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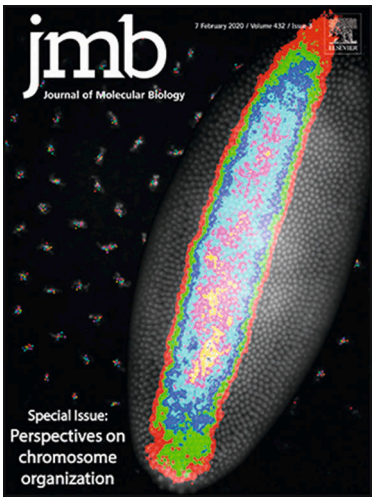

Review Article

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Membraneless organelles and condensates orchestrate innate immunity against viruses

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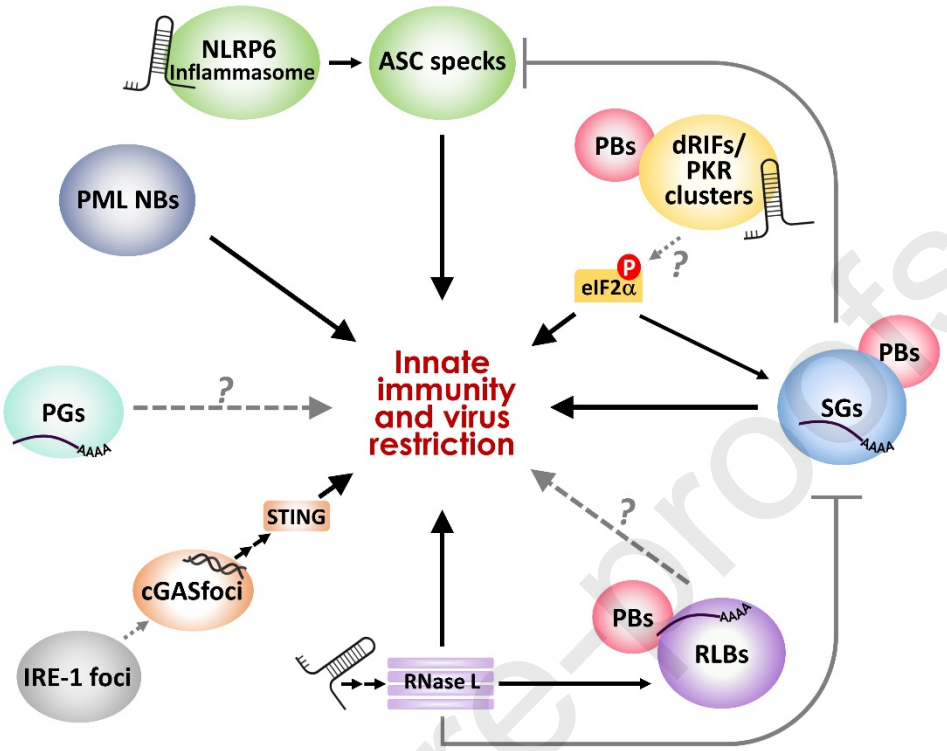

Research Highlights

- The immune activation of a number of Pattern Recognition Receptors upon virus infection involves their oligomerization and the formation of membraneless condensates.
- RNase L-dependent bodies, Paracrine Granules and Protein Kinase R clusters are novel virus-induced condensates.
- Disruption of membraneless condensates relevant to innate immunity by the action of viral proteins and nucleic acids is a frequent strategy for virus escape.
- Distinct membraneless condensates may be functionally linked, and insights on their coordinated formation are emerging.

Abstract

The cellular defense against viruses involves the assembly of oligomers, granules and membraneless organelles (MLOs) that govern the activation of several arms of the innate immune response. Upon interaction with specific pathogen-derived ligands, a number of Pattern Recognition Receptors (PRRs) undergo phase-separation thus triggering downstream signaling pathways. Among other relevant condensates, inflammasomes, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) specks, cyclic GMP-AMP synthase (cGAS) foci, protein kinase R (PKR) clusters, ribonuclease L-induced bodies (RLBs), stress granules (SGs), processing bodies (PBs) and promyelocytic leukemia protein nuclear bodies (PML NBs) play different roles in the immune response. In turn, viruses have evolved diverse strategies to evade the host defense. Viral DNA or RNA, as well as viral proteases or proteins carrying intrinsically disordered regions may interfere with condensate formation and function in multiple ways. In this review we discuss current and hypothetical mechanisms of viral escape that involve the disassembly, repurposing, or inactivation of membraneless condensates that govern innate immunity. We summarize emerging interconnections between these diverse condensates that ultimately determine the cellular outcome.

Keywords

Membraneless organelle, innate immunity, RNase L, RLBs, PML-NBs, Smaug, stress granule, sfRNA

Introduction

Cellular condensates and membraneless organelles (MLOs) play important roles in diverse processes. Particularly, the activation of several arms of the antiviral defense is linked to the condensation of innate immunity factors into specific cellular bodies. The formation of biocondensates and MLOs depends on multiple weak interactions between protein molecules and in addition, RNA molecules are frequently involved. Liquid-liquid phase separation (LLPS) processes were suggested to direct the formation of most cellular membraneless condensates, which can behave as liquid droplets, hydrogels, or may even contain several phases, including solid-like cores. Membraneless condensates may influence each other. For example the massive recruitment of a client or scaffold molecule in a given condensate may alter the function or assembly of another [1-4]. Intracellular condensates may provide molecular and cellular functions that are not present in the “diluted” surrounding phase. Biocondensates and MLOs can buffer the intracellular concentration of a given macromolecule, which demix above a saturating concentration, storing the “excess” in the condensed phase and keeping the cytosolic concentration constant. For example, endogenous double stranded RNA (dsRNA) may trigger strong inflammation and it was recently shown that

ribonucleoproteic condensates keep cytosolic dsRNA separated from RNA sensors [5-7]. In addition, cytosolic, nuclear as well as membrane-associated condensates can allow for reactions that are not favored in the diluted phase. Intermolecular interactions and chemical reactions can be facilitated by the higher local concentration of substrates inside or at the surface of condensates. Moreover, the physicochemical milieu is in general less aqueous than in the surrounding cytosol and this may influence biochemical processes and reactions [6, 8-10]. Thus, the formation of condensates may work as an ON/OFF switch, potentially generating dramatic changes in cell responses and fate, as needed when a viral infection threats cell survival.

