Wrapping up : nidovirus membrane structures and innate immunity

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Chapter 7

General discussion

DMV FORMATION OF ARTERIVIRUSES AND CORONAVIRUSES

The studies described in this thesis provide new insights regarding the biogenesis of nidovirus replication organelles (**Chapters 3-4**) as well as the cellular response to these unique membrane structures (**Chapter 6**). In **Chapter 3**, using EM and ET techniques, the formation of arterivirus ROs was investigated, both during EAV infection and in cell lines that express membrane-spanning viral nsps. Importantly, the DMVs formed after expression of EAV nsp2 and nsp3 (nsp2-3) share the same 3D membrane architecture as those formed during EAV infection. Moreover, co-expression of the EAV transmembrane protein nsp5 with nsp2-3 altered the size of the induced DMVs, but not their overall morphology, suggesting a regulatory rather than a "structural" role for nsp5 in DMV biogenesis. Also for the betacoronaviruses MERS-CoV and SARS-CoV, the expression of just two non-structural proteins (nsp3 and nsp4) suffices to induce the formation of DMVs (**Chapter 4**), whereas co-expressing the third transmembrane nsp (nsp6) did not seem to alter DMV morphology.

Using EM and ET, numerous membrane structures that provide novel insights into arterivirus and coronavirus DMV biogenesis were observed. In cells expressing EAV nsp2-3 or MERS-CoV nsp3-4, as well as in EAV-infected cells, we observed the accumulation of extensive double-membrane sheets, which we interpret as an intermediate stage of DMV biogenesis via an enwrapping mechanism (**Chapter 2**, Fig. 5). Double-membrane sheets were also found to accumulate when EAV nsp2-3 expressing cells were treated with IFN-β, which reduced the number of DMV positive cell profiles by about 27 percent. Assuming that IFN-β treatment somehow stalls or slows down DMV formation, this could be taken as additional support for double-membrane sheets being an intermediate stage in DMV biogenesis (**Chapter 6**). It should be noted though that both proposed mechanistic models for DMV formation, enwrapping and double-budding, would require the same fundamental membrane remodeling steps, albeit in a different order, and that in EAV-infected cells we also observed some structures that could be intermediates compatible with the double-budding model. The frequent observation of double-membrane sheets in different experimental setups suggests that enwrapping is a key mechanism for DMV biogenesis. Thus far, infected cells have been studied using ET for three other DMV-forming +RNA viruses, two picornaviruses (poliovirus and coxsackievirus B3 (CVB3)) and hepatitis C virus (HCV) (23, 56, 65). In the case of picornaviruses DMVs are formed later in infection and they are thought to arise by enwrapping of single-membrane tubules that are induced earlier during the infection, at the peak of RNA replication (56, 65). How the DMVs in HCV-infected cells are formed is currently unclear. One important distinction between nidovirus DMVs and those of picornaviruses and HCV is that in the case of those latter viruses the DMV interior occasionally was found to be connected to the cytosol. Although enwrapping may be a general mechanism for DMV biogenesis, the observed differences in terms of cytosolic connections could reflect fundamental differences that exist between different virus groups.

The findings in **Chapters 3 and 4** revealed striking similarities between DMV formation in cells infected with arteriviruses and coronaviruses. The nsps required for DMV

formation, arterivirus nsp2-3 and coronavirus nsp3-4, respectively, share a remarkable number of properties, despite considerable size differences (800-1400 amino acids (aa) in arteriviruses versus 2100-2500 aa in coronaviruses). First, their position in the viral replicase polyprotein, just upstream of the main protease, is comparable (see also **Chapter 2**, Fig. 1) (78, 344). The (predicted) membrane topology of arterivirus nsp2-3 and coronavirus nsp3-4 is similar as well. For example, arterivirus nsp2 and coronavirus nsp3 are mainly cytosolic. Furthermore, both arterivirus nsp3 and coronavirus nsp4 span the membrane four times, with the first transmembrane helix being adjacent to the N-terminal cleavage site and both proteins having two luminal domains, the N-terminal of which is the largest. Apart from their relative position in the polyprotein and membrane topology, they share some functional characteristics as well. Both arterivirus nsp2 and coronavirus nsp3 contain a papain-like protease (PLP) domain that processes the polyprotein junction at their C-terminus. Interestingly, although they belong to different protease classes (165, 190, 345, 346), both arterivirus PLP2 and coronavirus PLpro can also function as deubiquitinating enzyme (DUB) in order to inhibit type I IFN production, presumably by cleaving ubiquitin chains from cellular proteins, such as innate immune factors (165, 313, 347-350).

