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Non-structural protein 6: a hallmark of SARS-CoV-2 disease progression and its connection to lipid droplets

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SUMMARY The genome of SARS-CoV-2 has been heavily studied with the roles of many enzymatic, and structural proteins being well-defined. However, knowledge remains limited for the 16 non-structural proteins (NSPs) known to be encoded by the genome. Until recently, these NSPs were not considered to be of importance for the viral lifecycle. Recent findings have demonstrated that NSP6 is a key determinant of disease progression. It is a multifunctional protein involved in the stimulation of the formation of double-membrane vesicles (DMVs) from the endoplasmic reticulum (ER), inhibition of autophagosome expansion within the cell, and induction of pyroptosis along with additional functions. In addition, many (+) ssRNA viruses, including SARS-CoV-2, have been shown to manipulate lipid droplets (LDs), highly conserved intracellular organelles that are composed of neutral lipids, as substrates for energy and as platforms for their replication. This article will highlight the current knowledge on NSP6 encoded in the SARS-CoV-2 genome and the involvement of LDs in the SARS-CoV-2 lifecycle, focusing on 1) NSP6 and its integral role as a determinant of viral pathogenicity and 2) the relation of NSP6 with the functionality of LDs in relation to DMVs and SARS-CoV-2 pathogenesis. It is evident that NSP6 is a critical protein to target in order to disrupt the viral life cycle. Thus, understanding the molecular details of the NSPs and their roles in the viral pathway presents an opportunity to identify potential therapeutic targets. Therefore, further investigation into the involvement of NSPs in disease progression is essential for furthering our understanding of the SARS-CoV-2 infection cycle.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped, non-segmented, single-stranded positive-sense RNA ((+) ssRNA) virus. The viral genome encodes two large polyproteins, ORF1a and ORF1b, four structural proteins, and nine accessory proteins (1–3). The two open reading frames, ORF1a and ORF1b, are proteolytically cleaved to form 16 non-structural proteins (NSPs) (2, 4–6) (Fig. 1). There is a ribosome frameshifting site located at the junction between ORF1a and ORF1b. This frameshifting mechanism is conserved in all coronaviruses (CoVs) and is required for the synthesis of viral RNA-dependent RNA polymerase (RdRp), or NSP12, and downstream NSPs (7). The NSPs are crucial to viral RNA replication and immune evasion (1). The enzymatic activities and functional domains of NSPs are expected to be conserved between the various CoVs suggesting their significance in viral replication. However, there is a lack of primary sequence homology between the NSPs of different CoVs making them difficult to study; therefore, it is important to focus on their functional similarities to comprehend their evolutionary association and the adaption of CoVs to specific host species (6).

NSPs play pivotal roles in infection and pathogenesis. In particular, NSP6 is a key determinant of disease progression (8). The binding of NSP6 to NSP3 and NSP4 promotes the formation of DMVs in infected cells, giving rise to replication organelles (RO) where viral replication takes place (5, 9). Furthermore, NSP6 confers a protective role over viral production by blocking the development of endoplasmic reticulum (ER)-induced autophagosome/autolysosome vesicles (4, 5).

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In addition to NSP6, lipid droplets (LDs) are critical to the SARS-CoV-2 viral lifecycle. LDs are highly conserved intracellular organelles that are composed of neutral lipids such as triacylglycerols, cholesteryl esters, fatty acids, and a surrounding phospholipid monolayer (10, 11). LDs have been found in the cytosol, nucleus, and mitochondria with cytosolic LDs making up a large proportion. The commonly attributed function of LDs is to store fat as an energy source however they are implicated in many important processes such as lipid homeostasis, protein storage, immune response, and the production of proinflammatory molecules (10–13). Despite their involvement in key processes, when in excess, the LDs can be toxic for cells and the accumulation of cholesterol and free fatty acids can disturb organelle functions or trigger apoptotic cascades if the stress is sustained (14). Many (+) ssRNA viruses, including SARS-CoV-2, have been shown to manipulate LDs as substrates for energy and as platforms for their replication (13, 15).

