UJEMI PERSPECTIVES

SARS-CoV-2 lysosomal egress pathway

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SUMMARY The early stages of the SARS-CoV-2 lifecycle have been relatively well-defined. However, knowledge of how newly assembled SARS-CoV-2 particles egress from cells remains limited. Until recently, SARS-CoV-2 egress was misunderstood as being dependent on the biosynthetic secretory pathway commonly used by enveloped viruses. Recent findings have demonstrated that SARS-CoV-2 instead utilizes an unconventional lysosomal egress pathway. This pathway has been characterized as being dependent on the Arl8b GTPase for anterograde movement of the lysosome along microtubules to the plasma membrane. Viral hijacking of the lysosomal pathway leads to lysosome deacidification and deactivation of lysosomal hydrolases, which perturbs antigen presentation. However, many questions regarding the molecular details of this lysosome-mediated egress remain unanswered. This article will highlight the current knowledge gaps in the lysosomal-egress pathway, focusing on 1) how newly assembled SARS- CoV-2 particles are trafficked to lysosomes, 2) the mechanisms by which lysosome deacidification occurs, and 3) the reasons why deacidification of lysosomes is essential for successful egress. Through its disruption of lysosome function and perturbation of antigen presentation, the lysosomal egress pathway has implications for the clinical presentation of COVID-19. Furthermore, understanding the molecular details of the pathway presents an opportunity to develop novel therapeutic targets. Therefore, further investigation of the viral and host factors involved in this egress pathway is essential in understanding SARS-CoV-2 as a whole.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the COVID-19 pandemic, is an enveloped, single-stranded positive-sense RNA virus of the β-coronavirus family (1). SARS-CoV-2 can infect many different cell types, including cells of the pulmonary, cardiovascular, hepatic, gastrointestinal, central nervous, and immune systems (2). Particularly concerning the immune system, significant evidence supports that the disruption of the innate and adaptive immune response contributes to the clinical progression of COVID-19 (3). The multiorgan tropism of SARS-CoV-2 results in a heterogeneous array of organ injuries in COVID-19 patients (4), leading to disease manifestations that vary greatly from individual to individual (4). While some infected patients remain asymptomatic, others succumb to the disease.

To better understand the clinical presentation of COVID-19, a complete elucidation of the replicative cycle of SARS-CoV-2 is necessary. As there are currently no specific antiviral therapies against SARS-CoV-2, understanding the replication pathway will also aid in the development of targeted medical interventions and the production of novel therapeutics. To this point, the entry and replication pathways of SARS-CoV-2 have been described in molecular detail (5,6). However, knowledge of how newly assembled coronaviruses egress from cells remains limited. It was previously assumed that β-coronaviruses use a biosynthetic secretory pathway as an egress mechanism, a pathway commonly used by enveloped RNA viruses such as hepatitis C (7,8,9). This egress mechanism largely relies on the viral hijacking of the classical cell secretory pathway, whereby cargo is transported from the ER to the Golgi apparatus and eventually to the plasma membrane for release of new viral particles (9).

However, a recent study by Gosh *et al*. revealed that in contrast to the common biosynthetic secretory pathway, β-coronaviruses such as mouse hepatitis virus (MHV) and SARS-CoV-2 utilize lysosomal trafficking to egress from infected cells (Fig. 1) (2). This

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pathway is dependent on lysosomal exocytosis meditated by Arl8b, a small GTPase required for linking lysosomes to kinesin-1 for anterograde movement of lysosomes towards the plus end of microtubules (10). The KDEL receptor, a transmembrane protein that cycles between the ER/Golgi apparatus to retrieve ER proteins escaped to the Golgi, and its cargo, the ER chaperones GRP78/BIP and calreticulin, are selectively co-trafficked with the viruses to the lysosomes (2). Once at the cell periphery, the virus-containing lysosomes can fuse with the plasma membrane to release the new viral particles, which are co-released with the KDEL receptor and its cargo (2). Because of viral exploitation of the lysosomal pathway, late endosomes and lysosomes are deacidified, resulting in the inactivation of lysosomal proteases (2). The dysregulation of the lysosomal pathway has repercussions for normal host cell function such as antigen presentation, which relies on lysosomes to break down proteins into antigens (2). Thus, the disruption of lysosomal trafficking may be at the root of some of the clinical presentations of COVID-19 such as adaptive immune dysregulation. The molecular details behind this unconventional egress pathway thus warrant further investigation.