As described in **Chapters 3 and 4** of this thesis, nsp2-3 of EAV and nsp3-4 of both MERS-CoV and SARS-CoV also share the ability to induce DMV formation, adding another functional similarity between these distantly related proteins. Whether the roles of the third transmembrane protein in each virus (arterivirus nsp5 and coronavirus nsp6, respectively) are similar as well is still unclear. Findings in **Chapter 3** show that co-expression of EAV nsp5 (when present in a nsp5-7 precursor, as in infected cells (84)) can reduce DMV size. Although a role for arterivirus nsp6 and/or nsp7 in DMV formation cannot be formally excluded, the very hydrophobic nature of nsp5 suggests that it is this subunit that is responsible for the observed effects. MERS-CoV nsp6 did not affect DMV formation or size when it was co-expressed with nsp3-4 as an individual subunit. However, when MERS-CoV polyproteins encompassing the nsp3-6 region were expressed, polyprotein processing was found to be severely affected. In those nsp3-6 polyproteins, cleavage of the nsp3/4 junction remained intact, but the nsp4/5 and nsp5/6 cleavages were impaired. DMV formation was also impaired in those experiments, with cells showing the formation of elaborate cubic membranes (**Chapter 4**, Fig. 4), which can form for several reasons, including protein misfolding and/or overexpression of ER proteins leading to aggregates (193). Given the large impact of blocking the cleavage of the nsp3/4 junction on DMV formation (**Chapter 4**, Fig. 5), it is conceivable that impairment of the nsp4/5 cleavage could be equally sufficient to prevent DMV formation, and trigger cubic membrane formation instead. Expression of nsp3-5 polyproteins in which either M^{pro} in nsp5 is inactivated or the nsp4/5 junction is mutated may provide an answer to the question what triggers the formation of cubic membranes instead of DMVs. It is currently still unclear whether in infected cells MERS-CoV nsp6 is (mainly) present as a fully processed nsp or in the form of long-lived polyprotein fragments. In case of the latter, those processing intermediates could play a role in the formation of DMVs or the other types of membranous structures observed during coronavirus infection. So far, nsp6 has only been detected in infected cell lysates (by western blotting) for the

betacoronavirus mouse hepatitis virus (MHV) (184). In that case, nsp6 was observed as two distinct protein bands (23 and 27 kDa), suggesting either a post-translational modification, or perhaps the existence of a long-lived nsp6-7 precursor, given the relatively small size of nsp7. Moreover, also an nsp4-10 precursor was readily detected for MHV (184). Determining which membrane structures contain coronavirus nsp6, *e.g.* by immuno EM, should provide more insight and may help to clarify whether nsp6 plays a role in coronavirus RO formation.

Expression of multiple proteins as a large polyprotein is a very common strategy among +RNA viruses. Proteolytic processing of these precursors into smaller fragments is often tightly coordinated and can be directly related to subunit functionality, as was evident from interfering with the cleavage of the nsp3/4 junction in the case of MERS-CoV (**Chapter 4**). Other examples include the role of polyprotein cleavage in the temporal regulation of alphavirus replication (351, 352), and the decreased efficiency of DMV formation by HCV proteins after acceleration of the cleavage at the NS4B/NS5A junction (39). In the latter case, a clear relationship was observed between membrane structure formation and the degree of polyprotein cleavage. A similar functional requirement for cleavage(s) may also explain the existence of two distinct polyprotein processing pathways, as they are observed both in EAV-infected cells and pp1a expression systems (84). These processing pathways mainly differ with respect to the cleavage of either the N-terminal or the C-terminal junction of nsp5. The latter subunit may thus act as a membrane anchor for different adjacent nsps, a feature that may also play a role in RO biogenesis.