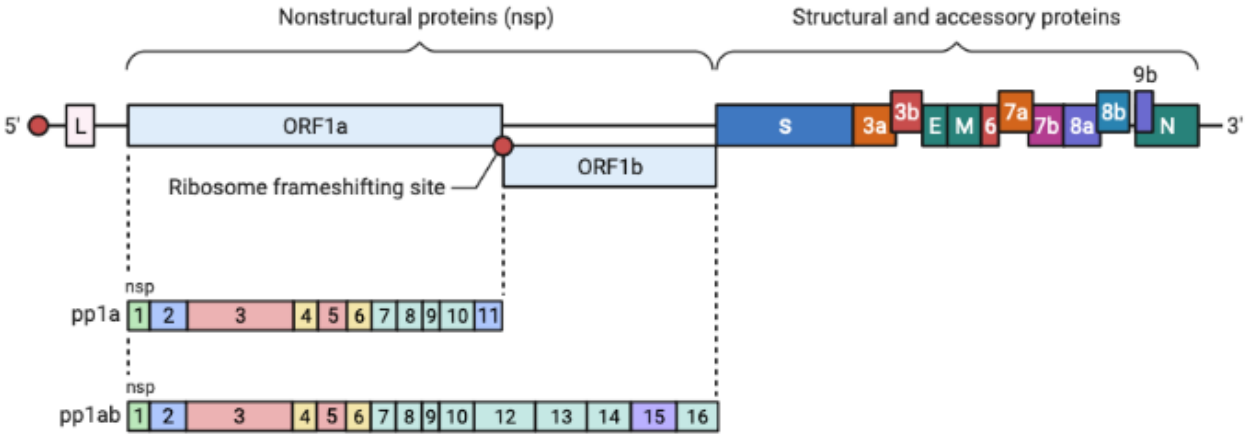

FIG. 1 Genomic organization of SARS-CoV. The RNA genome encodes NSPs and structural and accessory proteins. ORF1a and ORF1b together encode all 16 NSPs. The structural proteins include the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. A ribosome frameshifting site is highlighted at the junction between ORF1a and ORF1b and is required for the synthesis of RdRp and downstream NSPs. Created with BioRender.com.

PROPOSED RESEARCH QUESTIONS

The discovery of NSP6 and its many roles have been crucial to understanding the SARS-CoV-2 replication cycle. NSP6 is implicated in many critical steps of viral replication including the biogenesis of double membrane vesicles (DMVs) and is a key determinant of viral pathogenicity (8, 16). Additionally, SARS-CoV-2 has been reported to lead to increased LD accumulation in host cells to support its replication (15, 17). However, many questions regarding the underlying mechanisms and molecular details behind the NSPs and LDs remain unanswered. This knowledge is necessary to gain a more comprehensive understanding of the viral lifecycle in order to identify potential therapeutic avenues to combat the global pandemic. This article will build upon this knowledge gap by firstly, examining the role of NSP6 in viral pathogenicity and disease progression and secondly, examining NSP6 in the context of LDs and DMVs.

PROPOSED PROJECT NARRATIVE

What is the role of NSP6 in viral pathogenicity and disease progression?

NSP6 is a multifunctional protein involved in the stimulation of the formation of DMVs from the ER, inhibition of autophagosome expansion within the cell, and induction of pyroptosis along with additional functions (4, 16).

SARS-CoV-2, like other CoVs, builds a membrane-bound RO to enable RNA replication. The SARS-CoV-2 RO is composed of DMVs that are tethered to the ER by thin membrane connectors. Together with NSP3 and NSP4, NSP6 is involved in the assembly of the ROs by stimulating the rearrangement of host cell membranes and inducing the formation of DMVs (4, 18). These DMVs are generated by NSP3 and NSP4 whereas NSP6 zippers the ER

membranes and establishes the connection between the DMVs and the ER in order to facilitate essential communication between the structures (4, 9).

Furthermore, NSP6 also activates NLRP3-dependent cytokine production and induces pyroptosis. The induction of pyroptosis is done through the direct interaction of NSP6 with an accessory subunit of a v-ATPase which leads to the inhibition of lysosome acidification and causes stagnation of autophagic flux (16). Autophagy is a lysosome dependent degradation process that is an important host defense mechanism to counteract viral infection, which not only selects viral components for lysis, but also facilitates antigen processing and adaptive immune responses (19). In addition, lysosomes are essential for removing protein aggregates, damaged organelles, and internalized pathogens (20). Therefore, normal lysosomal acidification is of utmost importance for its degradative function. This inhibition of the lysosome-autophagy system triggers NLRP3 inflammasome activation and leads to the activation of NLRP3-dependent caspase-1 and the maturation of IL-1ß and IL-18. These events together ultimately result in the induction of pyroptosis (16).

It is evident that with its ER zippering function and ability to induce inflammatory cell death, NSP6 is a key determinant of SARS-CoV-2 pathogenicity and disease progression.

What is the connection of NSP6 to the functionality of lipid droplets and their relation to DMVs?

