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Interaction of the innate immune system with positive-strand RNA virus replication organelles

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ABSTRACT

The potential health risks associated with (re-)emerging positive-strand RNA (+RNA) viruses emphasizes the need for understanding host-pathogen interactions for these viruses. The innate immune system forms the first line of defense against pathogenic organisms like these and is responsible for detecting pathogen-associated molecular patterns (PAMPs). Viral RNA is a potent inducer of antiviral innate immune signaling, provoking an antiviral state by directing expression of interferons (IFNs) and pro-inflammatory cytokines. However, +RNA viruses developed various methods to avoid detection and downstream signaling, including isolation of viral RNA replication in membranous viral replication organelles (ROs). These structures therefore play a central role in infection, and consequently, loss of RO integrity might simultaneously result in impaired viral replication and enhanced antiviral signaling. This review summarizes the first indications that the innate immune system indeed has tools to disrupt viral ROs and other non- or aberrant-self membrane structures, and may do this by marking these membranes with proteins such as microtubule-associated protein 1A/1B-light chain 3 (LC3) and ubiquitin, resulting in the recruitment of IFN-inducible GTPases. Further studies should evaluate whether this process forms a general effector mechanism in +RNA virus infection, thereby creating the opportunity for development of novel antiviral therapies.

1. Introduction

The innate immune system forms the first line of defense against pathogens and its initial function is to recognize pathogen-associated molecular patterns (PAMPs) [1], which ultimately leads to induction of the antiviral state that effectively hampers spread of the infection. The adaptive immune system then kicks in to (in most cases) fully clear the virus and build up memory. Viral RNA is a very potent inducer of innate antiviral signaling [2,3]. Therefore, detection of viral RNA and the subsequent induction of antiviral effector mechanisms play an important part in the onset of an antiviral state in the context of RNA virus infections. The study of PAMP recognition and signaling by cytosolic and membrane bound sensors has intensified tremendously in the last decade. Several comprehensive reviews on this subject have been published lately [4,5]. Additionally, investigation of the involvement of intracellular organelle membranes of mitochondria, ER, and peroxisomes and their mutual interactions indicated that these are important signaling platforms in innate immunity [6,7]. Together, this has resulted in a better understanding of the details of innate immune responses that target RNA viruses.

In this review, we will focus on the interaction of the innate immune system with viruses that have a positive-strand RNA

Abbreviations: +RNA, positive-strand RNA; 5′ppp-RNA, 5′ tri-phosphorylated RNA; DENV, Dengue virus; DMV, double membrane vesicle; EAV, equine arteritis virus; eIF, eukaryotic translation initiation factor; EndoU, endonuclease; GBP, guanylate-binding protein; HCV, Hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; Inv, invaginated vesicle/spherule; IRF, interferon regulating factor; IRG, immunity-related p47 GTPase; ISG, interferon-stimulated gene; JAK/STAT, Janus kinase-signal transducer and activator of transcription; JEV, Japanese encephalitis virus; LC3, microtubule-associated protein 1A/1B-light chain 3; MAM, mitochondrion-associated membrane; MAVS, mitochondrial antiviral signaling; MDA 5, melanoma differentiation associated factor 5; MERS-CoV, Middle East respiratory coronavirus; MHV, mouse hepatitis virus; MNV, murine norovirus; Mx, myxovirus resistance proteins; MyD88, myeloid differentiation primary response gene 88; nsp, non-structural protein; OAS, 2′,5′-oligoadenylate synthetase; PAMPs, pathogen-associated molecular patterns; PE, phosphatidylethanolamine; PKR, protein kinase R; PLP, papain-like protease; PV, pathogen-containing vacuole; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I like receptor; RNP, ribonucleoprotein; RO, replication organelle; SARS-CoV, severe acute respiratory coronavirus; SG, stress granule; STING, stimulator of interferon genes; *T. gondii*, *Toxoplasma gondii*; TAG, targeting by AutophaGy proteins; TIR, Toll/interleukin-1 receptor; TLR, toll-like receptor; TRIF, TIR-domain- containing adapter-inducing interferon-β; VLIGs, very large IFN-inducible GTPases; WNV, West Nile virus.

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(+RNA) genome. In response to innate immune reactions, virtually all +RNA viruses interfere to delay antiviral innate immune signaling in several ways [1,8,9]. A key feature of immune evasion by +RNA viruses, and simultaneously the hallmark of +RNA virus infection, is rearrangement of host membranes into viral replication organelles (ROs). As these structures are thought to shield viral RNA from the host innate immune system and additionally seem to play a fundamental role in viral RNA replication, ROs in this sense seem to have a central and dual function in viral replication [10–14]. Therefore, disrupting integrity of ROs might simultaneously result in impaired viral replication and enhanced antiviral immune signaling, which would be a beneficial effect from an antiviral immunity point-of-view. However, whether host cells possess effector mechanisms to disrupt +RNA virus ROs has hardly been investigated yet, and only quite recently some studies have shed more light on this, which will be the focus of this review together with the body of literature that surrounds it.