Cumulative evidence supports that the cellular defense against viruses depends on the dynamic condensation of a growing number of proteins linked to different pathways of the innate immune response. Innate immunity involves numerous pattern recognition receptors (PRRs) that recognize a wide spectrum of viral-derived factors, either of nucleic-acid or proteinaceous nature and collectively termed pathogen-associated molecular patterns (PAMPs). An important cytosolic PAMP is dsRNA, which is recognized by protein kinase R (PKR), 2′,5′-oligoadenylate synthetases (OAS) and melanoma differentiation-associated protein 5 **(**MDA5) [11]. Host mRNAs normally carry a 2'-O-methylation in their 5'-cap and lack of this modification stimulates MDA5. In addition, cellular transcripts carrying dsRNA stretches escape immune recognition through A to I editing by adenosine deaminases acting on dsRNA (ADARs) [12]. In contrast, short dsRNAs with a 5′-tri- or 5′-di-phosphate end are relevant viral PAMPs recognized by retinoic acid-inducible gene-I (RIG-I) [13]. Cytosolic DNA is also strongly inflammatory and is detected by cyclic GMP-AMP (cGMP-AMP) synthase (cGAS), an important trigger of the immune response. Furthermore, fragments of nuclear or mitochondrial DNA released to the cytosol as a result of loss of membrane integrity during viral infection are important damage-associated molecular patterns (DAMPs) that add to the inflammatory processes [13, 14].

PKR, RIG-I-like receptors, cGAS, OAS and inflammasomes are important PRRs that respond to different stimuli and elicit partially overlapping responses including interferon (IFN) induction. Following PAMP recognition, PRRs typically oligomerize and may form filaments or supramolecular complexes, which involves profound structural changes that have been largely described [15]. Here we will focus on the formation of membraneless condensates, which are by definition orders of magnitude larger than oligomers. The condensation of cGAS was early identified as a key step for cytokine induction [16]. The formation of specific cytosolic specks linked to inflammasome activation is likewise well-stablished [17, 18].

Promyelocytic leukemia protein (PML) nuclear bodies (PML NB) are nuclear condensates that among other functions, were proposed to mediate virus restriction through multiple pathways [19]. The contribution of stress granules (SGs) to the antiviral defense was suggested several years ago and is currently a matter of debate [20-26]. More recently, additional MLOs both related and non-related to SGs are emerging as potentially important players in the cellular response to viral infection. Among others, pseudo SGs termed paracrine granules (PGs) are induced in non-infected cells exposed to paracrine signals delivered by virus-

infected cells [27]. Another example is provided by cytosolic granules induced upon cleavage of cellular transcripts by RNase L, which were recently described and termed RNase L-dependent bodies (RLBs) [4, 26]. It is as well remarkable the formation of PKR-containing condensates during the cell reaction to dsRNA, either artificial or generated during virus infections (Figure1) [28-30].

Viruses and their hosts have been interacting for an extremely long time, and antiviral responses and concomitant counteractive mechanisms have evolved from this dynamic interplay. Emerging examples of this antagonism involve the disruption, inactivation or repurposing of cellular condensates linked to innate immunity. Viral proteomes frequently contain a large proportion of low-complexity regions (LCRs) and intrinsically disordered regions (IDRs) [31]. LCRs and IDRs are key drivers of phase-separation and play important roles at several stages during virus infection. Virus replication typically involves the formation of cytosolic condensates termed viral factories whose formation depends on viral proteins carrying LCRs or IDRs. For example, JUNV N forms cytosolic clusters upon acute or persistent infection, as well as upon transient transfection of cultured cells (Figure 1) [32-39]. Viral factories can sequester cellular factors involved in the IFN response [40]. In addition, IDRs in viral proteins may direct their interaction with condensates formed by the host cell, putatively interfering with their dynamics or function. RNA-binding proteins (RBPs) are frequent in cellular condensates and moreover, RNA can affect their formation. Nucleic-acids derived from viruses, which include a wide variety of DNA and RNA molecules, may affect MLO and condensate formation. In this review, we will focus on apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) specks, cGAS foci, PKR foci, RLBs, PGs and PML NBs, and will direct the reader to comprehensive reviews that thoughtfully discuss the potential relevance of SG formation during viral infection [20-24, 26]. We will provide an updated description of MLOs, condensates and granules with important roles in innate immunity and will discuss emerging findings on how viruses can influence their assembly and/or function.

Inflammasomes and ASC specks

Inflammasomes are multimeric complexes that mediate the production of pro-inflammatory cytokines, which involves the proteolytic processing of cytokine precursors by caspases [41]. In turn, caspases are activated by self-cleavage, and this is facilitated by their aggregation in specific supramolecular assemblies that form upon a variety of stimuli. During the inflammatory response, inflammasomes serve as platforms for the proteolytic activation of specific caspases, which then cleave and activate cytokines that are secreted thereby stimulating antiviral responses. In addition, inflammasome activation may initiate pyroptosis, which is a form of caspase-dependent cell death different from apoptosis.

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are major PRRs that form inflammasomes. Upon recognition of cognate PAMPs or DAMPs, the binding of ATP to the NOD motif allows for NLR self-oligomerization. NLRs can be activated by pathogen-derived or cellular ligands generated during infections, as well as by signals that alert of disrupted cell homeostasis [42]. For example,

NLRP6 responds to viral and non-viral ligands that directly interact with the leucine-rich repeat motif (LRR) present in the NLRP6 C-terminus. More recently, the binding of NLRP6 to dsRNA was reported to direct its LLPS, and moreover LLPS of NLRP6 was shown to be critical for downstream signaling [17]. An internal IDR carrying four contiguous lysine residues mediates NLRP6 demixing and subsequent inflammasome activation. The biological significance is clear as experimental animals lacking this NLRP6 motif fail to overcome rotavirus or hepatitis virus infection [17]. The formation of mature inflammasomes involves the recruitment of additional protein factors and the adaptor molecule ASC, also known as PYCARD or TMS-1, is key to this pathway. ASC protein is mostly nuclear in resting cells and rapidly accumulates in the cytosol in association with inflammasome components, including caspases and NLRs. Ultimately, the condensation of a single cytosolic body termed ASC speck or pyroptosome occurs, and ASC speck formation is strictly required for caspase activation and pyroptosis (Figure 2) [18, 43].

A competition between ASC specks and SGs has been proposed [2, 3]. The physical interaction between NLRP3 and the RNA helicase termed DDX3/DDX3X is critical to initiate NLRP3 condensation and downstream ASC speck formation. DDX3 is also recruited to SGs, and SG formation reduces the availability of cytosolic DDX3. Thus, this RNA helicase can balance the assembly of ASC specks and SGs, which were reported to be mutually exclusive [2, 3]. Defective ASC speck formation affects caspase activation and pyroptosis and thus SG formation may limit inflammasome-dependent cell death [3]. Furthermore, of high importance for virus restriction, cytokine production may be reduced (Figure 2). Conversely, if SG formation is hampered, DDX3 can mediate NLRP3 inflammasome formation thus allowing for an effective antiviral response [2].