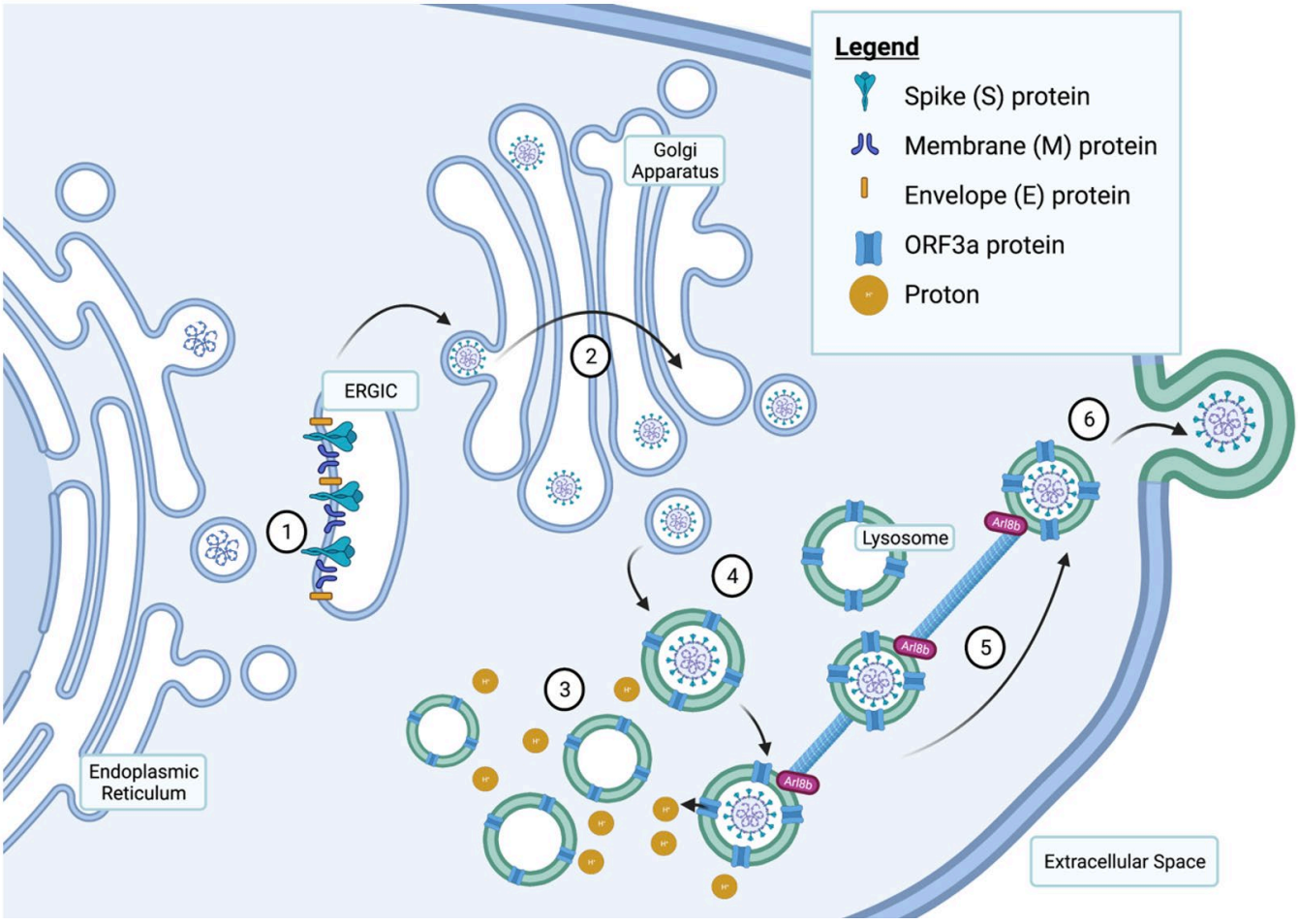

FIG. 1 Overview of proposed lysosomal egress pathway. (1) Assembly of SARS-CoV-2 particles at the ERGIC compartment. (2) Virus maturation through Golgi trafficking. (3) Deacidification of lysosomes via ORF3a viroporin. (4) Transport of viral particles from Golgi to lysosomes. (5) Arl8b-mediated anterograde movement of virus-packed lysosomes along microtubules. (6) Fusion of lysosome with plasma membrane and budding. Figure created with BioRender.com.