2. Positive-strand RNA viruses: societal impact, taxonomy, and virus-induced replication organelles

Positive-strand RNA viruses have caused multiple outbreaks during the past decade: severe acute respiratory coronavirus (SARS-CoV) infected more than 8000 individuals in 2003 of which almost 800 died [15]. Its close relative Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in Saudi Arabia in 2012. While SARS-CoV infections have not been reported in humans after 2004, MERS-CoV currently still regularly occurs in dromedary camels as well as in humans, and the virus displays a lethality rate of around 35% in the latter [16]. Zika virus, which received a lot of societal attention lately, is an example of a re-emerged +RNA virus with significant impact [17]. These outbreaks, and the lack of tailored antiviral strategies against them, clearly illustrate the potential health risks associated with (re-)emerging +RNA viruses, and emphasize the need for a precise understanding of host-pathogen interactions to facilitate development of novel antiviral therapies or vaccination methods. Over the last decades, many +RNA viruses have been extensively studied for their intriguing biology, including coronaviruses as those mentioned above, picornaviruses such as coxsackievirus and poliovirus, and flaviviruses such as West Nile virus (WNV), Hepatitis C virus (HCV), dengue virus (DENV), Zika virus, and Japanese encephalitis virus (JEV), as well as the family of togaviridae, including rubella virus and chikungunya virus.

One of the intriguing features that is shared by all +RNA viruses is the rearrangement of host membranes into viral ROs, whereby particular cellular organelles (depending on the virus) are used as membrane donors. Concerning their role in viral RNA replication, it is thought that the structures form a scaffold that improves efficiency of enzymatic reactions and provides a spatiotemporal regulation of the different stages in the virus life cycle. Besides this, it is thought that ROs have an important role in shielding viral RNA products from the RNA sensors of the innate immune system. ROs are therefore believed to have a dual role in +RNA virus infection and innate immune evasion, which will be elaborated on further in this review. Several comprehensive reviews have recently described the current knowledge of these structures [12,18–20], therefore we will only briefly summarize this below. Most studies until now focused on the architecture of structures induced by different viruses, and based on electron microscopy and electron tomography, ROs were categorized into two different classes: the invaginated vesicle/spherule (Inv) type, and the double membrane vesicle (DMV) type (Fig. 1).

Inv ROs are predominantly observed during infection with alphaviruses, such as Semliki Forest virus and Sindbis virus, nodaviridae such as Flock House virus, bromoviridae such as bromovirus, and flaviviridae such as rubella virus, DENV, and WNV [19,21]. Although the location and dynamics of the formation of Inv ROs varies per virus, the result is a single membrane invagination of which the content is connected to the cytosol. In addition, replicase proteins and newly synthesized viral RNA were observed in these spherules, strongly suggesting that Inv ROs are sites of viral RNA replication [22–24]. Furthermore, ribosomes are found in close proximity of some Inv ROs [25], suggesting that viral RNA replication and translation are spatially separated. A similar organization of Inv ROs was found for Flock House virus, rubella virus, DENV, and WNV. Moreover, a recent study visualized virus budding in close association with Inv ROs, suggesting a direct role for these structures in coordinating virus assembly [26].

Alternatively, DMV ROs are known to be formed by enteroviruses such as coxsackievirus B3 and poliovirus, coronaviruses such as SARS-CoV and MERS-CoV, arteriviruses such as equine arteritis virus (EAV), and the flavivirus HCV [19,27]. Besides DMVs, for most of these viruses other kinds of structures are found during infection, mostly in close proximity to the DMVs themselves, such as single membrane vesicles, multi-membrane vesicles, vesicle packets, tubular structures or zippered ER membrane. As for Inv ROs, replicases and viral RNA of poliovirus, EAV, and coxsackievirus B3 were demonstrated to localize to DMVs, suggesting that these structures might also support viral RNA replication. Likewise, for HCV replicase proteins and viral RNA were predominantly found in DMVs, and the number of DMVs positively correlated to the amount of viral RNA produced, suggesting that DMVs indeed serve as sites of RNA replication. However, for coronaviruses, the number

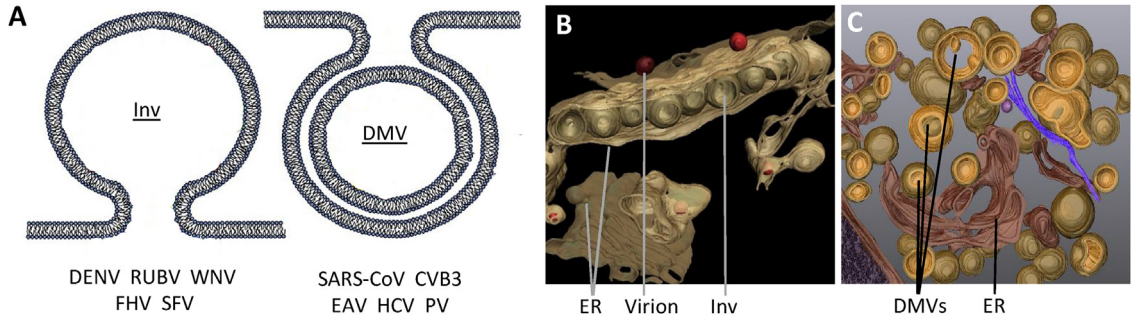

Fig. 1. Viral replication organelles. (A) Schematic representation of viral replication organelles (ROs), modified from [18]. Membranous structures that form ROs can be categorized as the invaginated vesicle/spherule (Inv) type (left), and the double membrane vesicle (DMV) type (right). Inv ROs are predominantly observed during infection with alphaviruses, such as Semliki Forest virus (SFV), nodaviridae such as flock house virus (FHV), and flaviviridae such as rubella virus (RUBV), dengue virus (DENV), and West Nile virus (WNV). Alternatively, double membrane vesicle (DMV) type ROs are formed by enteroviruses coxsackievirus B3 (CVB3) and poliovirus (PV), coronaviruses severe acute respiratory coronavirus (SARS-CoV) and equine arteritis virus (EAV), and the flavivirus hepatitis C virus (HCV). (B) 3D reconstruction of Inv ROs upon DENV infection, modified from [21] (C) 3D reconstruction of DMV ROs upon HCV infection modified from [27].