INNATE IMMUNE RESPONSES TARGETING DMVS

Whether cells have the means to counteract the hijacking of cellular membranes by +RNA viruses was explored for EAV in **Chapter 6**. Treatment of cells expressing EAV nsp2-3 with IFN-β resulted in a decrease of the fraction of cells showing double-membrane structures, leading to the general conclusion that the type I IFN response can indeed impair the formation of arterivirus ROs. Moreover, large numbers of double-membrane sheets accumulated in IFN-β-treated cells, adding to the notion that these may be intermediates of DMV biogenesis. Interestingly, for HCV at least one ISG has already been shown to affect DMV formation (326). This concerns CH25H, a host factor that could however not be linked to EAV DMV biogenesis (**Chapter 6**) suggesting the existence of multiple mechanisms by which the innate immune response targets the formation of ROs by different viruses. Also two other ISGs (PLSCR1 and viperin) that exert their antiviral effects through affecting lipid metabolism were found not to be involved in impairing EAV DMV formation (**Chapter 6**). **Chapter 6** briefly discusses possible future approaches to unravel the mechanism underlying the inhibition of DMV formation by IFN-β treatment, including a hypothesis pertaining to LC3-mediated tagging of aberrant intracellular membrane structures and the effector function of immunity-related GTPases (336, 337). Below I will outline two other hypotheses by which the innate immune response could inhibit RO formation and suggest approaches for future studies.

Could the membranes of viral ROs function as a PAMP?

The host cell's effector mechanism that inhibits DMV formation likely depends on recognition of a pathogen-associated molecular pattern (PAMP). One possibility would be that the innate immune system targets the tightly apposed membranes that are so typical of this class of viral RO structures. Another attractive option is that this pattern finds its basis in the lipid composition of the RO membranes. For example, for several +RNA viruses the phosphatidylinositol 4-kinases have been identified as important host factors in their replication (42-44), which are thought to drive the accumulation of phosphatidylinositol 4-phosphate (PI4P) lipids at sites of RO formation. Detection of increased local levels of PI4P lipids by the innate immune system could thus activate a cellular effector mechanism. Such 'membrane-associated' PAMPs could be what is recognized and/or targeted by the innate immune system to impair DMV formation. The observation that DMV formation by expression of nsp2-3 did not induce the production of IFN-β (**Chapter 6**) suggests that EAV nsp2-3 induced DMVs do not function as such PAMPs. However, it could very well be that this type of membrane-associated PAMP is only involved in effector mechanisms of the innate immune response, and not during the initial sensing of infection, as those molecular patterns also play important roles in normal cellular metabolism. For example, the formation of double membranes is a central part of the autophagy pathway (353) and PI4P lipids play a key role in several cellular signaling events (354). Recognition of such elements by the innate immune response during normal cell homeostasis would likely be detrimental for the cell in the absence of viral infection, but during an antiviral state induced by type I interferon it might be beneficial to antagonize such mechanisms to inhibit virus replication.

An innate immune effector mechanism targeting such membrane-associated PAMPs could either be activated to inhibit other players in virus replication (e.g. viral proteins or RNA), or target the membranes directly. The antiviral state induced by the innate immune response not only targets virus replication directly, but also greatly alters cellular metabolism to limit virus replication, which in turn could influence the integrity of viral ROs (355). A lipidomics approach using membrane fractions of cells expressing EAV nsp2-3 (or EAV-infected cells) with or without IFN-β treatment, could provide insight into changes in the lipid composition of RO membranes. Such changes could be the result of an innate effector mechanism directly targeting the RO membrane. Chemical compounds or inactivation of genes involved in lipid metabolism could then be used in attempts to mimic the effect of IFN-β treatment on specific parts of lipid metabolism, in order to determine whether such changes indeed play a role in antagonizing DMV formation.

Does ubiquitin play a role in the innate immune response targeted at ROs?