Excessive inflammation upon virus infection may be causative of severe pathology, as in the case of COVID-19 and other diseases where cytokine storms are major etiological drivers. A relevant outcome is that ASC speck condensation has potential as therapeutic target [44]. On the other hand, viruses evolved strategies to counteract this pathway and evidence begins to emerge that viruses can influence inflammasome assembly and ASC speck formation to escape innate immunity. Sendai virus V protein (SeV V) is a recent example [45]. SeV V directly binds NLRP3, thereby inhibiting NLRP3 self-oligomerization and affecting ASC speck formation, which remains dispersed in the cytosol and nucleus. As a consequence of defective ASC speck formation, caspase 1 is not fully activated and pro-interleukin 1beta (IL-1B) is not cleaved, thus damping IL-1B secretion. Importantly, this viral mechanism contributes to SeV pathogenicity in vivo and the inhibitory effect of V proteins is conserved in other members of the *Paramyxovirinae* subfamily, specifically Nipah virus (NiV) and human parainfluenza virus type 2 (HPIV-2). In all cases viral V proteins bind NLRP3 blocking NLRP3 self-oligomerization and ASC speck formation. Information regarding relevant V protein residues is pending [45].

A similar pathway was identified earlier in the pathogenesis of influenza A virus (IAV) [46]. IAV NS1 protein directly binds NLRP3 thus blocking ASC speck formation. The inhibitory effect is conserved in IAV strains linked to several pandemics, including H1N1, H5N1 and H7N9. The NS1 molecular determinants have been identified and both the NS1 RNA binding domain and the TRIM25-binding domain mediate the inhibitory effect

[46]. More recently, it was shown that the NS1 C terminus of 2009 pandemic H1N1 suppresses ASC ubiquitination, which is required for full activation of the NLRP3 inflammasome [47]. Other viruses affect inflammasome activation by alternative or overlapping pathways, underscoring the evolutionary pressure to evade this arm of innate immunity [46, 47].

cGAS foci

An important antiviral response is governed by LLPS of cGAS. Cytosolic DNA binds and stimulates cGAS enzymatic activity thereby initiating the biosynthesis of cGMP-AMP, a second messenger that in turn activates a protein factor termed stimulator of IFN genes (STING), which is normally associated to the endoplasmic reticulum (ER). STING activation directs the expression of several pro-inflammatory cytokines, including type I IFN (IFN-I) (Figure 2). Several years ago, the Z. Chen's laboratory described the formation of cytosolic foci containing cGAS and DNA [16]. cGAS foci are stable enough to resist mild detergent extraction, and display the typical behavior of dynamic MLOs. They sporadically fuse and rapidly exchange cGAS molecules with the cytosol, as neatly shown by fluorescence recovery after photobleaching (FRAP) analysis.

The formation of cGAS condensates follows the same scheme as that of many other MLOs and depends on multiple intermolecular interactions. cGAS binds DNA through multivalent contacts. Both the disordered N-terminus and the structured C-terminus –which contains the catalytic domain– recognize DNA, without sequence requirements. The "monomers" in the cGAS condensates are likely polyvalent tetramers that when bound to long DNA molecules can generate a large cGAS-DNA network.

The formation of droplets of cGAS together with DNA can be recapitulated *in vitro* and GTP and ATP –the substrates that are converted into cGMP-AMP– are straightaway incorporated into the droplets. cGAS condensation correlates with its activation and it was proposed that the phase separation of cGAS allows for a switch-like response. When DNA concentration in the cytosol exceeds a critical level, cGAS molecules demix and cGMP-AMP synthesis starts. As expected, longer DNA molecules induce cGAS phase separation and catalysis at lower concentration than short DNA molecules and thus, the immune activation is adjusted to different inputs [16].

Given the central role of this pathway in the antiviral response, it may be anticipated that viruses evolved counteracting strategies and examples where cGAS phase separation is altered by viruses start to emerge (Figure 2). A significant one is provided by herpesviruses. The proteome of these viruses includes a family of structurally related proteins termed tegument proteins, which are located underneath the viral envelope. VP22 and ORF52 are tegument proteins from alpha- and gamma- herpesvirus and can be incorporated into cGAS-DNA droplets, thereby displacing cGAS and dramatically affecting its activation, downstream STING stimulation and cytokine production [48, 49]. This mechanism of immune evasion is active in Kaposi

sarcoma-associated herpesvirus (KSHV), an important pathogen causative of several tumors. KSHV ORF52, also known as KSHV inhibitor of cGAS (KicGAS) contains disordered regions that drive self-oligomerization, which in turn facilitates the cooperative binding to dsDNA and the subsequent demixing of KicGAS-DNA complexes in specific droplets that exclude cellular cGAS. Defective phase separation of KicGAS mutants and variants correlates with poor cGAS inhibition [49]. Independent work demonstrated that ORF52 and VP22 from several other viruses similarly disrupt cGAS-DNA droplets. In all cases, the binding of ORF52 or VP22 proteins to DNA followed by their demixing interrupts the interaction of cGAS with DNA, thus affecting cGAS activation. Viral particles contain numerous units of tegument proteins that can neutralize the cGAS pathway immediately after their release inside cells and thus, this conserved mechanism of immune evasion may act early during infection [48].

Besides DNA, cGAS can bind RNA and moreover, cGAS phase separation can be induced by RNA molecules. However, cGAS accumulation in a separated phase is not enough to activate cGAS enzymatic activity and RNA fails to stimulate cGMP-AMP synthesis, which strictly requires DNA [50]. *It has been suggested that the formation of cGAS condensates directed by cellular RNA in the absence of dsDNA might modulate the innate immune response (reviewed in [51]).* Whether viral-derived RNAs can influence cGAS phase separation and activation remains an open question.

It was also proposed that cGAS condensation involves G3BP1, a key cellular factor that plays a pivotal role in the assembly of several cytosolic condensates linked to innate immunity and stress responses [1, 52, 53]. Independent reports have shown the formation of primary condensates of G3BP1 and cGAS where the binding to DNA is stimulated. G3BP1 deficiency seriously affects cGAS condensation, its activation by DNA and the ensuing cytokine production [54-56]. G3BP is an important SG-forming factor targeted by numerous viruses and moreover, cGAS was reported to be present in SGs in specific cases. Viruses can affect G3BP1 levels [20] and this may have consequences on the condensation of both SGs and cGAS foci, thus simultaneously affecting two arms of the cellular response.

More recently, cGAS activation was shown to be stimulated by collided ribosomes, which occur under a variety of cell insults [57]. For example, the ER-stress sensor inositol requiring enzyme 1 (IRE1) is activated by increasing levels of unfolded proteins in the ER lumen, thus directing the endonucleolytic cleavage of mRNAs and thereby alleviating the load of nascent proteins that enter the reticulum. This mechanism is termed regulated IRE1-dependent decay (RIDD) and acts quite selectively on endomotifs that consist on small stem-loops carrying specific sequences. IRE1 is an integral ER membrane protein and can form condensates termed IRE1 foci, which remain associated to the ER. The endoribonuclease activity in the IRE1 foci allows for a less-selective mRNA cleavage and this mode of action is termed RIDD lacking endomotif, o RIDDLE [58]. Translating mRNAs can be targeted by RIDD or RIDDLE and this may provoke the accumulation of ribosomes upstream of the cleavage site [59], potentially influencing cGAS activation. RNase L provides another pathway for the endonucleolytic cleavage of polysome-engaged mRNAs [60-62], thus hypothetically provoking ribosome collisions that might stimulate cGAS activation. Whether the physical interaction of cGAS with

collided ribosomes is linked to cGAS foci formation is currently unknown. In connection with this putative mechanism, abortive polypeptides generated as a consequence of ribosome stalling can form cytosolic aggregates [63], which may nucleate additional macromolecules thus promoting their condensation. Finally, whether stimulation of cGAS by stalled ribosomes is functional upon virus infection is a tempting hypothesis, as IRE1 and RNase L respond to the presence of viral RNAs and their translation at the ER.