of DMVs is not necessarily proportional to replication capacity [28,29]. Also, whereas SARS-CoV dsRNA (a replication intermediate) was found inside DMVs, replicase proteins were more abundant in surrounding convoluted membranes, raising questions regarding the spatial organization of SARS-CoV RNA replication. The lack of clear connections between the inside of corona- and arterivirus-induced DMVs and the cytosol (where the RNA products have to go for translation and particle formation) further complicates our current understanding of coronavirus DMV functionality [30,31]. Together, these data continue to cause debate on the exact function of DMVs and other features of DMV ROs. The notion that expression of combinations of non-structural viral proteins (nsps) for some of these viruses mimics the formation of membrane alterations as observed during infection confirms the viral induction of these structures and opens possibilities for detailed study of this particular feature of the infection [32–34].

3. Innate immune recognition and responses targeted towards viral RNA replication, transcription, and translation

3.1. Innate immune detection of viral RNA establishes an antiviral state

Rapid production of interferons (IFNs) and pro-inflammatory cytokines is an important consequence of virus detection, as it contributes to an antiviral state in both the infected host cell and the (un)infected surrounding cells. In addition, IFNs play an essential role in coordinating the antiviral adaptive immune response, which has been reviewed elsewhere [35]. Three types of IFNs have been described. Type I IFNs consist of 13 subtypes of IFN-α and a single subtype of IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ and IFN-ω. Type II IFN only contains one subtype of IFN-γ, and type III IFNs comprise of IFN-λ1 through −λ4. Whereas it is known that most cell types produce type I IFNs in response to viral infection, type II IFNs are only produced after antigenic stimulation of an expanding group of certain immune cells, including T-cells, natural killer cells, dendritic cells, and macrophages [36,37]. In contrast, little is known about type III IFN production *in vitro* and *in vivo*, although it is believed that most cell types that produce type I IFNs are capable of producing type III IFNs as well [38]. Most cells are able to respond to IFN-I and −II, whereas IFN-III receptors are mainly found on epithelial cells [39]. The protective role of IFNs during viral infection is for example illustrated by inhibitory effects of IFN-α, IFN-β, and IFN-γ on SARS-CoV replication *in vitro* and *in vivo* [40–43]. Importantly, correct timing and amount of IFN and subsequent pro-inflammatory cytokine expression is essential for an effective antiviral immune response, as delayed and/or elevated induction may stimulate immunopathological outcomes [35].

Below we will focus on the interactions of the innate immune system with the RNA replication stages of +RNA virus infection, including exposure of viral RNA to the cytosol after the unpacking of virus particles, and formation of ROs by the virus. We will also discuss examples of viral evasion surrounding these processes. IFN-I and –III production is triggered after host cells detect viral RNA, primarily using cytosolic RIG-I like Receptors (RLRs) and membrane-bound Toll-like receptors (TLRs). RLRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated factor 5 (MDA5) have been studied extensively (reviewed in [44,45]). Besides their role in RNA metabolism, RIG-I and MDA5 recognize viral RNA by binding phosphorylated 5′ termini (5′ppp-RNA) in combination with dsRNA or ssRNA motifs. Detection of viral RNA activates RLRs and triggers downstream signaling through the mitochondrial antiviral signaling (MAVS) adaptor, which is localized on the outer mitochondrial membrane [46–49]. The importance of MAVS is underscored by the observation that silencing of this protein by RNA interference abolishes expression of type I IFNs [46]. Subsequently, MAVS recruits various adaptor molecules such as Stimulator of interferon genes (STING) and TNF receptor-associated factors, resulting in the formation of large signaling complexes [50]. Ultimately, this leads to activation of kinase complexes IKKε/TBK1 and IKKα/IKKβ/IKKγ, resulting in activation of interferon regulating factor 3 (IRF3), 7 (IRF7) and NF-κB. These transcription factors then translocate to the nucleus and initiate the expression of IFNs and pro-inflammatory cytokines.

Besides the role of RLRs, other RNA sensing molecules have been shown to participate in antiviral responses. For example, of the 10 types of TLRs described in humans, endosomal TLRs 3, 7, and 8 are known to have a role in viral RNA detection. A detailed overview on the role of TLRs in antiviral signaling can be found elsewhere [51]. Briefly, TLRs interact with various adaptor molecules after stimulation, and the combination of recruited adaptors influences downstream signaling events. TLR3 eventually recruits common adaptor molecule TIR-domain-containing adapter-inducing interferon-β (TRIF), whereas TLRs 7 and 8 engage myeloid differentiation primary response gene 88 (MyD88). As in RLR signaling, this leads to activation of kinase complexes IKKε/TBK1 and IKKα/IKKβ/IKKγ. Furthermore, Protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (OAS) have been demonstrated to induce antiviral activities upon binding of dsRNA, as is described in detail elsewhere [45]. Well-established examples of antiviral activity provoked by PKR include inhibition of translation and inflammasome activation, and OAS activates RNase L to initiate degradation of host and viral RNA. In addition, PKR and OAS have been suggested to amplify RLR-mediated antiviral immune signaling [45].

