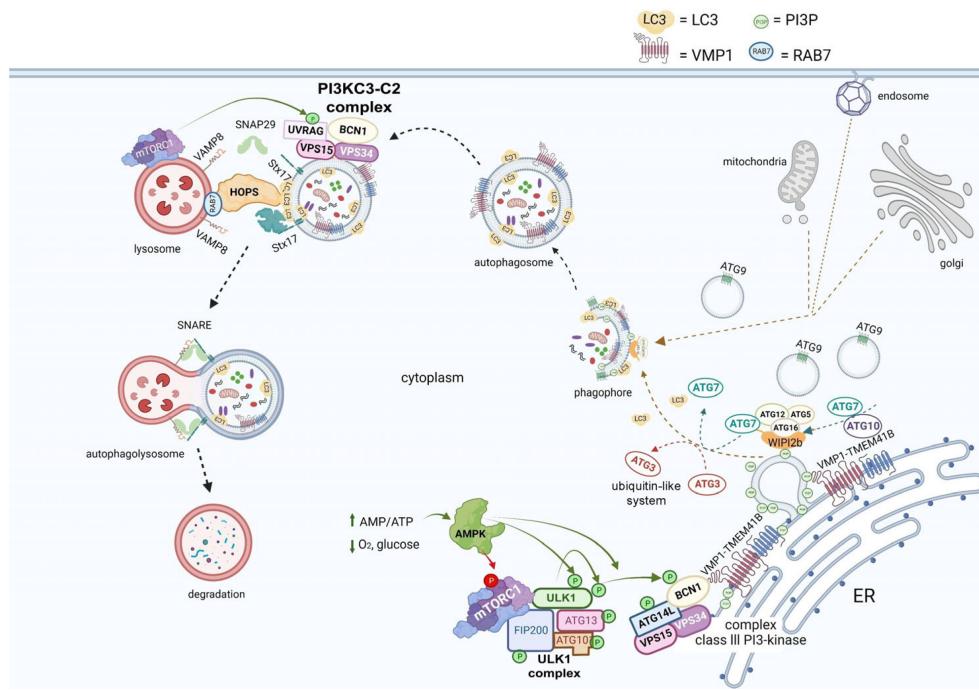


Figure 2. Autophagy overview diagram flow. Autophagy can be initiated by a lack of nutrients, implying amino acid, glucose, or O₂ depletion, or an AMP/ATP increase, as a result. When AMPK is activated, it inhibits the mTORC1 complex and activates the ULK1 complex through phosphorylation. Autophagosome biogenesis is mediated by ULK1 activation and translocation to ER. Thus, the class III PI3-kinase complex is activated by the phosphorylation of BECN1 and ATG14L; this process leads to a PI3P production increase through catalytic subunit VPS34 activation; transmembrane protein VMP1 interacts with BECN1 and WIPI2b protein is recruited. WIPI recruits ATG16L-ATG5-ATG12, a ubiquitin-like system, on the isolation membrane, mediating LC3 lipidation on the membrane. LC3 plays a crucial role in the whole autophagy process. The transmembrane protein ATG9 is implied in vesicular trafficking towards the phagosome maturation area. Once the autophagosome is mature, the second complex, PI3KC3-C2, replaces ATG14L by UVAG, which is activated by mTORC1 and participates in autophagosome-lysosome fusion. This fusion is regulated by the HOPS complex. STX17 interacts with lysosomal VAMP8 through SNAP29, constituting the SNAREs, forming the autolysosome structure. The complexes highlighted in this figure are as follows: complex ULK1 (ULK1-ATG13-FIP200-ATG101); complex class III PI3-kinase (BECN1-ATG14L-VPS34-VPS15); complex PI3KC3-C2 (BECN1-UVRAG-VPS34-VPS15).