PKR clusters

PKR is a key factor in the cellular defense against viruses [64, 65]. This kinase is activated by dsRNA and the alpha subunit of the translation initiation factor 2 (eIF2alpha) is the main downstream target. As a consequence of eIF2alfa phosphorylation, most cellular and viral mRNAs are translationally repressed. Simultaneously, transcripts that carry upstream open reading frames (uORFs) or particular internal ribosome entry sites (IREs) are translationally activated. Both uORFs and IREs are frequent in viral mRNAs and all this leads to a profound reprogramming of the translatome. PKR is constitutively expressed but remains inactive in resting cells and its transcription is enhanced by IFN. PKR kinase activity is stimulated by trans-autophosphorylation, which is a common theme among stress sensors including IRE1 and the four eIF2alpha kinases known to date, namely PKR; general control nonderepressible 2 (GCN2); heme-regulated eIF2 alpha kinase (HRI) and protein kinase R-like endoplasmic reticulum kinase (PERK), each of them responding to specific inputs. In the case of PKR, the binding of dsRNA to its dsRNA binding domains (dsRBDs) or the direct interaction of PKR with specific cellular proteins, as for example PKR activating protein (PACT), induce a conformational change that ultimately triggers PKR activation. Once activated, PKR phosphorylates eIF2alpha and likely a few other targets thereby initiating concerted responses including translation inhibition, caspase activation and IFN induction [64, 65].

Early evidence of PKR aggregation was provided by both *in vivo* and *in vitro* studies [66-69]. More recently, two independent works by the laboratories of R Parker and D Acosta-Alvear showed that PKR forms cytosolic clusters, also termed dsRNA-induced foci (dRIFs), when A549 or H4 cells are exposed to polyIC, which is a synthetic analog of dsRNA (Figure 1) [28, 29]. Binding to dsRNA triggers PKR demixing and the size of PKR condensates correlates with the concentration and length of polyIC. PKR foci are also induced by increased levels of cellular dsRNA, as for example transcripts from ALU inverted repeats.

PolyIC-induced PKR clusters/dRIFs undergo fusion and fission as usually described for a wide variety of membraneless condensates. Importantly, PKR foci do not overlap with SGs or RLBs, which can also form upon exposure to polyIC. In addition, PKR condensates do not contain G3BP1 nor polyadenylated RNA. As expected from the fact that they lack mRNAs, foci containing either endogenous or transfected PKR do not dissolve upon polysome stabilization by cycloheximide, further stressing their difference with SGs. PKR clusters are also different from NLRP6 inflammasomes, whose condensation is similarly stimulated by dsRNA [17, 28, 29]. Their relationship with Processing Bodies (PBs) was also investigated. PKR foci sporadically

contact PBs and can contain specific PB markers [28, 29] (Figure 2). The functional significance of this association has not been elucidated and among other observations linked to these recent findings, DCP1a –a major PB component– was shown to enhance PKR activation [70].

PolyIC-induced dRIFs contain additional dsRNA binding proteins (dsRBPs) and strikingly, PKR is not required for their assembly, rather, PKR is recruited to pre-formed foci [28]. PKR kinase activity is not required either and moreover, genetic or pharmacological inactivation of PKR kinase activity enhances cluster formation and PKR residence times evaluated by FRAP. FRAP analysis further indicated that the foci contain two pools of PKR molecules. A fraction with high turnover and an immobile fraction that corresponds to PKR molecules strongly associated [28-30]. While additional studies are being performed in this exciting new area, a preliminary model can be proposed. Inactive PKR, altogether with other dsRBPs are recruited to dsRNA molecules, which serve as a scaffold to initiate condensation. Then, PKR activation by trans-phosphorylation is facilitated as a consequence of the close contact between clustered PKR molecules. PKR phosphorylation is known to weaken its affinity for dsRNA and thus fully active phospho-PKR is released to the cytosol. This may be key to convey the signal, as the eIF2 complex, which includes three subunits –alpha, beta and gamma– cannot enter the PKR clusters due to steric hindrance and is thus phosphorylated in the cytosol surrounding the PKR foci [29]. At the same time, the release of phosphorylated PKR may allow for new inactive PKR molecules to be recruited to the foci, followed by its activation and thereby maintaining the signal in an ON state. This hypothetical mechanism and how PKR foci formation is coordinated with the assembly of additional complexes similarly induced by dsRNA remain to be investigated. Further consequences of PKR clustering are expected to be unveiled. For example, it has been proposed that PKR foci are reservoirs of PKR and that regulation of their formation would allow for a fine tuning of eIF2alpha phosphorylation [29].

The relevance of PKR foci formation upon virus infection is incipiently known. Viral replication and viral RNA accumulation temporally correlate with PKR clustering upon infection of H4 cells with a measles virus strain that strongly induces PKR activation [28, 29]. Related examples are provided by New World arenaviruses. Acute infection of A549 cells with JUNV Candid strain induces P-PKR clusters that include dsRNA and JUNV N protein [71]. PKR also colocalizes with viral replication complexes of Tacaribe virus (TCRV). JUNV and TCRV stimulate PKR to different levels, thus opening further questions on the role of PKR clusters in the control of PKR activation by viruses [38, 72, 73]. Among other open possibilities, PKR compartmentalization might provide with a mechanism to isolate PKR from viral inhibitory factors, which can affect PKR stimulation in multiple ways. On the other hand, viral factors may be recruited to PKR clusters thus directly affecting the activation and/or the release of activated PKR molecules [38, 65, 73]. The presence of additional PRRs such as NLRP1 in polyIC-induced dRIFs is as well intriguing and suggests that multiple antiviral pathways are coordinately controlled in these bodies. In summary, the relevance of PKR clusters to the antiviral response and whether viruses evolved mechanisms to control their formation and dynamics are promissory research lines with wide perspectives.

RNase L oligomers and RNase L-dependent bodies

Besides PKR, dsRNA activates additional viral restriction factors that promote innate immunity. Whereas PKR is directly activated by cellular or viral dsRNA, RNase L activity is indirectly controlled. In resting cells RNase L is present in a latent state ("L" state for "latent") and is allosterically stimulated by a number of 2′,5′-oligoadenylates (2-5A) synthesized by 2-5A synthetases (OAS), which are in turn activated by direct binding to dsRNA. When bound to 2-5A, RNase L becomes active thus initiating the cleavage of cellular and viral single-stranded RNAs with low sequence specificity and a mild preference for UU and UA dinucleotides. RNase L induces the rapid decay of most cellular transcripts, including those engaged in active translation, thus triggering an important translational reprograming. In addition, it has been proposed that structured RNA fragments that resist RNase L can act as PAMPs for dsRNA sensors. The cellular outcome of RNase L activation is variable and depends on multiple mechanisms, including transcriptional activation of IFN, degradation of coding and non-coding RNAs and nuclear export blockage (Figure 2) [60, 61, 74].