PROPOSED RESEARCH QUESTIONS

The discovery of the lysosome-dependent egress pathway is an important step in the complete understanding of the SARS-CoV-2 replication cycle. This egress pathway relies on Arl8b for anterograde movement of the lysosome along the microtubules to the cell membrane, and the viral hijacking of the lysosomal pathway leads to deacidification of the lysosomes and subsequent deactivation of lysosomal enzymes. However, many questions regarding the underlying mechanisms and molecular details behind this pathway remain unanswered. We

must gain a comprehensive understanding of the egress pathway to develop novel antiviral therapies and to better understand the clinical presentation of patients with COVID-19. This article will discuss the largest knowledge gaps in the egress pathway and attempt to bridge some of these gaps. First, I will propose processes by which newly assembled viruses are trafficked to lysosomes. Second, I will discuss viral factors that may be involved in lysosome deacidification. Finally, I will examine the reasons for which lysosomal deacidification is necessary for successful lysosome-mediated egress.

PROPOSED PROJECT NARRATIVE

What is the mechanism behind the transport of viral particles to lysosomes? The transport mechanism that targets newly assembled viral particles from their assembly site at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) to the lysosomes remains to be described. Knowledge of the host and viral factors involved in this transport mechanism is essential for the development of novel therapeutics targeting the egress pathway. It has been shown that nucleocapsid-bound viral RNA buds into the ERGIC, which is decorated with S, E, and M structural proteins, to form the viral envelope (5). The newly assembled virus then passes through the Golgi apparatus for modification and maturation (6). After this, it is not yet known how the mature virus is trafficked to the lysosomes for egress. At least two trafficking routes are worthy of investigation: the mannose-6-phosphate pathway and the Rab7 GTPase-regulated maturation of late endosomes/MVBs into lysosomes (Fig. 2).