The production and release of IFNs and pro-inflammatory cytokines contributes to an antiviral state in both infected host cells and in (un)infected surrounding cells. Despite the presence of multiple IFN and receptor types [52], the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway is utilized by all IFNs to establish expression of interferon-stimulated genes (ISGs). The observation that a deficiency in separate IFN receptors did not affect disease progression during murine SARS-CoV infection, in contrast to a deficiency in common signaling molecule STAT1, illustrates this high degree of redundancy [53]. At the moment, hundreds of ISGs have been identified. However, an exact function has only been clarified for a relatively low number of the corresponding proteins, a description of which can be found elsewhere [54–56]. Nonetheless, these studies suggest that ISGs exhibit overlapping inhibitory activity towards most components of virus replication, including virus entry, uncoating, translation of viral proteins, RNA replication, and egress [54]. In summary, host cells detect viral RNA by several detection mechanisms to establish an antiviral state via the induction of IFNs and pro-inflammatory cytokines, leading to expression of ISGs.

3.2. Antiviral innate immune signaling is spatially organized

As mentioned earlier, MAVS localized on mitochondria is a key player in antiviral signaling. Therefore, studies demonstrating that the MAVS adaptor molecule STING is located on the ER implied that earlier identified contacts between mitochondrial membranes and ER membranes (mitochondrion-associated membranes: MAMs) were possibly involved in antiviral signaling [57]. In support of this theory, expression of a MAM-enriched marker protein followed by cell fractionation revealed that MAVS localizes to MAMs, which was supported by immunofluorescence assays of MAVS and MAM-enriched proteins [58]. Additionally, immunoprecipitation analysis of the MAM fraction of Sendai virus-infected hepatocytes confirmed that MAM-localized MAVS interacts with RIG-I and signaling cofactor TRAF3 [58], indicating that MAMs are involved in the RLR-mediated antiviral immune signaling pathway.

Therefore, MAMs function as important signaling platforms that govern expression of type I and III IFNs. Interestingly, MAVS is also expressed on peroxisomal membranes, and interacts with stimulated RLRs during viral infection [59]. However, in contrast to its mitochondrial counterpart, peroxisomal MAVS was found to induce expression of ISGs by an IFN-independent mechanism, and this relatively rapid process seems to provide short-term protection until the IFN-dependent expression of ISGs mediated by other viral RNA-sensing mechanisms is established [59]. Interestingly, recent studies found that direct contacts between peroxisomes and ER membranes were involved in maintaining peroxisomal function [60–62], raising the question whether these contact sites might also regulate antiviral immunity-related processes. In summary, peroxisomal and mitochondrial membranes and their contact-sites with the ER are important regions for converting viral RNA detection by RLRs to downstream events required for establishing an antiviral state.

Besides MAMs, increasing evidence suggest a role for stress granules (SGs) in antiviral immune signaling. SGs are non-membranous compartments in the cell that contain aggregates of ribonucleoprotein (RNP) and mRNA, and are formed as a physiological response to various stress stimuli that cause temporal stalling of mRNA translation, suggestively preventing accumulation [63]. Consistent with the function of this structure, inhibition of translation through the inactivation of eukaryotic translation initiation factor (eIF)2a by phosphorylation is an important cue for SG formation [63]. Considering that various RNA virus infections result in shutdown of host translation by inactivation of eIF2a by for example PKR, it is now known that formation of SGs occurs to different extends during infection with various RNA viruses, including HCV, DENV, and SFV [63,64]. Interestingly, multiple lines of evidence have demonstrated that SGs in encephalomyocarditis virus- or influenza virus-infected cells contain multiple RNA sensing molecules that were found to contribute to IFN production *in vitro*, including RIG-I, MDA5, PKR, OAS, RNaseL, and dsRNA [65,66]. However, it should be noted that the contribution of SGs to antiviral signaling differs per virus, as SG-localized MDA5, an RLR that greatly contributes to IFN induction upon encephalomyocarditis virus infection, was not found to support IFN expression upon infection with this virus [67]. Nonetheless, these findings suggest an important role for SGs as detection platforms for viral RNA. Taken together, increasing evidence suggests that the antiviral innate immune signaling cascade is spatially organized, and since different structures in the cell seem to be specialized in a particular aspect of this process, it can be hypothesized that interaction between these structures plays a fundamental role in orchestrating the antiviral innate immune response.

4. Positive-strand RNA viruses interfere with antiviral innate immune signaling

4.1. Shielding viral RNA species away from innate immune sensors

Positive-strand RNA viruses developed multiple mechanisms to impair RNA recognition and antiviral signaling (also reviewed in [8]). As mentioned above, the presence of viral RNA in ROs suggests a role for ROs in shielding viral RNA from the innate immune detection machinery. While there is no unequivocal evidence supporting this theory, the finding that isolated viral RNA-containing membrane alterations only become sensitive to nuclease treatment after membrane disruption by nonionic detergents supports this hypothesis [68–71]. In addition, a positive correlation between cytosolic exposure of viral RNA and IFN induction was recently found by an *in* vitro comparison of JEV and DENV infections [72]. Another important strategy by which RNA viruses avoid recognition is their modification of viral RNA molecules to mimic eukaryotic mRNA. For example, the formation of a 5′ cap is catalyzed by viral phosphatases and methyltransferases in some viral families such as the coronaviruses. From work on mouse hepatitis virus (MHV) it became clear that coronaviruses need to take care of N-linked as well as 2′O-linked methylation on the CAP structure, in order to avoid recognition by innate immune sensors [73]. Mimicry of eukaryotic RNA in this way was shown to be an important immune evasion strategy by +RNA viruses such as SARS-CoV and JEV [74,75]. A recent report indicated that coronaviruses also evade dsRNA mediated recognition by PKR and OAS by using their endonuclease (EndoU), which cleaves free viral (and cellular) RNA that is somehow exposed to the cytosol, in order to prevent antiviral signaling [76].