The stimulation of this pathway is linked to two aggregation events that occur both upstream and downstream of RNase L activation. First, the activation of RNase L requires its oligomerization. Once activated, the endonucleolytic cleavage of mRNAs paradoxically directs the formation of specific bodies containing polyadenylated RNA termed RNase L-depending bodies or RLBs (Figure 1).

RNase L oligomers

The first evidence that RNase L oligomerizes was provided by *in vitro* experiments. At sufficient concentrations, RNase L does not require 2-5A for activation and shows cooperativity [75]. The presence of 2-5A further induces cooperativity and RNase L dimers associate into high-order oligomers. Structural analysis allowed to identify the residues that contact 2-5A, along with the molecular determinants for RNase L oligomerization. Importantly, RNase L oligomerization and cooperativity generate a switch-like response, such that a modest increase in 2-5A levels upon virus infection can significantly stimulate RNase L activity, thus triggering a rapid reaction. Whether RNase L oligomerization correlates with the formation of cytosolic condensates of microscopic scale is currently unknown. This seems quite likely considering that the closely related endonuclease IRE1 forms foci with strong RNase activity [58, 76].

RNase L-dependent bodies (RLBs)

These yet mysterious bodies condense as a consequence of RNase L activity (Figure 2) [4, 26, 60]. RLBs contain PABP and polyadenylated RNA but are clearly different from SGs. Rather, RLBs form instead of SGs when RNase L is activated. SGs cannot form if mRNAs are massively cleaved by RNase L and moreover, pre-formed SGs dissolve upon RNase L activation. RLBs condense in MEFs carrying a non-

phosphorylatable mutant version of eIF2alpha and do not require PKR for their assembly [4, 26, 60]. Also unlike SGs, which dissolve upon exposure to translational inhibitors that capture mRNAs in polysomes, RLBs do not respond to this type of drugs, and is not yet clear whether RLBs contain intact mRNA molecules or cleavage products. Also different from SGs is their lack of requirement for G3BP1, although this protein is present in RLBs and used as an RLB marker.

Similar to other condensates and MLOs described here, RLBs are highly dynamic and both G3BP1 and PABP rapidly exchange with the cytosol. The RLB proteome was determined and RLB-resident proteins are linked to mRNA metabolism and translation. Like several other MLOs containing mRNAs, they are in close contact with PBs although the relevance of this association remains unclear [26, 30]. As expected, the formation of RLBs is triggered by viruses. Specifically, the infection of A549 cells with dengue virus or SARS-CoV-2 induces RLBs [26, 77, 78]. Whether RLB formation is a byproduct of RNase L activity or an important factor in the antiviral response remains unknown. For example, translating ribosomes may halt at RNase L cleavage sites thus resulting in collided ribosomes. Whether RLBs contain stalled ribosomes that may stimulate cGAS activity thus connecting two important arms of the antiviral response is an exciting question (Figure 2).

Stress Granules

Stress granules are transiently induced upon acute translation shutdown and are typically observed after eIF2alpha phosphorylation triggered by stress insults or viral infections [79-83]. The biological significance of SG formation is a matter of intense research. It has been initially proposed that SGs serve as platforms for the activation of several PRRs, as SGs appear to facilitate the activation of RIG-I and PKR as well as IRF3, IRF7, TBK1, and STING in a number of viral infections. This model has been extensively reviewed [20-24, 31, 84-88] (Figure 2). However, the requirement of SGs in the antiviral response is not universal and examples are emerging where SG formation can be uncoupled from RIG-I and PKR activation. Specifically, RIG-I dependent cytokine production upon yellow fever virus (YFV) infection of cultured Huh7 cells is not affected by the simultaneous knockdown of the SG assembly factors TIAR, TIA-1, G3BP1, and G3BP2, which significantly reduces SG condensation. In addition, although SG forms upon YFV infection, SG disruption doesn´t affect YFV replication [89]. Furthermore, it has been shown in other experimental models that SG integrity is seriously affected by RNase L activation [4], which normally occurs upon RNA virus infection, further supporting the notion that SGs are not indispensable for virus restriction.

Viral proteins, proteases as well as viral transcripts were reported to affect SG formation and dynamics [20-24, 31, 84-88]. SG assembly and disassembly require chaperones, molecular motors and adaptors, which may be usurped by viruses in multiple ways [80, 90-92]. SG inhibition by viruses can release RNA binding proteins that facilitate viral translation and/or replication [20-24, 31, 84-87]. Future work aimed to investigate the contribution of loss-of-function consequences, as for example defective PRR activation, and of gain-of-

function mechanisms, as for example release of pro-viral factors upon SG disruption will shed light on this complex subject.

Paracrine Granules

The description of these cytosolic condensates containing G3BP is currently limited (Figure 2). A pioneering report shows that PGs form in bystander cells upon infection of Crandell-Rees feline kidney (CRFK) cells with feline calicivirus (FCV) [27]. Remarkably, virus-free supernatant from infected cells is able to induce PGs in CRFK or U2OS cells, strongly suggesting that infected cells deliver paracrine signals that trigger PG formation, and that PGs do not contain viral proteins or nucleic acids. PGs are highly reversible and dissolve immediately after stimulus withdrawal. Although PG formation correlates with acute translational silencing, PGs are able to condense in cells carrying eIF2alpha mutations that abrogate its phosphorylation. PGs are clearly different from SGs. Several translation initiation factors that are present in SGs are absent from PGs and conversely, PGs contain proteins from large ribosome subunits, which are excluded from SGs. Another important observation is that similarly to PKR foci and RLBs, PGs are resistant to polysome-stabilizing drugs such as cycloheximide and moreover, cycloheximide induces PG enlargement, all this suggesting the presence of polysomes. However, PGs share some components with SGs, including G3BP1 –which is also present in RLBs–, although G3BP1 is not required for the formation of PGs nor RLBs, in striking contrast to the relevant role of this protein in SG assembly. The molecular mechanism that drives PG formation has not been elucidated. As expected, the PG proteome is enriched in proteins carrying IDRs that may help their condensation. In addition, PGs contain numerous mRNAs and RNA binding proteins [27].

As discussed, condensation may generate an environment or surface where selected protein factors can be recruited or activated thus facilitating specific cellular pathways. A number of PRRs such as PKR, RIG-I and others appears to be absent from PGs, weakening the speculation of their role as platforms for innate immunity activation. Whether PGs contribute to virus restriction is yet unknown and the biological relevance of PGs remains unclear. Another important avenue for future research is the identity of the paracrine signal that induces their assembly. Among other potential mediators, extracellular RNAs either naked or packed in extracellular vesicles are emerging as important cell-cell communication factors thus opening wide perspectives [93].