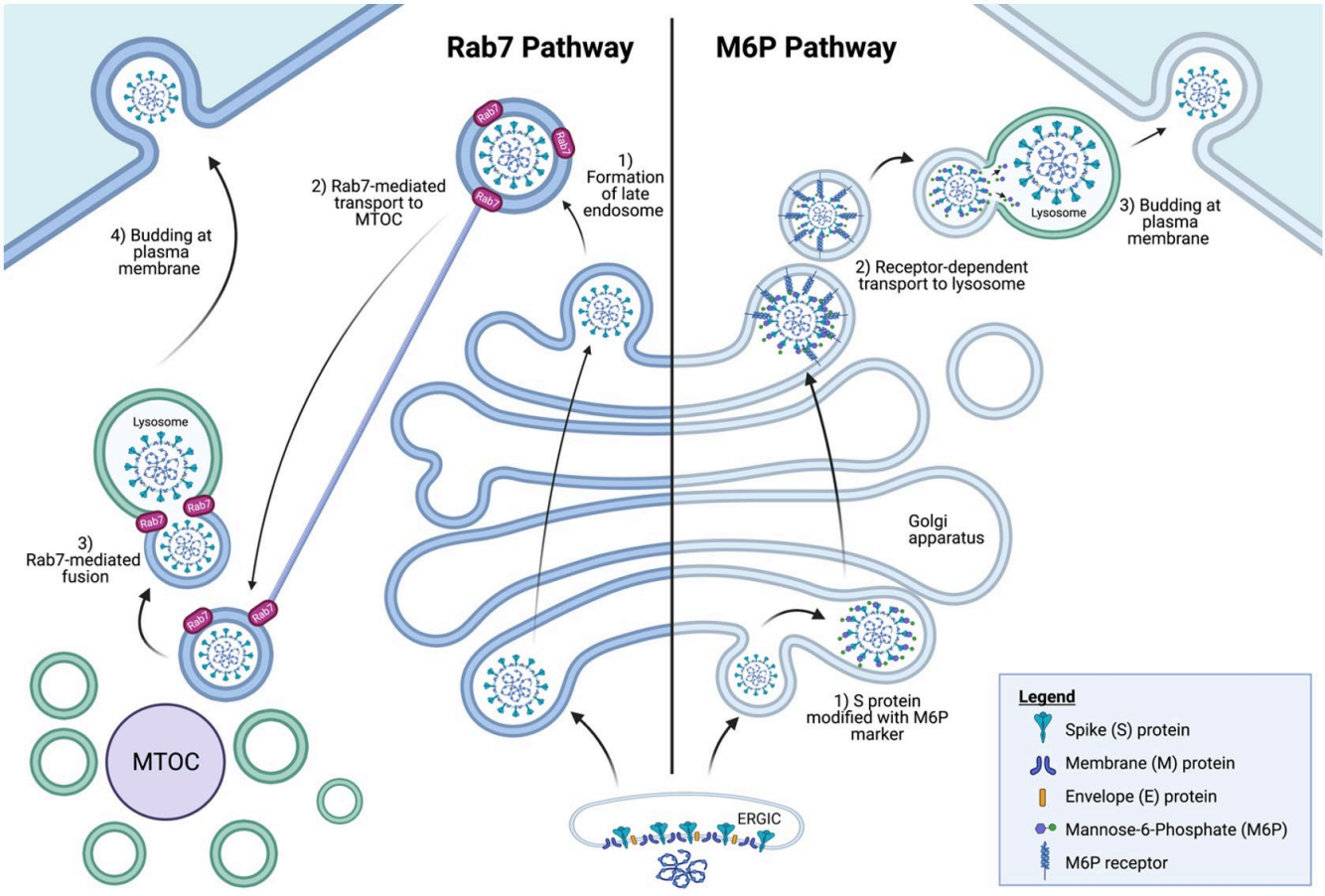

FIG. 2 Potential mechanisms for targeting viral particles to lysosomes. Two potential transport mechanisms are highlighted. Left: Rab7-mediated formation of the late endosome, followed by retrograde transport to the MTOC for Rab7-regulated late endosome and lysosome fusion. Right: mannose-6-phosphate modification of the SARS-CoV-2 spike protein leads to M6P-receptor dependent transport to lysosomes. Figure created with BioRender.com.

In normal cell activity, lysosomal proteins are trafficked from the Golgi apparatus to the lysosomes via the mannose-6-phosphate (M6P) pathway. Lysosomal hydrolases are synthesized in the endoplasmic reticulum and are cotranslationally glycosylated on specific asparagine residues (11). At the *cis*-Golgi face, these glycosylated hydrolases are recognized by UDP-N-acetylglucosamine-1-phosphotransferase, a phosphotransferase that initiates the reaction that results in the placement of the M6P marker on N-linked oligosaccharides on the hydrolases(12). Next, in the *trans*-Golgi network, the M6P marker is recognized by two independent receptors, forming a ligand-receptor complex that is packaged into clathrin-coated vesicles for transport to lysosomes (12). Overall, the M6P pathway results in the transport of proteins from the Golgi apparatus to the lysosomes. Thus, the utilization of this pathway by newly assembled SARS-CoV-2 particles could be the mechanism by which the virus is moved from the Golgi to the lysosomes. In fact, mass spectrometry analysis of glycosylation of the SARS-CoV-2 spike protein (S) revealed the presence of M6P on the S1 subunit on the ChAdOx1 nCoV-19 S protein, with some evidence for this modification also on the S1 protein of the virus itself (13). Thus, confirming the addition of M6P on the S1 subunit N-terminus certainly warrants further investigation, as this could be the mechanism by which the virus is transported from the Golgi apparatus to the lysosomes.

Another possible pathway that newly assembled viruses could utilize for transport from the ERGIC to the lysosomes is the Rab7-regulated fusion of late endosomes or multivesicular bodies (MVBs) with lysosomes. Rab7 is a small GTPase of the Rab family that is localized mainly to late endosomes (14). It plays a critical role in the maturation of early endosomes to late endosomes, the biogenesis of lysosomes, and the fusion of late endosomes with lysosomes (14). The acquisition of Rab7 on late endosomes coincides with the loss of Rab5, a marker of an early sorting endosome (15). This Rab5/Rab7 switch is characterized by changes in the fusion machinery of the endosome, giving it the ability to recognize and fuse with other late endosomes as well as lysosomes (15). In addition to regulating the fusion of late endosomes with lysosomes, Rab7 is also implicated in the transport of late endosomes to the microtubule organizing center (MTOC). Through the recruitment of RILP, Rab7 mediates the attachment of late endosomes to the dynein-dynactin complex, which ultimately leads to the transport of the late endosome towards the minus-end of the microtubule towards the MTOC (14). Once late endosomes are localized near the MTOC, the fusion of the late endosome and the lysosome is possible because of the fusion machinery the late endosome acquired during the Rab5/Rab7 switch (14). Thus, newly assembled viruses may be transported from the ERGIC to lysosomes via late endosomes/MVBs, in a Rab7-dependent pathway. In fact, a depletion of Rab7 in MHV-infected cells using CID1067700, a competitive inhibitor, decreased viral egress in a dose-dependent manner, by 100-fold at 40μM and 1000-fold at 400μM (2). This data supports an essential role for Rab7 in the SARS-CoV-2 lysosomal egress pathway as a regulator of the transport of newly assembled viral particles from the ERGIC to the lysosome in late endosomes and/or as a contributor to the biogenesis of lysosomes necessary for egress.