Autophagy is induced in mammalian cells by mTORC1 inhibition and AMPK activation, which activate autophagy when a lack of nutrients or energy is detected. The ULK1 complex, ULK1-ATG13-FIP200-ATG101, drives the pre-autophagosomal structure. Then, the class III PI3K-kinase complex, BCN1-ATG14L-VPS34-VPS15, mediates

membrane phosphorylation and two ubiquitin-like systems involving ATG7, ATG12, ATG10, ATG5, ATG16, and ATG3, which allows the recruitment of Atg8 family proteins, promoting membrane expansion and cargo sequestration [7][28][29]. Two transmembrane proteins are involved in this sophisticated membrane trafficking process, ATG9 and (VMP1) [30][31][32]. VMP1 is a well-described transmembrane protein essential for mammalian cell autophagy [7][30]. TMEM41B is a transmembrane protein recently linked to autophagy that shares a conserved VTT domain with VMP1, and, together, they belong to the conserved DedA family of half transporters [33]. Finally, soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), such as STX17, facilitate the fusion between autophagosomes and lysosomes, forming the autolysosome structure, where cargoes are degraded and recycled into the cytosol. The autophagosomal fusion is regulated by the homotypic fusion and protein sorting (HOPS) complex. Then, mTOR phosphorylates UVrag and activates the PI3KC3-C2 complex to produce a lysosomal pool of PI3P. In this way, mTOR controls this process and regulates tubular initiation and maintenance. Macroautophagy can be non-selective or selective. In selective autophagy, one specific cellular component is recognized and sequestered in autophagosomes [34]. Examples of this mechanism are mitophagy, lipophagy, zymophagy, and xenophagy, the latter being a pathway by which the cell sequesters and degrades external invading microorganisms, including viruses, as a protective defense mechanism [35] (Figure 2).

Non-canonical autophagy describes other processes that use the autophagy molecular machinery, such as phagocytosis, inflammatory signaling, and secretion. One of the most relevant non-canonical autophagy pathways is the recently described secretory autophagy [36]. Secretory autophagy has been related to unconventional secretion, which links autophagy with an anabolic condition where cytosolic proteins lack an N-terminal signal peptide, which is the main peptide needed to undergo the conventional secretion pathway through the ER and Golgi apparatus. These are cytosolic proteins secreted from the cells to perform their biological functions. This process is associated with the canonical autophagic pathway as it shares regulation factors (ATGs), which also lead to the formation of autophagic membranes. These autophagic regulating factors include ULKs, BCN1, LC3s, and GABARAPs (analogs of the yeast Atg8).

3. Autophagy and the Viral Replication Cycle

There is considerable evidence suggesting that autophagy could play a role in assisting with the viral replication cycle at different stages.

As is well known, the SARS-CoV-2 virus enters the host by binding its receptor-binding domain (RBD), located in the spike protein, to the ACE2 host cell receptor. Moreover, a bioinformatic analysis showed that two integrin-binding regions are present in the cytoplasmic C-terminal tail of the host ACE2 protein. Additionally, the viral S protein has an arginine–glycine–aspartic acid (RGD) motif-binding domain that links to integrins, suggesting that integrins could be used as co-receptors for viral entry [37].

Furthermore, one specific type of short linear motif (SLiM) has been found to be present in the tails of integrin $\beta 3$ and ACE2. Thus, these structures from the autophagic machinery could be used to facilitate viral attachment, entry, and replication (as highlighted in the first step of Figure 3). Additionally, LC3-interacting region motifs (LIRs) have

been identified in the tails of integrin $\beta 3$ and ACE2. These LIRs are involved in the autophagic pathway through interaction with LC3, a marker of autophagosomes [38]. These LIR motifs also facilitate viral attachment, entry, and replication. Several viruses, including coronaviruses (CoVs), take advantage of cellular autophagy to facilitate their own replication. SARS-CoV-2 mediates its replication through a dependent or independent ATG5 pathway using specific double-membrane vesicles that can be considered similar to autophagosomes [39].