4.2. Active interference with innate immune signaling by viral proteins

In addition to avoidance of recognition, +RNA viruses actively interfere with antiviral signaling components to impair expression of IFNs and pro- inflammatory cytokines. Very often, the viral proteases that are expressed by +RNA viruses, and which mostly have a primary function in cleavage of viral replicase polyproteins, seem to have a prominent role in this. For example, HCV NS3/4A and DENV NS4A cleave MAVS [47,77–82], and the DENV protease complex and HCV NS4B inhibit adaptor molecule STING by cleaving it [83–87]. Additionally, human CoV NL63 and SARS-CoV nsp3 possess a papain-like protease (PLP) domain that prevents dimerization of STING and its complex formation with MAVS and IKKε, and almost all nidovirus-encoded PLPs have been shown to display deubiquitinating activity [88]. Several have been suggested to facilitate deubiquitination of innate immune factors such as STING, RIG-I, TBK1 and IRF3 in order to halt innate immune signaling [8]. Moreover, in collaboration with others, our lab demonstrated that EAV and MERS-CoV virus mutants that lack the DUB activity of their PLPs while retaining polyprotein cleavage functions suppress IFN production less efficiently during infection ([89,90] and our unpublished data). Furthermore, viral proteins such as DENV NS2B/3 and the SARS-CoV membrane glycoprotein interfere with complex formation of IKKε/TBK1 and IKKα/IKKβ/IKKγ or downstream transcription factors, thereby effectively inhibiting both RLR and TLR signaling [91,92]. Interestingly, accessory proteins encoded by open reading frames of CoVs have been shown to be involved in inhibition of IRF3, IRF7, and NF-κB by undefined mechanisms [93,94]. In addition, several methods are employed by +RNA viruses to interfere with JAK/STAT signaling. For instance, JEV NS5 and WNV NS4 B inhibit phosphorylation and activation of JAK1, and suppressors of JAK1 are induced by viruses such as WNV, JEV, and chikungunya virus [95–99]. However, STAT1 and STAT2 are most heavily targeted. JEV NS5, WNV and DENV NS4B, and JEV NS4A have been shown to inhibit STAT activation. In addition, DENV NS5 promotes proteasomal degradation of STAT2. Interestingly, SARS-CoV inhibits STAT1 signaling in three different ways. Firstly, SARS-CoV nsp1 binds STAT1 to inhibit its phosphorylation [100]. Secondly, the accessory protein encoded by SARS-CoV open reading frame 6 prevents nuclear transport of STAT1 [93,101]. Thirdly, SARS-CoV PLP induces expression of E3 ubiquitin ligase E2–25k, which promotes proteasomal degradation of extracellular signal-regulated kinase 1, a protein responsible for activation of STAT1 [102]. To our knowledge, no studies have been published (yet) about specific interference of +RNA viruses with IFN-II production.

4.3. Dysregulation in the spatial organization of the antiviral innate immune system

Interference of +RNA viruses with antiviral signaling also has consequences for the spatial organization of the antiviral innate

immune system. For instance, since SGs suggestively have an antiviral role, interfering with SG formation might constitute an opportunity for viruses to hamper the innate immune response. Indeed, various proteins of poliovirus, coxsackievirus, encephalomyocarditis virus, DENV, WNV, MERS-CoV, chikungunya virus, and JEV have been shown to prevent the formation of SGs and thereby suppress IFN production [63,66,103–109]. Thus, dysregulation of SGs seems to be a common strategy for +RNA viruses in order to dampen the innate antiviral immune response. In addition, the finding that MAMs are involved in the RLR-mediated antiviral immune signaling pathway shedded new light on the immunosuppressive role of viral proteases. By performing colocalization analysis with a MAM-enriched marker protein and cell fractionation experiments in both uninfected and HCV-infected cells, it was found that NS3/4A also localizes to MAMs [58]. Intriguingly, despite the clear function for peroxisomal MAVS in antiviral signaling, cleaved MAVS was found solely in MAM-enriched cell fractions. In addition, recent findings suggest that MAMs are also physically disrupted during DENV infection [110]. Thus, these data indicate that MAMs are critical locations for antiviral signaling and have an important role in expression of type I and III IFNs. Moreover, increasing evidence suggests that at least some +RNA viruses in fact occupy or hijack MAM-membranes during infection, as MAMs of HCV-infected cells were found to contain proteins involved in virus assembly and fully assembled virions [111]. It remains to be investigated whether MAMs also serve as platforms for viral assembly of other +RNA viruses. In addition, MAM disruption as a consequence of DENV infection was followed by the formation of convoluted membranes at the same location, which supported DENV replication [110]. Moreover, promoting the formation of convoluted membranes further repressed the IFN response, underscoring the importance of MAM disruption in both the replication and immune evasion of +RNA viruses [110]. Taken together, these studies underscore the importance of MAMs as key antiviral signaling platforms, and disruption of MAMs constitutes an effective immune evasion strategy for +RNA viruses. In conclusion, +RNA viruses developed divergent methods to delay antiviral innate immune signaling at multiple levels. In addition, interference of +RNA viruses with antiviral signaling leads to spatial disorganization of the antiviral innate immune system, and future studies will hopefully elucidate the consequences of this phenomenon in the context of other cellular compartments and viruses.