What is the mechanism behind the deacidification of lysosomes? The alkalization of acidic organelles is not uncommon in the replication cycles of enveloped viruses. For example, the influenza A virus matrix 2 (M2) protein and the hepatitis C virus p7 protein have both been shown to reduce the acidity of intracellular vesicles (16,17). Generally, the virus-induced deacidification of acidic organelles is mediated by viroporins, small hydrophobic viral proteins that oligomerize in host cell membranes to form hydrophilic pores (18). Depending on the specificity of the pore, viroporins can disrupt several physiological properties of the host cell or organelle, including pH (18). Thus, to understand the mechanism underlying the deacidification of lysosomes in SARS-CoV-2 infected cells, it is important to investigate the viroporins that exist in the viral proteome. Indeed, evidence has suggested ion channel activity for the SARS-CoV envelope (E), ORF3a, and ORF8a proteins (19). The ORF3a protein presents the most compelling case as a possible modulator of lysosomal deacidification in SARS-CoV-2 infected cells (Fig. 3).

ORF3a is a 31-kDa homodimer accessory protein specific to human and animal isolates of SARS-CoV-2 (19,20). The protein consists of three transmembrane spanning α-helices and a cytoplasmic domain of two β-sheets connected by a short α-helix (19). Through

extensive dimer-dimer interactions, the homodimer assembles in membranes as a homotetramer (19). ORF3a has been shown to localize to several subcellular locations, including the Golgi apparatus and the plasma membrane, the late endosomes and lysosomes, but rarely to the endoplasmic reticulum and the mitochondria (20-22). Localization of ORF3a to the late endosomes and lysosomes is significant for the viroporin's potential role in lysosome deacidification. Furthermore, it has been demonstrated that the SARS-CoV ORF3a protein is directly involved in modulating viral release (23). For instance, when ORF3a expression was suppressed in SARS-CoV infected FRhk-4 cells, viral infection and RNA replication were not affected, however, viral titers and genomic RNA of virus released into culture media were significantly reduced (23). These results suggest a direct role of ORF3a in viral egress.