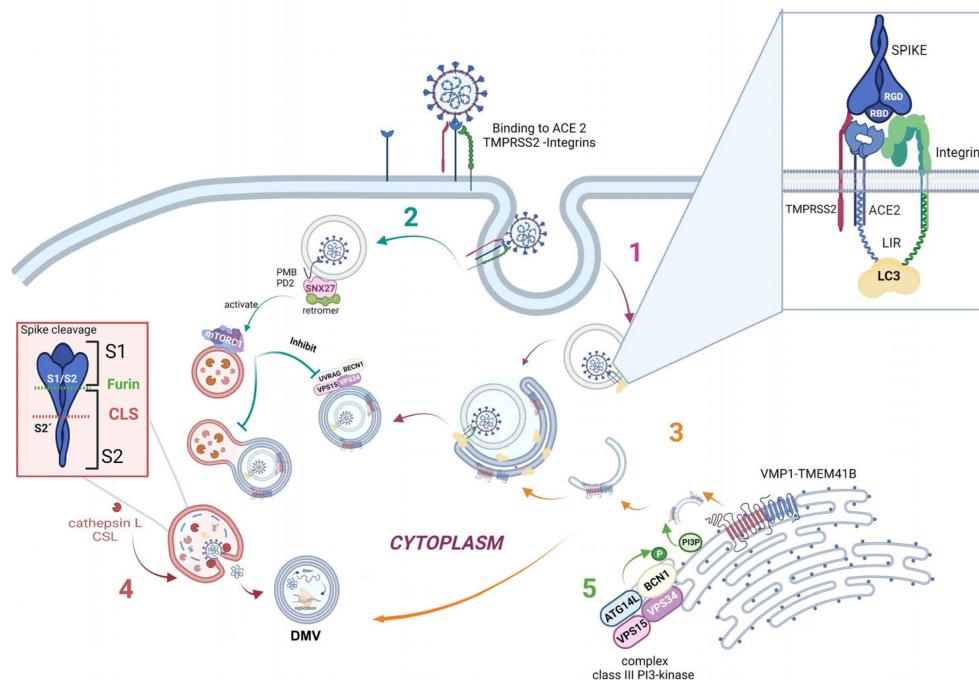


Figure 3. Schematic modeling of the crosstalk between autophagy and SARS-CoV-2 replication pathways.
 1—Autophagy may help virus endosomal entry through LC3. 2—Autophagy may be down-regulated by the activation of SNX27 present in the endosomes. 3—VMP1 and TMEM41B trigger autophagosome formation to aid viral replication. 4—The protease Cathepsin L allows viral RNA release to the cytosol, before viral replication into DMVs. 5—The complex class III PI3-kinase through VPS34 is necessary for SARS-CoV-2 infection and replication.

The virus enters the cell via the endosomal pathway. The spike protein is composed of two subunits: one binding subunit (S1) and one fusion subunit (S2). First, the S1 subunit binds to the host cell receptor ACE2. This union induces conformational changes in protein S. Second, the S2 subunit is activated. This activation occurs through two consecutive cleavage steps: a cleavage between the S1 and S2 domains of protein S by Furin is produced, and it then undergoes further cleavage at the S2' site, which promotes the unmasking and activation of the fusion peptide [40]. To activate the spike protein's fusion potential, a second cleavage performed by the host's proteases is required. The cleavage can occur at different stages of the virus infection cycle by different host proteases, such as Furin (convertase), TMPRSS2 (cell surface protease), and Cathepsin L (lysosomal protease) [41][42]. Cathepsin L, which links the virus cycle to the autophagy process, acts at a low pH, degrades cargo, and maintains autolysosome homeostasis and autophagic flux. By cleaving the spike protein at S2', it mediates virus membrane and autolysosome fusion, thus facilitating the release of viral RNA into the host cell. Smieszek et al., demonstrated that the inhibition of Cathepsin L could significantly reduce the entry of viruses into host cells [43]. It has also been

shown that TMPRSS2, Furin, and Cathepsin L proteases have cumulative effects to activate virus entry and increase the pathogenicity of SARS-CoV-2 [44][45] (Figure 3, pathway 4).

Once the virus has entered the cell, it uses the autophagy machinery for its own benefit through viral proteins, NSPs. Recently, as shown in **Figure 3**, pathway 3, transmembrane proteins related to autophagy, i.e., VMP1 and TMEM41B, have been reported as critical host factors at the early stages of viral infection [45]. VMP1 and TMEM41B both contribute to different stages of DMV formation. Ji et al., have revealed that DMV biogenesis is impaired in VMP1 and TMEM41B knockout cells. Analysis using transmission electron microscopy revealed that the formation of DMVs was substantially inhibited in cells lacking these autophagy proteins. Hence, by inhibiting VMP1 and TMEM41B expression, the virus is unable to hide from the immune sensors in DMVs, thus reducing its protection from the immune system [46][47]. Schneider et al., showed that TMEM41B participates in the transport of lipids to the membrane and that, together with VMP1, they are involved in the remodeling of the ER to form double-membrane vesicles (DMV). Scudellari compared the double-membrane spheres to bubbles being blown by the endoplasmic reticulum [20]. These DMVs may act as replication organelles (RO), which might provide a safe place for viral RNA to be replicated and translated, protecting it from innate immune sensors in the cell, similar to other β coronaviruses. Therefore, these structures play a central role in infection, and, consequently, the loss of RO integrity due to the lack of VMP1 or TMEM41B could lead simultaneously to altered viral replication and enhanced antiviral signaling, as viral RNA is a very potent inducer of innate antiviral signaling [48].