In order to understand the relation between NSP6 and LDs, the main roles of NSP6 in DMV formation must first be examined. To begin with, NSP6 acts as a filter in RO-ER communication allowing lipid flow but restricting access of ER luminal proteins to the DMVs; this is accomplished through the formation of a zippered double-membrane compartment that maintains full membrane continuity but restricts luminal continuity with the ER (9, 21). In addition to linking the DMVs to the ER, the connectors mediate the association of ROs with LDs. At any given time 40% of ROs are associated with LDs. The contact of the DMVs with the LDs mediated by the LD-tethering complex is perfect to deliver lipids synthesized in the ER to the DMVs but exclude “undesired” ER proteins. It has been observed, that when LD biogenesis is inhibited, the viral load decreases significantly, pro-inflammatory mediator production is reduced, and cell death is prevented indicating that SARS-CoV-2 replication is dependent on LDs (9, 15). This indicates that the LDs may have a potential role in supplying fatty acids to fuel DMV growth.

Furthermore. NSP6 positions and organizes DMV clusters. In the absence of NSP6, short and tubular connections were observed between the DMVs and ER along with the presence of a clearly detectable lumen. On the other hand, in the presence of NSP6, DMV clusters were connected with the ER through longer sheet-like zippered domains. The number of DMVs per connection also varied in the presence of NSP6 with an average of about 3 DMVs per tubular connection without NSP6 and of about 15 DMVs per zippered connection with NSP6. It was also observed that the DMVs had a more regular shape and appeared more uniform in size and were packed more densely in each cluster when NSP6 was present (9). Thus, the presence of NSP6 is necessary for the DMV clusters to establish a good connection with the ER which will be used for lipid delivery and ultimately impacts viral replication.

In addition, a study conducted by Nardacci et al. analyzed the presence of LDs in SARS-CoV-2. They observed that infected cells displayed a significant increase in the number of cells with LDs in their cytoplasm with time of infection, from 24 to 48 hours post-infection (22). The LDs were often found in contact with mitochondria; this proximity is necessary for ATP production via ß-oxidation (22, 23). Another study by Baek et al, observed similar results along with the disappearance of LDs in the late stage of viral infection as a result of LD lipolysis. They concluded that the decline of LDs was causally correlated with intracellular lipolysis via LD-associated lipases through activation of cyclic AMP/protein kinase A signaling pathways. Their results demonstrate that lipase-mediated intracellular LD lipolysis is often exploited by RNA viruses to facilitate their replication (17). The combined results of these studies suggest that LDs accumulate during the early stages of viral infection and are used as an energy source during the late stage of viral infection.

SARS-CoV-2 appears to favour a lipid-rich environment with NSP6 facilitating the selective process of obtaining the LD-derived lipids. LDs are key organelles in the viral replication cycle and therefore, presents a potential strategy to interfere with viral replication

by blocking lipid biogenesis or lipolysis. However, greater research into the use of pharmacological inhibitors of LD-associated lipases is necessary to inform the use of such interventions against SARS-CoV-2.

POTENTIAL IMPACT/CONCLUSIONS

The genome of SARS-CoV-2 encodes 16 NSPs which have important functions in viral replication (1). Specifically, NSP6 is a multifunctional protein implicated in multiple steps of the viral replication cycle including the formation of DMVs, inhibition of autophagasome expansion, and induction of pyroptosis (4, 16). Additionally, SARS-CoV-2 has been shown to manipulate LDs as substrates for energy and thus LDs are critical to the viral lifecycle (17, 22). Moreover, it is NSP6 that establishes the connection between the DMVs and the ER and allows for the selective transfer of LD-derived lipids (4, 9).

Understanding the molecular details of the NSPs, their roles, and the interactions of the NSPs with each other as well as with other cellular components in the viral pathway presents an opportunity to gain insight into how specific functions are modulated and provide crucial insight into the SARS-CoV-2 lifecycle. In addition, several studies have demonstrated that targeting host lipid metabolism by statins, suppresses the viral replication of many (+) RNA viruses such as hepatitis C, dengueviruses, and influenza A; therefore, interfering with key lipid metabolic pathway enzymes could represent a therapeutic perspective (22, 24).

Despite the advances in understanding the role of NSPs and LDs in SARS-CoV-2 pathogenesis, there remains a vast amount of knowledge to be gained. Elucidating the structures and specific functions of each of the NSPs will provide crucial insight into the viral lifecycle. Furthermore, current research focuses on cytosolic LDs but a future path of research should draw attention to the potential functions associated with nuclear LDs and their association with the SARS-CoV-2 lifecycle. A potential LD buildup in the nucleus could impact transcriptional regulation of genes and therefore be implicated in pathogenesis. Additionally, lipid modulating drugs can be used to inhibit cytosolic LD biogenesis, however it is unclear whether nuclear LDs would be impacted and thus warrants further investigation. Further research in these key areas is critical to gain a deeper understanding of the SARS-CoV-2 lifecycle and its mechanisms of infection that are essential in developing effective therapeutics to combat the global pandemic.

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