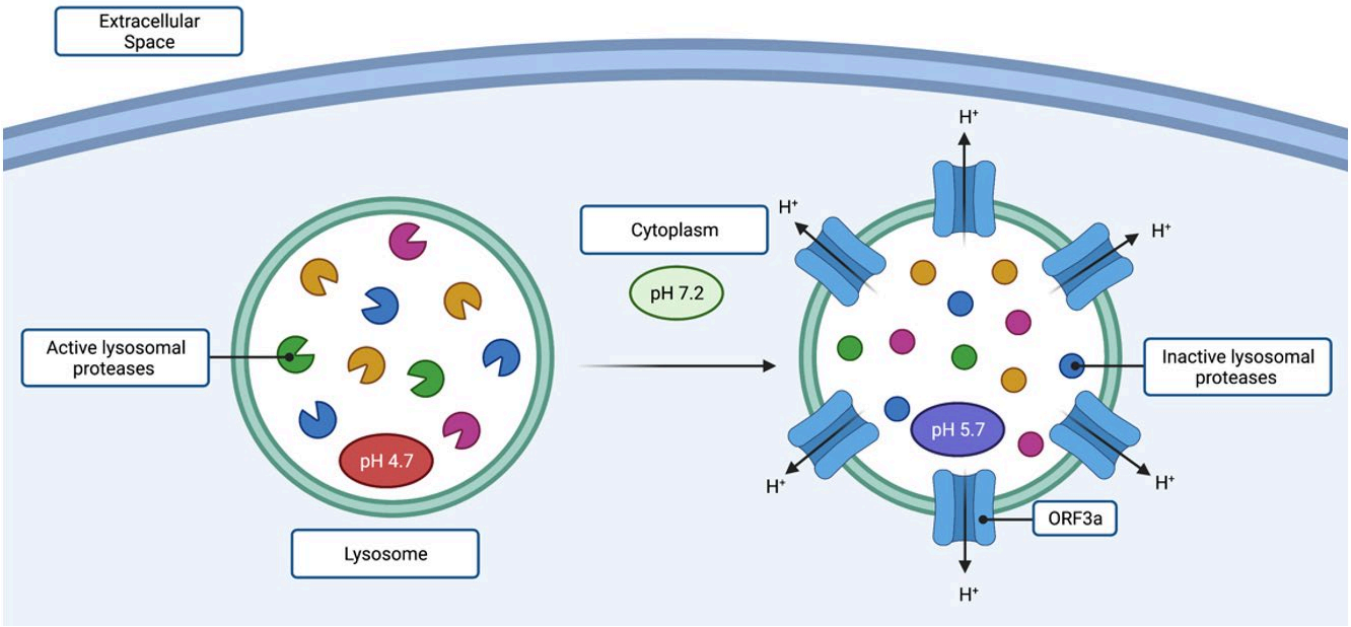

FIG. 3 ORF3a-mediated lysosome deacidification. Proposed mechanism of lysosome deacidification. SARS-CoV-2 viroporin ORF3a is targeted to the lysosomal membrane, where it acts as a proton channel to transport protons out of the lysosomal lumen. Consequently, acid-dependent lysosomal proteases are inactivated. Figure created with BioRender.com.

Furthermore, ORF3a has been shown to disrupt lysosomal protease function and fusion of autophagosomes with lysosomes during SARS-CoV-2 infection (20). For example, DQ-BSA is a bovine serum albumin labelled with a self-quenched fluorophore that is normally endocytosed and fluoresces upon cleavage by lysosomal proteases (24). Upon treatment of ORF3a-GFP expressing cells with DQ-BSA, it was found that the number of DQ-BSA fluorescent puncta was significantly reduced in ORF3a expressing cells compared to control cells (20). Furthermore, Galectin-3 is a protein that specifically labels damaged endosomes and lysosomes (25). In ORF3a expressing cells, a significant increase in Galectin 3 puncta was observed compared to control cells (20). Because lysosomal protease activity relies on a pH of less than 5, ORF3a's role in disrupting lysosomal protease function could potentially be explained by ORF3a's ability to increase the lysosomal pH beyond what is optimal for lysosomal protease activity.

Further, it is worth noting that late endosome localized ORF3a has been shown to disrupt autophagy. The protein sequesters the homotypic fusion and protein sorting (HOPS) component VPS39, impairing the ability of the HOPS complex to interact with the STX17 SNARE protein on the autophagosome (20). Ultimately, this prevents the fusion of autophagosomes with lysosomes (20). One possible explanation for this role of the ORF3a protein is to protect the viral particles that are packaged in lysosomes for egress. The fusion of a virus-containing lysosome with an autophagosome would disrupt the release of infectious

viral particles from the host cell. Thus, by inhibiting autophagy, ORF3a could aid in the release of viral particles via the lysosomes.

Therefore, due to the transmembrane nature of the ORF3a protein, its localization on late endosomes and lysosomes, its direct involvement in viral egress and its role in disrupting lysosomal protease function and the fusion of lysosomes with autophagosomes, the potential role of the ORF3a protein in lysosome deacidification warrants further investigation.

Why is the deacidification of lysosomes necessary? Although the deacidification of lysosomes in SARS-CoV-2 infected cells has been reported, the reason why this increase in pH is necessary for successfully viral egress has not been established. Lysosomal deacidification may be necessary to prevent the degradation of viral particles in the lysosome and to prevent the occurrence of a premature fusion event. Furthermore, lysosomal deacidification disrupts antigen presentation, which acts as an immune evasion mechanism and may play a role in the immune dysregulation characteristic of COVID-19 patients (Fig. 4).