SARS-CoV-2 mediates its replication through a dependent ATG5 pathway using specific DMVs that can be considered similar to autophagosomes. Mutations in the NSP6 protein with a positive influence on autophagosome production suggest a potential link with autophagy [49]. Thus, researchers hypothesize that some of these DMVs could be related to autophagy structures, and, more specifically, to autophagosomes. Researchers are certain that, in the near future, it will be found that well-described autophagy markers colocalize with these DMVs in SARS-CoV-2-infected cells.

Another connecting pathway between the viral replication cycle and autophagy is represented by SNX27, one of the sorting nexin (SNX) family members, which down-regulates autophagy by increasing the level of mTORC1 signaling [50]. **Figure 3**, pathway 2 shows how SNX27 regulates the traffic of endosomal receptors towards recycling endosomes. Kim et al., found that mTORC1 acts as a signal integrator at the lysosome and can act as an inhibitor of later stages of autophagy, suppressing phosphorylation on UVAG, which is a component of VPS 34 complex II. In this way, it avoids autophagosome and endosome maturation [50]. These events are relevant for the viral cycle, given that the virus enters the cell via directly fusing to the membranes in the cell surface pathway or via the endocytic pathway through endosome/lysosome-mediated endocytosis. Interestingly, it has recently been found that the ACE2 receptor possesses a type I PDZ binding motif (PBM) and can therefore interact with a PDZ domain-containing protein such as SNX27. A recent study has shown SNX27 to be critical for ACE2 cell surface regulation, and SNX27 prevents ACE2-bonded viral particles from entering the lysosome, down-regulating the endocytic viral entry pathway and therefore serving as a viral trafficking regulator [51].

Regarding autophagy machinery, a class III PI3-kinase that produces PI3P has a role in cellular trafficking and in the nucleation step in both canonical and non-canonical autophagy. In fact, inhibition of VPS34 kinase activity by VPS34-IN1, a well-known inhibitor for this kinase, reduced PI3P production and suppressed SARS-CoV-2 infection and replication in ex vivo human lung tissues [52] (Figure 3, pathway 5).

The features mentioned above provide consistent evidence that the autophagy machinery is actively involved in the viral entry and replication process of SARS-CoV-2 infection and therefore could be used as potential therapeutic targets to battle infection and prevent viral entry and replication at different steps.

4. SARS-CoV-2 Infection and Its Effects on Autophagy

Although strong evidence points toward the SARS-CoV-2 virus having an inhibiting role in some stages of autophagy, paradoxically, it has been suggested that the virus enhances autophagy in other steps of this process. Li et al., explored the regulatory role of the SARS-CoV-2 spike protein in infected cells and attempted to elucidate the molecular mechanism of SARS-CoV-2-induced inflammation. They found that SARS-CoV-2 inhibits the PI3K/AKT/mTOR pathway by upregulating intracellular reactive oxygen species (ROS) levels, and, in this way, promotes the autophagic response. Subsequently, SARS-CoV-2-induced autophagy triggers inflammatory responses and apoptosis in infected cells [53].

Recent studies suggest that SARS-CoV-2 inhibits autophagy at different stages, limiting the autophagic flux to suppress viral clearance by selective autophagy, known as virophagy, a process mediated by autophagy receptors that recognize and sequester viral components inside autophagosomes. To avoid virus inactivation, SARS-CoV-2 uses the autophagy machinery for its benefit [14]. Autophagy also regulates adaptive immunity through antigen presentation. Gassen and colleagues found that SARS-CoV-2 modulates cellular metabolism and reduces autophagy; therefore, the induction of autophagy limits SARS-CoV-2 propagation [16]. Autophagy machinery proteins and viral proteins interact, leading SARS-CoV-2 to successfully survive and complete the replication cycle in the infected host cells.