Interestingly, the PLP2-DUB protease in EAV nsp2 has been shown to have immune evasive activity (338). When EAV PLP2 was mutated in the virus in a way that prevented ubiquitin binding without affecting nsp2-3 cleavage (which is essential for infection), this resulted in increased IFN-β production in infected primary cells (165). The decreased in number of cell sections displaying double-membrane structures and the morphological changes

occurring after IFN-β treatment were, although consistently observed, rather modest (**Chapter 6**; Figs 4, 6). This could be interpreted as only limited support for the inhibitory effect of IFN signaling on RO formation in the infected cell or organism. It is however important to realize that viruses in general exhibit multiple activities that counteract or evade the antiviral responses of the cell.

The presence of a DUB in arterivirus nsp2 (and coronavirus nsp3) could also help to evade innate immune effector responses that target membrane structure formation if these would rely on post-translational modifications involving ubiquitin or ubiquitin-like modifiers. The cell could inhibit DMV formation by flagging relevant viral proteins involved with specific ubiquitin chain types (or ubiquitin-like modifiers), to either degrade them or inhibit their function. Such tagging could also result in the degradation of induced membrane structures containing these flagged proteins. This could resemble the use of ubiquitin as a flag in the degradation of intracellular bacteria by selective autophagy (139), or the labeling of aberrant membrane structures with LC3 to degrade them via immunity related GTPases (discussed in **Chapter 6**) (336, 337). On the other hand, the double-membrane sheets that are induced by EAV nsp2-3 expression after IFN-β treatment suggest that a block in DMV biogenesis is a more likely explanation for this phenomenon than the enhanced degradation of already formed structures.

The DUB function of EAV PLP2 might obviously counteract mechanisms as outlined in the previous paragraph, although apparently not in full as IFN-β treatment still interfered with the DMV formation induced by expression of EAV nsp2-3 containing an active PLP2-DUB (**Chapter 6**). To further test the role of the DUB activity in the context of the innate immune response against DMV formation, several cell lines inducibly expressing nsp2-3GFP carrying a DUB knock-out mutation (T312A/I313V/I353R) in PLP2 were created (165), in order to evaluate the effect of IFN-β treatment on DMV formation compared to the wild-type nsp2-3 construct (**Chapter 6**). If ubiquitin-based signaling would be involved and EAV PLP2 can no longer remove ubiquitin chains from nsp2-3, the observed effects of IFN-β treatment on DMV formation should become much more severe. In each of those cell lines nsp2-3GFP-DUB k/o expression levels decreased after IFN-β treatment, unlike what was observed in cell expressing the wild-type nsp2-3 (Fig. 1). This may have resulted from a decrease in transcription and/or translation of nsp2-3GFP mRNA; however, as the decrease was observed only upon expression of the DUB k/o construct, this data suggests that nsp2-3 was indeed directly targeted for ubiquitin-dependent degradation after IFN-β treatment. Due to the DUB function of nsp2 this was likely counteracted when expressing wild-type nsp2-3, resulting in unchanged nsp2-3 protein levels after IFN-β treatment.

The effect on nsp2-3 levels of mutations that knock out the PLP2-DUB function renders nsp2-3 DUB knock-out cell lines unsuitable for the kind of EM analysis used in **Chapter 6**. Any direct effect of IFN-β treatment on membrane structures, as observed in the wild-type nsp2-3-expressing cells, would be impossible to separate from the reduced or impaired formation of membrane structures that could simply result from the lower nsp2-3 expression level in DUB knock-out nsp2-3-producing cells treated with IFN-β. Determining the reason for this decrease could provide further insight into the innate immune response