5. Specific responses towards viral replication organelles

Assuming that ROs impair antiviral signaling by shielding viral RNA from the host, it is tempting to hypothesize the existence of host cell mechanisms aimed to disrupt ROs, thereby exposing viral RNA and promoting antiviral signaling. The fact that +RNA viruses display such elaborate activities to inhibit viral RNA recognition and subsequent signaling, as detailed in the former paragraphs, supports the view that disruption of RO integrity is a situation these viruses anticipate dealing with. However, evidence suggesting targeting of ROs by the innate immune system is still scarce, although it was reported that HCV ROs are attacked by the ISG Viperin [94]. Additionally, 25-hydroxycholesterol, a product produced by the ISG cholesterol 25-hydroxylase also modifies HCV replication organelles [112,113]. Recently, our lab published a study suggesting that IFN-β signaling also negatively influences arterivirus ROs, since significantly less were formed and remaining structures showed drastically different morphology after IFN-β treatment. The results suggested that the treatment interrupted biogenesis of these membrane structures rather than breaking down structures that were already made [114]. Interestingly, neither Viperin nor 25-hydroxycholesterol was involved in the observed effects, and the mechanistic details therefore have to be further investigated.

5.1. The innate immune system disrupts pathogen-containing membrane rearrangements to expose PAMPs

Other recent data suggest that there may indeed be specific mechanisms by which the innate immune system tags and disrupts so-called non- or aberrant-self membrane structures in the cell [115], which could include viral ROs. Most data originate from studies focusing on bacteria, fungi, or parasites that reside in rearranged membranes, known as pathogen-containing vacuoles (PVs), which prevent detection of cytosolic innate immune sensors in a similar way as viral ROs are thought to do. Multiple studies found that enzymes capable of disrupting PVs are GTPases, such as effector immunity-related p47 GTPases (IRGs) and guanylate-binding proteins (GBPs), which are part of a family which we will refer to as IFN-inducible GTPases [116]. Expression of IRGs and GBPs is induced upon IFN-γ stimulation, and leads to their accumulation on PVs. Subsequently, activation of these GTPases due to the exchange of GDP for GTP results in membrane disruption as a consequence of their dynamin-like activity [117]. To prevent aspecific disruption, host cell membranes contain a set of proteins that inhibit GTPase activity, known as ''guard proteins'' [118]. Well-studied pathogens in the context of this process are the protozoan parasite *Toxoplasma gondii* (*T. gondii*), the bacterium *Chlamydia trachomatis* and the microsporidian *Encephalitozoon cuniculi* [119–125]. Although it remains unclear for most of these pathogens how IFN-inducible GTPases are recruited towards PVs, recent studies on *T. gondii* infection demonstrated that host cells label these membrane structures, for example with various forms of microtubule-associated protein 1A/1B-light chain 3 (LC3) to initiate this process [122–125] (Fig. 2). LC3 is a well-known factor in the autophagy pathway, and several forms of LC3 exist in the human proteome, which seem to have overlapping as well as distinct functions. The human genome encodes homologs LC3A, LC3B, and LC3C and LC3-like homologs GABARAP, GABARAPL1, GABARAPL2, and GABARAPL3. We will refer to all of these as LC3 unless stated otherwise. LC3 was initially discovered as an essential protein for autophagosome formation, which requires a covalent interaction between cytosolic LC3 (LC3-I) and the phospholipid phosphatidylethanolamine (PE), after which LC3 is referred to as LC3-II or lipidated LC3 [126]. However, recent studies have now revealed that labeling of (foreign) membrane compartments by LC3 conjugation can also have various autophagy-unrelated consequences, as has been reviewed elsewhere [127,128]. Interestingly, multiple studies demonstrated that labeling of LC3 on PVs recruits IFN-inducible GTPases upon IFN-γ expression during *T. gondii* infection, resulting in exposure of *T. gondii* to the host cell cytosol, thereby triggering anti-bacterial immune responses [122,123,125]. Considering the function of LC3 in autophagosome formation, Jayoung Choi and co-workers [124] termed this process Targeting by AutophaGy proteins (TAG). In addition, a similar role was recently found for ubiquitin, the versatile regulator of numerous important cellular processes, and also a well-known autophagy-related protein [129]. A study on *T. gondii* and *Chlamydia trachomatis* infection demonstrated that PVs are also recognized and labeled by the ubiquitination pathway upon IFN-γ expression, resulting in recruitment and activation of IFN-inducible GTPases [130]. Importantly, it was shown that virulent strains of *T. gondii* and *Chlamydia trachomatis* interfere with the deposition of IFN-inducible GTPases on PVs, underscoring the importance of this mechanism in clearance of associated infections [119,131,132]. Collectively, these studies demonstrate the existence of various innate immune responses that result in tagging and subsequent disruption of non- or aberrant-self