Regarding SARS-CoV proteins, Mohamud and colleagues have shown that NSP3 can cleave the serine/threonine unc-51-like kinase (ULK1) and prevent the formation of the autophagy initiation complex in the absence of nutrients. Another recent study showed that different viral proteins targeted and inhibited autophagy to avoid viral clearance and to block the antiviral functions of autophagy [54]. SARS-CoV-2 uses autophagy to its benefit, hijacking the autophagy mechanism in the host cell to improve viral replication and to avoid the immune response and extracellular release. Viral proteins ORF3a and ORF7a were shown to cause the accumulation of autophagosomes [55]. Specifically, ORF3a interacts with autophagy-related protein UVAG, suppressing autophagosome maturation and therefore the autophagy flux.

In two other studies, it was demonstrated that ORF3a interacts with VPS39, colocalizing with lysosomes. In this way, it impairs the binding of HOPS with RAB7, avoiding the regulation of the fusion of autophagosomes with lysosomes [56][57].

ORF7a generates a dysfunctional deacidified lysosome; therefore, autophagosomal degradation is interrupted and the virus can exit the host cell [55].

Nsp15 modulates autophagy regulation hypothetically by interfering with the mTOR pathway, in this way facilitating SARS-CoV-2 replication [55].

Non-structural protein NSP6 interacts with autophagy in different ways. It can join ER membranes, stimulating the rearrangement of its membranes and facilitating phagophore formation. This is another example of how SARS-CoV-2 uses the autophagy machinery to form DMVs to hide from the immune system and to replicate RNA. Moreover, it was found that viral protein NSP6 impairs autophagic flux, inhibiting autophagy at a late stage and impairing lysosomal acidification by targeting ATP6AP1, a vacuolar ATPase proton pump component. Consequently, the inflammasome is activated through NLRP3. To confirm that NSP6 elicits pyroptosis, Sun and colleagues experimentally overexpressed NSP6 and found that NLRP3/ASC Caspase-1-dependent activation released IL1 β and IL18 in lung epithelial cells, thus being a crucial factor in viral pathogenicity [58].

Clinical Implication

Autophagy would be involved in the entry, endo, and release, as well as the transcription and translation of the viral particles in the host cell [59] (He et al. 2022). Also, autophagy could be involved in the systemic inflammatory response and post-COVID-19 syndrome. Selective autophagy, specially mitophagy, was reported to be induced by SARS-CoV2 proteins, modulating inflammatory response. Secretory autophagy would also be involved in the development of the thrombotic immune-inflammatory syndrome seen in a significant number of COVID-19 clinical courses that lead to severe illness and even death.

However, autophagy not only relates to SARS-CoV2 infection by participating in its viral replication cycle. Studies suggest that, surprisingly, reciprocal dysregulation of autophagy by the viral infection itself would be one of the mechanisms of viral survival and tissue damage, given the antimicrobial functions of autophagy, aiding with viral clearance through xenophagy, and its immunological role in battling infection and regulating excessive inflammation [60] (Deretic 2021). Autophagy's role as a balancer of beneficial and detrimental effects of immunity and inflammation becomes disrupted by viral effects over autophagy's complex machinery.(Deretic 2021; Levine et al, 2011) [60][61]. Dysregulation of autophagy could imply disinhibition of different molecular processes stimulating the release of pro-inflammatory cytokines and interleukins. These mediators favor an exaggerated inflammatory response overall, leading to a thrombotic immunoinflammatory state that correlates with a more severe clinical illness of COVID-19. In fact, one of the described cargos of unconventional autophagy-associated secretion pathways is the export of cytosolic protein IL1 β , a proinflammatory cytokine, which has a central role in inducing pro-inflammatory signaling (Ponpuak et al. 2015) [62]. A possible link between this process and the cytokine storm that characterizes the immunoinflammatory state seen in patients with severe COVID-19 could be further explored.

Consequently, identifying different autophagic biomarkers could help correlate with severity of illness, and thus work as a biological marker for prognosis of disease. Many viruses induce direct and indirect mechanisms

explaining most of the short-term complications of the disease have correlations with alterations in autophagy. Long-term post-COVID syndromes may be also related to dysfunctional autophagy. Besides, the association between interindividual markers of short- and long-term prognosis and dysfunctional autophagy offers many gaps for further investigation. More research is needed to clarify the involvement of these abnormalities in disease infection and clinical evolution.

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