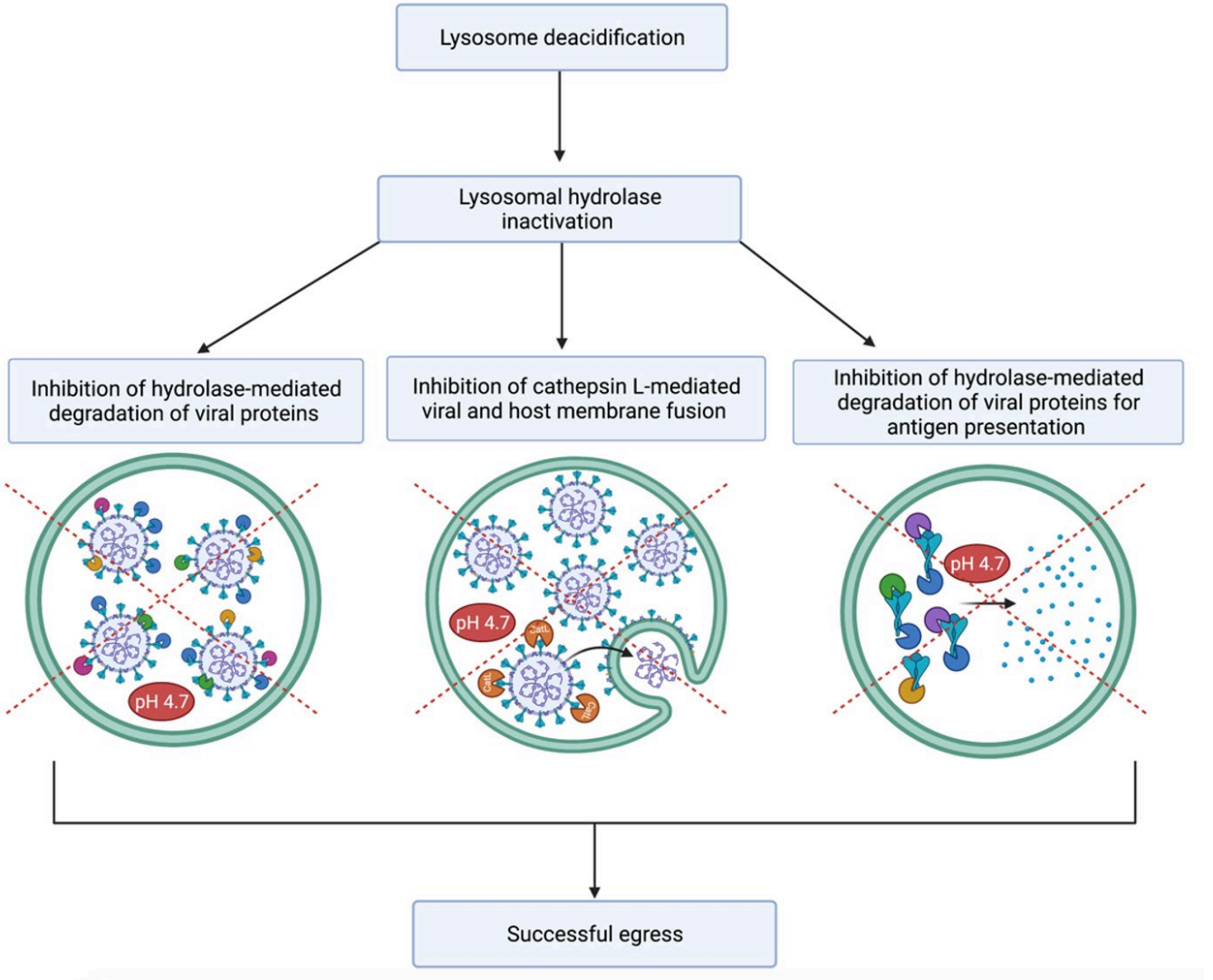

FIG. 4 Lysosomal deacidification is necessary for successful egress. Deacidification of lysosomes inactivates lysosomal hydrolases, which is necessary for lysosome-mediated egress because it 1) inhibits hydrolase-mediated degradation of viral proteins, 2) inhibits the cathepsin L-mediated fusion of the viral and host envelopes and release of the viral genome, and 3) disrupts antigen presentation by inhibiting the degradation of proteins into antigen fragments. Figure created with BioRender.com.

The primary role of the lysosome in the cell is digestion (26). Thus, to successfully utilize the lysosomal pathway for egress, the virus needs a mechanism to prevent its digestion, such as deacidification of the lysosomal lumen. The lysosome's characteristic acidic pH is necessary for the activity of over 60 hydrolytic enzymes contained within the organelle that function to degrade molecules into their constituent components (12). Specifically, a pH of between 4.5 and 5.0 must be maintained for these hydrolases to successfully perform their digestive functions (26). It has been demonstrated that lysosomes of cells infected with β-coronaviruses display a mean pH of 5.7 (with a range of 5.0-6.4), compared to control cells with a mean lysosomal pH of 4.7 (ranging from 4.2-5.2) (2). This significant increase of one full pH unit is sufficient to prevent the activity of lysosomal proteases (27). Therefore, by increasing the pH of the lysosomes, newly assembled viral particles can successfully hijack the lysosomal pathway for egress without being degraded inside the organelles.