Processing Bodies

PBs are constitutive MLOs that contain repressed mRNAs. PBs are not centers for RNA decay, which occurs in the cytosol, and it is accepted that mRNAs in PBs can enter translation [94, 95]. Whereas a direct role for PBs in the activation of innate immune pathways seems unlikely, they may be involved in

translational reprograming upon virus infection. Specifically, PBs and related MLOs formed by a RBP termed Smaug1/Samd4a, which is stimulated by IFN and restrict hepatitis B virus (HBV) infection, were proposed to control specific sets of mRNAs encoding respectively regulatory functions and metabolic enzymes [96-98]. Relevantly, T cell activation depends on the reprogramming of the energetic metabolism [99], thus opening a new field to be explored. Similarly, purinosomes and G-bodies are membraneless condensates that contain multiple enzymes from specific metabolic pathways [100] and their relevance to the immune metabolism remains largely unknown.

Several viruses interfere with PB assembly, and PB dissolution may release specific PB components that are required for virus translation or replication. Among the first findings, infection with the positive-strand RNA poliovirus (PV) was reported to rapidly induce PB disassembly through viral proteases that cleave specific PB proteins [101]. Similarly, 2A protease from enterovirus (EV) 71 initiate PB dissolution thus releasing DDX6 and 4ET, two factors that interact with EV71 RNA and facilitate virus replication [102]. With an opposite effect, other viruses protect and usurp PBs for its own use. Negative-strand RNA virus snatch 5' cap structures from cellular mRNAs and cap-snatching occurs in PBs. Briefly, transcription by viral RNA-dependent RNA polymerase requires 5'capped RNA primers. Small fragments from capped mRNAs, usually 10-20 nt long, serve this purpose and are incorporated into viral transcripts. Several years ago, nucleocapsid protein and PBs were shown to be involved in cap-snatching by hantavirus [103]. More recently, cap-snatching by additional animal and vegetal bunyavirus was reported to occur in both PBs and SGs and particularly, in PB-SG contacts [104]. The involvement of SG and PBs in cap-snatching is a striking example of how viruses can repurpose cellular organelles for its own benefit.

Promyelocytic leukemia protein nuclear bodies

Unlike the above discussed cytoplasmic MLOs and condensates, PML bodies reside inside the nucleus. PML NBs play an important role in the antiviral cellular defense. As described below, most virus infections provoke PML NB dissolution, and in a few examples the formation of PML NB is enhanced, as occurs upon rabies virus (RABV) or JUNV infections (Figure 1) [105]. PML is the main PML NB component and is conjugated to small ubiquitin-like modifier (SUMO). Early models have proposed that the interaction between SUMO-conjugated PML and SUMO-interacting motifs (SIMs) present in PML contributes both to the initial nucleation steps and to the recruitment of additional PML NB proteins. In addition, PML has several dimerization domains located at the N-terminus, all of which are required for PML NBs formation [106]. At least seven PML isoforms have been described with differences at the C-terminus, and these variants multimerize to form the PML NB shell. The different C-terminal regions likely contribute to the fine-tuning of PML NB composition, and some isoforms were proposed to recruit specific proteins, nucleic acids or even lipids [106, 107].

PML NBs are implicated in intrinsic immunity against viruses by sensing DNA, RNA or protein complexes [108, 109]. PML NBs activate several pathways that converge in the sequestration of viral proteins, chromatinization of viral DNA with specific histone variants and epigenetic marks, as well as SUMOylation of viral and cellular proteins, which may alter their stability, function or phase-separation propensity [19, 110]. PML NBs also promote IFN signaling, IFN-induced global cellular SUMOylation and IFN-induced apoptosis (Figure 2) [111].

The interplay between PML NBs and innate immunity was discovered with the observation that IFN treatment induces the upregulation of several PML NB proteins, including PML and Sp100, and enhances the antiviral activity of PML proteins. In addition, PML depletion and PML NB disassembly reduce the capacity of IFNs to protect from viral infections [112]. Specifically, PML associates with transcription factors that control IFN and ISG expression, resulting in their stabilization and enhanced promoter occupancy [113, 114]. PML stimulates an extended spectrum of cytokines and among others, the production of IL-1β and IL-6 markedly decreases in PML-deficient cells. Remarkably, individual PML isoforms play specific roles. The PML isoform II (PML II) seems to be of particular importance, as it directly associates with key transcription factors such as IFN regulatory factor 3 (IRF3) and signal transducer and activator of transcription 1 (STAT1), thus promoting their recruitment to IFN-β and ISG promoters, respectively [115]. PML isoform IV (PML IV) prevents IRF3 degradation, thus enhancing IFN-β production during vesicular stomatitis virus (VSV) infection [116]. The antiviral role of PML was also reported against RNA viruses such as the lymphocytic choriomeningitis virus (LCMV), rabies virus (RABV), dengue virus (DENV) and JUNV [117-120]. The relevance of specific PML isoforms was identified in a few examples and overexpression of PML-III/IV but no other PML isoforms suppressed the replication of influenza viruses, RABV, PV, encephalomyocarditis virus (EMCV), EV71, DENV and Zika virus (ZIKV) [105, 119, 121-123].

As in the case of other immune-relevant condensates described in this work, viruses have evolved effector proteins that antagonize PML NB-dependent intrinsic immunity [109]. In most cases, antagonistic factors are products of viral immediate early genes. In particular, nuclear-replicating viruses target PML NBs as a critical step for establishing productive infection. Early studies showed that DNA viruses including simian virus 40 (SV40), herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV), and some adenoviruses (ADV), all induce the relocalization of PML NB-residing proteins, resulting in the disassembly of the bodies and thus suggesting that PML NB disruption contributes to viral transcription and replication [124]. More recently, the disruption of PML NBs was described upon infection with influenza virus, rotavirus (RV), DENV, ZIKV and other flaviviruses, enteroviruses and retroviruses [105, 123, 125, 126]. Collectively, all these findings indicate that PML NBs modulate cellular antiviral responses by a variety of mechanisms. Sequestration of viral genomes or viral proteins, interference with viral replication and stimulation of the IFN pathway all have been reported in different infection models. As a result of selective pressure, viruses evolved strategies that depend on PML-NB disruption and/or repurposing of their functions to escape the cellular defense.

Viral RNAs affect cellular condensates

As described along this review, viral proteins carrying IDRs as well as viral proteases can affect the formation of cellular condensates and MLOs, with direct consequences in virus replication and antiviral responses. In addition, genomic RNA and viral transcripts can interact with several RBPs, sequestering them and/or modulating their activity as scaffolds [20-25, 31, 65]. Another type of viral RNA of growing importance in the control of cellular condensates are the subgenomic RNA fragments (sfRNA) typical of flaviviruses. All flaviviruses, both those that are restricted to insects and those that are transmitted from insects to vertebrates produce non-coding sfRNAs [127-131]. The sfRNAs correspond to the 3' most distal part of the viral genome and are generated in high amounts as a consequence of incomplete 5'-3' degradation of genomic RNA. This involves exonucleolytic digestion by vertebrate XRN1 or by the insect ortholog Pacman. These 5'-3' exoribonucleases are largely processive on non-structured RNA but stall at structured motifs. The 3'UTR of flaviviruses typically includes knots and pseudoknots that interrupt the progress of 5'-3' exoribonucleases thus leaving undigested highly structured sfRNAs of 200-600 bases in length. These sfRNAs can remain bound to XRN1 thus globally reducing RNA degradation [129].