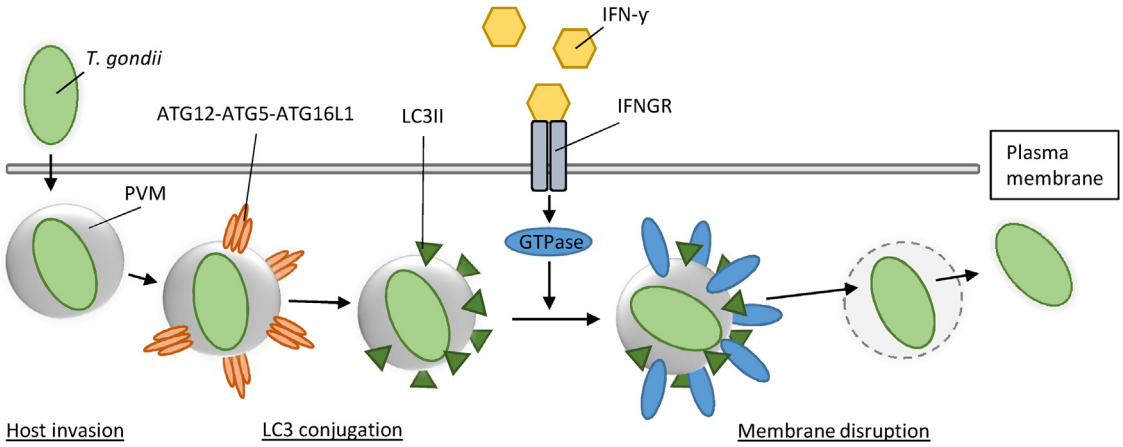

Fig. 2. LC3 recruits IFN-γ inducible GTPases to disrupt the parasitophorus vacuole of *Toxoplasma gondii*. Upon invasion of the host cell, *Toxoplasma gondii* (*T. gondii*) resides in a host-derived membranous structure, termed the parasitophorus vacuole membrane (PVM), to evade immune detection. Recent studies demonstrate that LC3 is conjugated to the PVM by the LC3 conjugation system, leading to recruitment of IFN-γ inducible GTPases to the PVM upon IFN-γ stimulation. As a consequence, activation of IFN-γ inducible GTPases leads to membrane disruption of the PVM, and exposure of *T. gondii* to the innate immune system.

membrane structures in the cell, thereby exposing PAMPs and promoting immune signaling. Importantly, the findings suggest that IFN-mediated membrane disruption might be a common principle in clearance of pathogens that use rearranged membranes to support replication and avoid innate immune detection.

5.2. Indications for disruption of +RNA virus ROs by the innate immune system

Like PVs, ROs induced by +RNA viruses are membranous compartments that constitute an environment for efficient replication, simultaneously avoiding immune detection by the host. Therefore, similar mechanisms might be aimed towards viral ROs. The observation that ROs induced by various +RNA viruses consist of a double membrane initially suggested a role for the autophagy machinery in the formation of these structures. However, only nonlipidated LC3, and not an intact autophagy pathway, was found to be essential for viral RO formation and viral replication upon infection with EAV and MHV [133,134], thereby contradicting this hypothesis. Interestingly, lipidated LC3 was found to support IFN-γ mediated *protection* against murine norovirus (MNV) infection by autophagy-unrelated means [135]. Furthermore, the induction of viral membranous structures by expression of MNV nsps led to a high colocalization between ATG16L1, a protein involved in LC3 conjugation, and the MNV polymerase. Since ATG16L1 determines the site for LC3 conjugation [136], and the MNV polymerase is associated with ROs, this further supports the possibility that LC3 conjugation occurs on ROs. Other supporting *in vitro* experiments show that conditional KO of *ATG4B* inhibits replication of MNV and *T. gondii* more effectively compared to wildtype cells [123,135]. In contrast to the multiple autophagins involved in pre-processing of LC3 for conjugation, ATG4B is the only human homolog of yeast *ATG4* that efficiently deconjugates LC3 from membranes [137], and therefore plays a major role in negatively regulating the fraction of conjugated LC3. Several groups have reported enhanced conjugation of LC3 to membranes during knockdown of *ATG4B* in various cell types [138–141], including those used by aforementioned studies [123,135]. Thus, it is likely that increased lipidated LC3 was present on ROs and PVMs during experiments in ATG4B-deficient