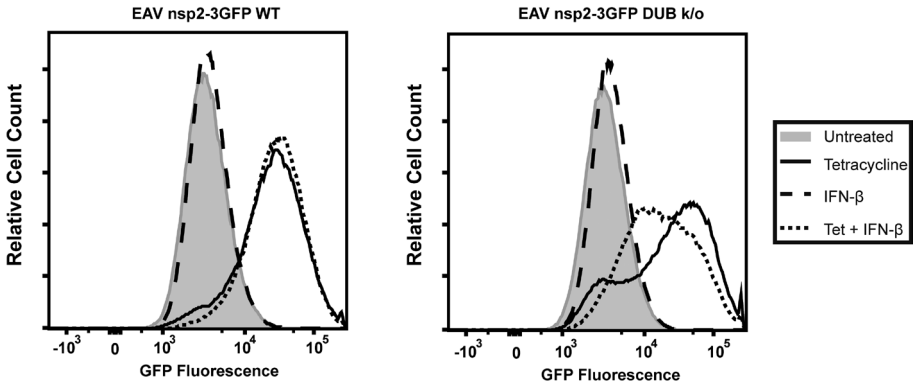

Fig. 1. EAV nsp2-3GFP DUB k/o protein levels decrease after IFN-β treatment. *HuH-7/tetR/HA-nsp2-3GFP (either wildtype PLP2 or the DUB k/o (T312A/I313V/I353R) (165)) cells were analyzed by flow cytometry after 24 hours of indicated treatments. For further experimental details, see methods section of* **Chapter 6**.

targeting DMV formation. One ISG that might be involved in this effect is ISG12a, which is an adapter for the SKP2 ubiquitin ligase and was shown to target HCV NS5A (one of the trans-membrane proteins required for HCV DMV formation) for degradation by K48 linked ubiquitination (356). If EAV nsp2-3 is indeed targeted directly by ISG12a (or any other ISG), mutating the lysine(s) targeted for ubiquitination in the nsp2-3 DUB knock-out construct could help reveal ubiquitin-dependent mechanisms involved in the inhibition of DMV formation.

CONCLUDING REMARKS

+RNA virus replication organelles have been an intriguing research topic over the last decades. Significant efforts were made to characterize the architecture and biogenesis of these membrane structures, which appear to be at the hub of cytoplasmic replication of +RNA viruses. Multiple hypotheses and models have been postulated regarding the structure-function relationships and design of replication organelles. For spherule-forming viruses, such models are relatively simple. Spherules by default provide a semi-closed compartment for efficient RNA replication, presumably away from RNA sensors such as RIG-I and MDA5, and newly synthesized genomes can exit through the relatively narrow openings that connect these compartments with the cytoplasm. For some DMV-forming viruses, such as HCV and picornaviruses, openings between the interior of some of their DMVs and the cytosol have been described. This provides a basis for a similar structure-function model, although the timeline of RO morphogenesis in general, and the appearance of open and closed structures in particular, remains to be investigated in more detail. Moreover, in the case of picornaviruses, DMV formation was most pronounced only after

the exponential phase of viral RNA replication (56, 65), raising the question whether DMVs actually have a key role in RNA replication in the first place.

The closed nature of nidovirus DMVs still confounds theories easily applicable to other +RNA viruses. The interior of arterivirus and coronavirus DMVs labels strongly for double-stranded RNA (20, 21), similar to what has been observed for other +RNA viruses. Recent EM studies (M. Bárcena *et al.*, personal communication) show that RNA replication is predominantly associated with DMVs, expanding the enigma of these closed vesicles. If the interior of closed DMVs is the site of replication, then newly made genomes and mRNAs, which need to be transported to the cytosol for translation and encapsidation, would either have to be translocated across two membranes through a protein channel or be released when a DMV would open up. Alternatively, virus replication could take place on the cytosolic face of the DMV outer membrane. In that scenario, however, most of the postulated benefits of compartmentalization and shielding of RNA replication intermediates inside vesicular structures would likely be minimal. Replication could also take place on both sides of the double membrane, but in that case RNA synthesis occurring on the inside would seem pointless if DMVs would remain closed.

Despite two decades of research on nidovirus-induced ROs, it remains very difficult to assess how important the reorganization of ER membranes, specifically into DMVs, really is. In some recent studies, the number of DMVs formed during coronavirus replication did not correlate very well with the efficiency of RNA replication, viral fitness, or pathogenesis (131, 357). On the other hand, our finding that the innate immune response targets DMV formation is promising, and suggests that membrane structure formation is a relevant step during virus infection that is "worth counteracting". Moreover, the knowledge gained on the viral proteins involved in arterivirus and coronavirus DMV formation will help to identify host factors involved in RO biogenesis, which in turn may aid to unravel why nidoviruses induce the formation of such extensive and unique membrane structures in infected cells.