Of importance, the formation, dynamics and viscosity of ribonucleoproteic condensates are influenced by the length and structure of RNA molecules in a complex pattern [132]. Long RNA molecules may help demixing, as they expose multiple sites for RBP recruitment, thus facilitating the interaction between bound protein molecules. In contrast, short RNAs with low valence for RBP binding may act as molecular decoys, promoting condensate dissolution [28, 29, 83, 132]. Double-stranded RNA may induce condensation, and long polyIC molecules are stronger inducers of dRIFs than short molecules. SGs contain more long than short mRNAs and can be dissolved by an excess of tRNA molecules. In addition, single-stranded RNA fragments were proposed to reduce the condensation of nuclear and cytosolic bodies [7, 133]. As reviewed in this work, the formation of relevant condensates including SGs, inflammasomes and PKR foci involves single or double stranded RNA molecules, and the effect of sfRNAs in these and other MLOs is incipiently known. DENV-2 sfRNAs were proposed to neutralize several RBPs that dramatically affect SG formation, including G3BP1, G3BP2 and Caprin. Titration of these proteins by DENV-2 sfRNAs correlates with defective ISG expression [134]. Similarly, the SG factor Fragil X Mental Retardation Protein (FMRP) binds to ZIKV sfRNA and this interaction antagonizes the antiviral effect of FMRP [135]. Whether reduced immune response by DENV-2 or ZIKV sfRNAs correlates with altered formation of RNA-dependent condensates, such as SGs, RLBs, NLRP6 inflammasomes or PKR foci remains unknown.

Finally, sfRNAs were shown to interact with a reduced number of PB components, potentially affecting PB formation, dynamics or liquidity. Specifically, ZIKV sfRNAs bind to DDX6/RCK and EDC3, two viral restriction factors that localize at PBs [129]. The DDX6/RCK insect ortholog Me31B similarly binds to sfRNAs from ZIKV and West Nile virus (WNV) [136]. Me31B restricts ZIKV replication in mosquito cells and

ZIKV sfRNAs suppress the antiviral response. Whether viral escape by ZIKV sfRNAs involves altered PB formation remains to be further investigated.

Conclusions

The physical interaction of a number of PRRs with their respective PAMPs initiates their demixing from the cytosol and the formation of specific membraneless condensates. The distinct physicochemical milieu inside these bodies, as well as the higher concentration of key proteins and activators altogether trigger major signaling pathways that act synergistically to control viral replication. Viruses have evolved numerous ways to counteract cell defense strategies and the disruption, interference and re-purposing of host condensates and MLOs are frequent pathogenic mechanisms. Future research on the molecular determinants underlying phase-separation of immune-relevant proteins and their interaction with viral components will contribute to understand critical pathogen-host interactions, thus helping to identify potential therapeutic targets.

A number of membraneless condensates involved in innate immunity are functionally interdependent and can physically interact. Several of them share triggers, scaffolds and client macromolecules and can influence each other in several manners (Figure 2). Protein factors that are simultaneously or alternatively present in distinct cellular bodies may contribute to the coordination and physical contact between them. Among others, G3BP1 is a paradigmatic example. G3BP1 is activated by binding to RNA and this affects the formation of both SGs and cGAS foci, besides being present in other bodies including RLBs and PGs. In contrast, DDX3 helicase switches between SG assembly and ASC speck condensation, balancing inflammation and cell death. RNase L activity dissolves SGs and induces the formation of RLBs, and may hypothetically affect cGAS activation. The role of PKR foci in PKR activation and SG formation is yet unclear. The interplay between these different bodies is emerging and future work will provide valuable information on the molecular networks that govern their simultaneous, successive or mutually exclusive formation, which ultimately determines the cellular outcome upon virus infection.

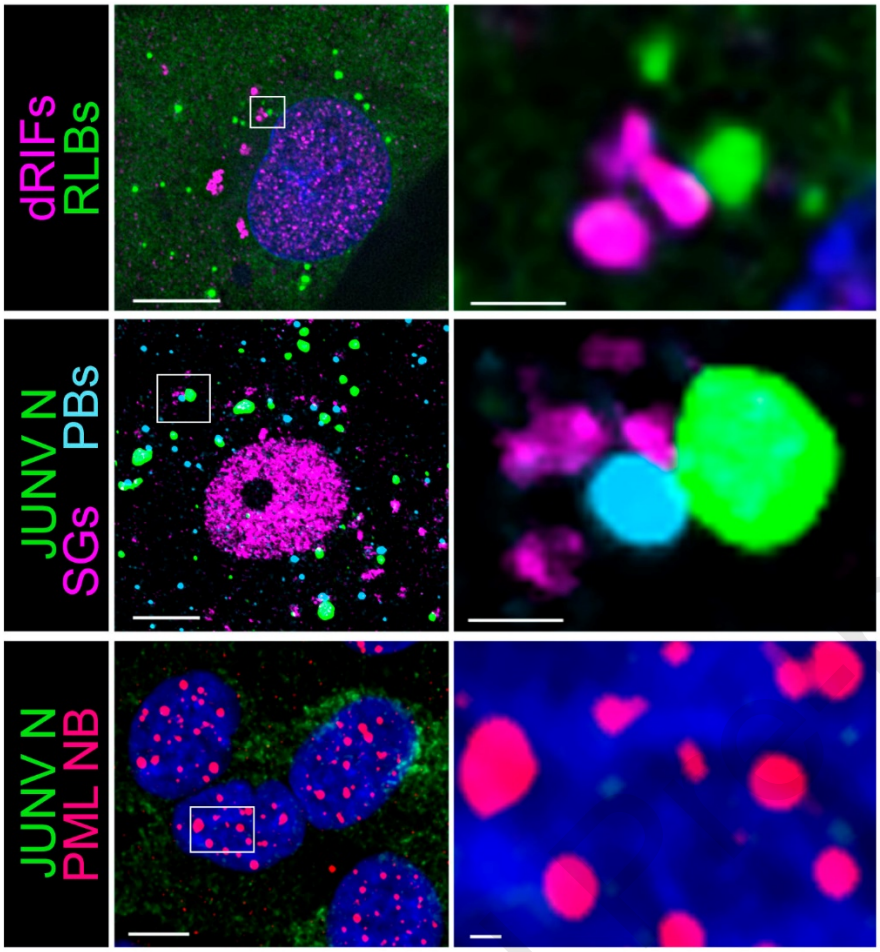

Figure 1. Examples of membraneless condensates linked to viral infections. Upper panel, dRIFs (PKR, magenta) and RLBs (G3BP, green) form in A549 cells exposed to polyIC as described [28]. Middle, JUNV nucleocapsid protein (N, green) in persistently infected Vero cells form clusters that sporadically contact SGs induced by exposure to arsenite (TIA1, magenta) as well as PBs (RCK/p54, cyan). Bottom, PML NBs (PML, red) are induced in A549 cells upon acute JUNV infection (N, green) as previously described [120]. Nuclei identified by DAPI staining are in blue. Scale bars: 10 μm in left panels, 1 μm for magnified images.