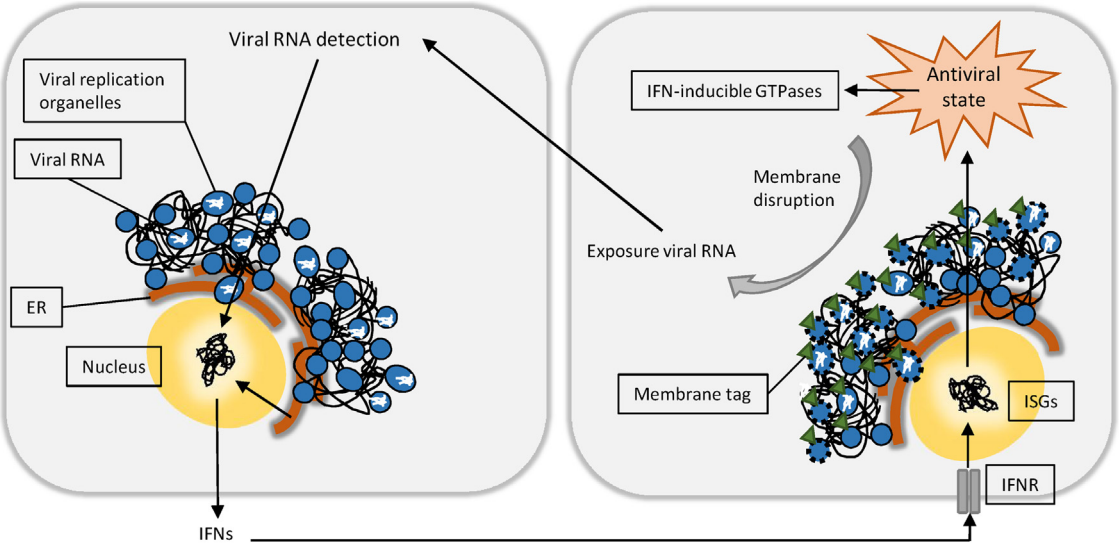

Fig. 3. A possible role for IFN-inducible GTPases in the targeting of viral replication organelles. Upon infection, +RNA viruses hamper IFN and ISG induction at multiple levels to decelerate innate immune detection. Viral replication organelles (ROs) are believed to have a dual role in +RNA virus infection and innate immune evasion, as they facilitate efficient RNA replication while shielding viral RNA from the host. Based on current knowledge, we hypothesize the existence of host cell IFN-inducible GTPases aimed to disrupt ROs, thereby exposing viral RNA and promoting antiviral signaling. IFNR: interferon receptor.

cells, thereby explaining the enhanced inhibition of replication. Interestingly, findings in other studies are contradictory in the sense that only LC3-I was observed on ROs of MNV, EAV, and MHV-infected cells [133–135]. On this note, it is worth mentioning that LC3-I does not support IFN-γ mediated inhibition of viral replication, as IFN-γ mediated inhibition of MNV-infected cells does not occur in the case of ATG7 deficiency, one of the proteins responsible for LC3 conjugation [135]. In conclusion, although there is no unequivocal evidence regarding the presence of conjugated LC3 on +RNA virus induced ROs, the studies described above provoke the hypothesis that membrane disruption of viral ROs mediated by IFN-γ inducible GTPases may be part of the innate immune response against +RNA viruses (Figure 3).

5.3. A general role for IFN-inducible GTPases in the antiviral innate immune response?

Interestingly, current knowledge of IFN-inducible GTPases suggests that membrane disruption of ROs by IFN-γ inducible GTPases might be part of a common antiviral mechanism that utilizes IFN-inducible GTPases to combat viral infection. Aside from IRGs and GBPs, other families of dynamin-like IFN-inducible GTPases exist, such as myxovirus resistance proteins (Mx) and the very large IFN-inducible GTPases (VLIGs) [116]. These enzymes are also known to have antiviral properties against a variety of viruses (reviewed in [116]). For example, the protective role of Mx proteins has been demonstrated for orthomyxoviruses such as influenza, lentiviruses such as human immunodeficiency virus (HIV) 1, and, interestingly, several +RNA viruses belonging to the picornaviridae and togaviridae [116]. Moreover, based on evolutionary studies, it was recently suggested that Mx proteins mediate protection against even a wider variety of viruses [142]. In contrast to IFN-γ inducible IRGs and GBPs, expression of Mx proteins is induced by IFNs type I and III, implying that all IFN types are capable of inducing IFN-inducible GTPases [116]. Therefore, all cell types known to respond to IFNs could theoretically induce a variety of IFN-inducible GTPases in response to viral infection. In the case of +RNA viruses, it is intriguing that multiple studies demonstrated how MxB suppresses replication of HIV-1 by targeting the viral capsid protein [143–145], given the parallels between retroviral capsids and +RNA virus ROs [146]. In conclusion, these findings again suggest a general function for IFN-inducible GTPase effectors in the targeting of viral intracellular membrane structures that function to shield away viral components away from the cytosol.

6. Concluding remarks

Rearrangement of host membranes into ROs is considered a hallmark of +RNA virus infection. ROs are believed to have at least a dual role in +RNA virus infection and innate immune evasion, as they facilitate efficient RNA replication while shielding viral RNA from the host antiviral response machinery. As viral RNA is a very potent inducer of antiviral signaling, we focused on how both the biochemical and spatial organization allows the innate immune system to convert detection of viral RNA to an antiviral state. In addition, we described part of the divergent methods by which +RNA viruses impair antiviral signaling surrounding their RNA replication, transcription, and translation in order to establish infection. At last, based on recent studies that demonstrated how IFN-γ inducible GTPases are capable of disrupting PVs, we discussed the possibility of a general function of IFN-inducible GTPases in the targeting of viral ROs. In summary, upon infection, +RNA viruses hamper IFN and ISG induction at multiple levels to decelerate antiviral innate immune signaling. In this process, the formation of ROs enables rapid viral RNA replication while masking it from the host. However, in case of sufficient activation of IFN-inducible GTPases by IFN-I and –III originating from various cell types, or IFN-II produced by specialized immune cells, disruption of ROs by IFN-inducible GTPases may result in enhanced exposure of viral RNA, thereby amplifying the innate immune response and ensuring efficient clearance of the virus. However, considering the differences in morphology and origin of ROs, the variety of infection dynamics within the category of +RNA viruses, and a lack of knowledge regarding the exact mechanism(s) of disruption of non/aberrant-self membrane structures by IFN-inducible GTPases and possibly other factors, extensive further research is required to elucidate whether this process plays a general role in +RNA virus infection, and might open possibilities for development of novel antiviral therapies.

Conflict of interest

None

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