Another reason why lysosomal deacidification is necessary for successful egress is because it prevents the premature cathepsin-mediated fusion of the viral and cellular membranes. Preventing this fusion event is critical for the successful egress of the virus because it impedes the release of the viral genome into already infected cells. It has been demonstrated that one of the entry mechanisms of SARS-CoV-2 is dependent on the activity of the lysosomal protease cathepsin L (CatL) (28). In this entry pathway, the SARS-CoV-2 spike protein (S) binds the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell membrane, and the virus is subsequently endocytosed (28). Once inside the endosome, CatL cleaves the S1/S2 boundary of the spike protein (28). This proteolysis event is necessary to reveal the fusion peptide of the S2 domain, which mediates fusion of the viral and cellular membranes upon extensive conformational changes (6). This fusion event ultimately leads to the release of the viral genome into the host cell cytoplasm to initiate viral replication. Therefore, for SARS-CoV-2 to utilize the lysosomal pathway for release, a mechanism must be put in place to prevent this fusion event from occurring during egress. By deacidifying the lysosomal lumen, the pH is increased beyond what is optimal for CatL function. Thus, CatL is unable to cleave the S1/S2 boundary of the spike protein, and the fusion event is impeded.

Further, deacidification of the lysosomes and subsequent inactivation of lysosomal proteases disrupts antigen presentation, which may explain some of the immune dysregulation observed in COVID-19 patients. Lysosomes are critical in the process of antigen presentation, as both MHC-I and MHC-II-restricted antigen processing pathways rely on lysosomal proteases for the breakdown of proteins into antigen fragments (29). It was demonstrated that the altered lysosomal pathway in β-coronavirus infected cells results in the perturbation of antigen presentation, which can lead to viral immune evasion as well as an altered host immune response (2). Immune dysregulation, such as lymphopenia, is commonly observed in the clinical presentation of patients with severe COVID-19 (3). Therefore, the inactivation of lysosomal proteases via lysosome deacidification warrants further investigation as it may explain some of the clinical manifestations of COVID-19.

CONCLUSIONS

A complete understanding of the molecular details of the SARS-CoV-2 replication cycle is vital for the comprehension of the pathology of the virus and for the development of novel therapeutics to combat the disease. The discovery of the unconventional SARS-CoV-2 lysosomal egress presents an opportunity to explain some aspects of the clinical presentations of patients with COVID-19 and opens the door to new therapeutic targets. However, these explanations and interventions will only be possible once a more complete understanding of the pathway is obtained. It is therefore essential that more research focusing on this pathway be performed.

Significant features of the lysosome-mediated egress pathway include transport of the viral particles from the ERGIC compartment to the lysosomes and the deacidification of the lysosomes. The molecular details of these features remain undescribed. Newly assembled viral particles may be directly targeted to lysosomes via the mannose-6-phosphate pathway, yet it is equally plausible that this transport is mediated by the Rab7-regulated transport of cargo from late endosomes to lysosomes. Furthermore, the virally encoded ORF3a protein presents a compelling case as the mediator of lysosome deacidification. It is critical that the

viral and host factors involved in these key steps of the egress pathway be identified as they may be important novel therapeutic targets.

The involvement of the SARS-CoV-2 lysosomal egress pathway in COVID-19 pathogenesis remains to be described. However, the disruption of lysosome function has major implications on host cell health. Lysosomes participate in several critical cellular processes beyond degradation, including gene regulation, immunity, plasma membrane repair, metabolic signalling, cell adhesion, and migration (30). Furthermore, disruption of lysosome function is implicated in the pathogenesis of several diseases, such as lysosomal storage diseases and cancer as well as neurodegenerative and metabolic disorders (30). Therefore, assessing the long-term impact of lysosome disruption on cellular health is critical for understanding the implications of the lysosomal egress pathway on the clinical presentation of COVID-19 patients. Additionally, it will be important to evaluate the specificity of the lysosomal egress pathway to understand if lysosomal disruption occurs on a multicellular level, or if it is cell-type specific. It has already been demonstrated that the altered lysosomal function observed in β-coronavirus infection results in a perturbation of antigen presentation (2). Moreover, significant evidence indicates that a dysregulated immune response contributes to the clinical presentation of patients with severe COVID-19 infections, with lymphopenia being the most consistent laboratory abnormality in these patients (3). Therefore, an investigation of the lysosomal egress pathway is an opportunity to explain some of the clinical manifestations of COVID-19, particularly the disrupted immune response.

As the COVID-19 pandemic continues to wreak havoc on the world, investigations of the molecular details of the viral replication cycle must be prioritized. In particular, understanding the lysosomal egress pathway is an opportunity to explain the pathogenesis of COVID-19 and bring to light new therapeutic targets. The lessons learned from these investigations will not only aid in putting an end to the current pandemic but could be helpful in future β-coronavirus outbreaks.

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