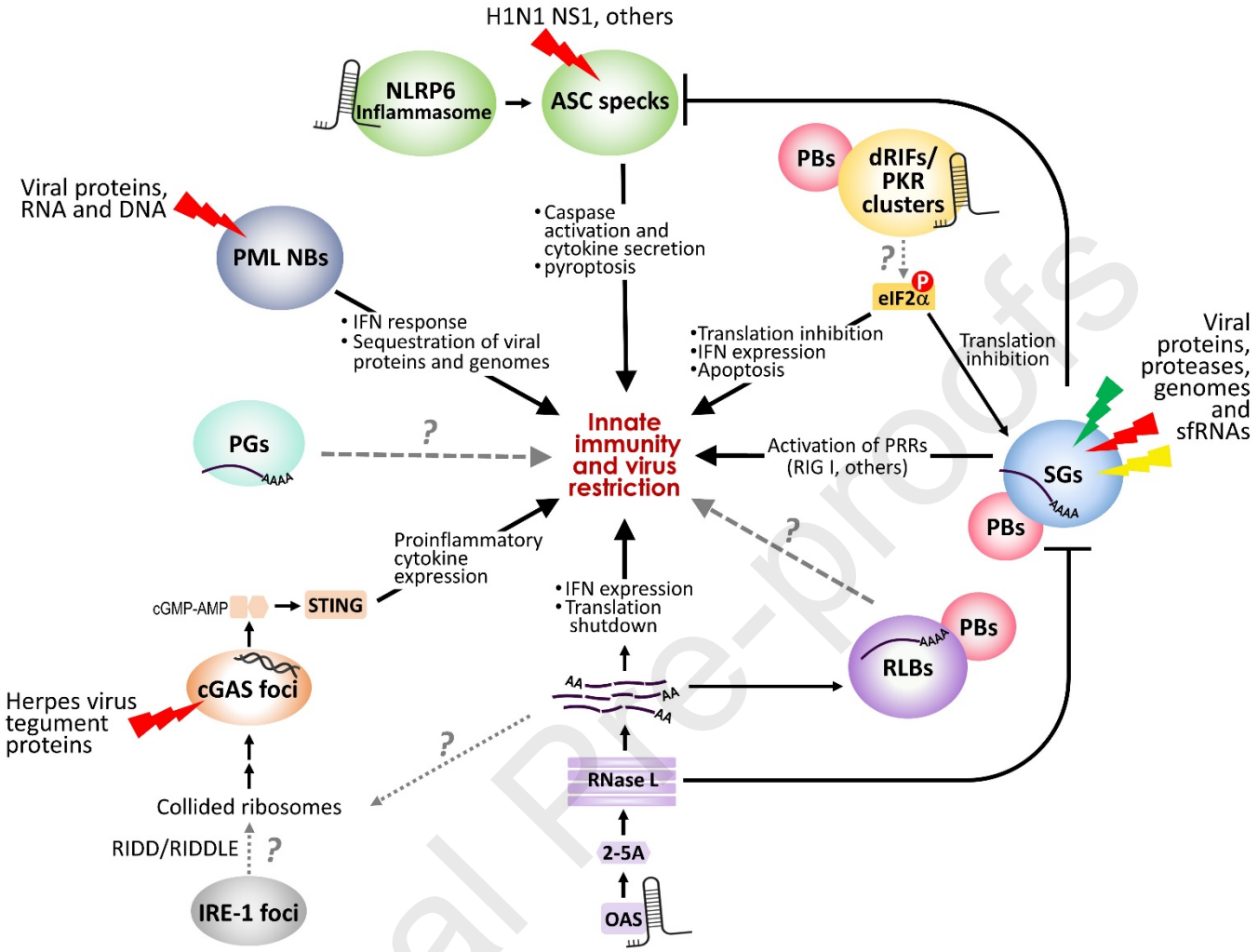

Figure 2. Summary and hypothetical model

Several PRRs form membraneless condensates upon interaction with their cognate ligands. Clockwise from top: The interaction of NLRP6 with dsRNA or several other ligands stimulates the condensation of inflammasomes and ASC specks. Among other viral proteins, NS1 from influenza H1N1 virus inhibits ASC speck formation thus controlling cytokine production. Cellular or viral dsRNA nucleates PKR and additional dsRNA binding proteins in specific droplets termed PKR clusters or dRIFs. Hypothetically, PKR is activated in dRIFs and released to the cytosol to allow for eIF2alpha phosphorylation (see text). dRIFs contain additional PRRs, such as NLRP1 (not depicted) and whether their activation is linked to the recruitment to dRIFs is currently unknown. Downstream of PKR activation, eIF2alpha phosphorylation induces the assembly of SGs, which typically form as a consequence of massive translation shutdown. SGs may act as platforms for the activation of RIG-I and other PRRs, although the requirement of SGs for immune activation is not universal (see text). SG formation opposes ASC speck condensation by retaining DDX3 and thus controlling cell fate. SGs are affected by numerous viral factors, including proteases that cleave

SG assembly factors. In addition, viral genomes and sfRNAs may influence SG and PB formation. PBs sporadically contact PKR clusters, SGs and RLBs with yet unknown implications. OAS is activated by dsRNA and the resulting 2-5A stimulates RNase L activation, which involves its oligomerization. Active RNase L directs the endonucleolytic cleavage of most cellular transcripts and RNase L-resistant RNA fragments may stimulate RNA sensors which in turn induce IFN expression. RLBs are the consequence of RNase L activity and their contribution to viral restriction remains unclear. cGAS phase-separates upon binding to cytosolic DNA, thus stimulating cAMP-GMP synthetase activity and triggering cytokine expression downstream of STING. IDRs present in ORF52/KicGAS, VP22 and related tegument proteins from several herpesviruses interfere with cGAS foci formation and cGAS activation, thus blocking the STING pathway. IRE1 forms foci during the unfolded protein response and cleaves translating mRNAs, potentially generating collided ribosomes. The stimulation of cGAS by collided ribosomes downstream of IRE1 or RNase L is entirely speculative. Paracrine granules (PGs) are induced by unknown molecules secreted by infected cells and like SGs and RLBs, contain polyadenylated RNA. The role of PGs during virus infection remains unknown. PML NBs are constitutive and bidirectionally affected by virus infection. PML NBs sequester viral proteins and nucleic acids and stimulate the IFN response.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: