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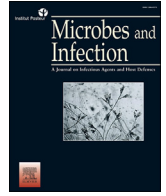
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Review

Biomolecular condensates with liquid properties formed during viral infections

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ABSTRACT

During a viral infection, several membraneless compartments with liquid properties are formed. They can be of viral origin concentrating viral proteins and nucleic acids, and harboring essential stages of the viral cycle, or of cellular origin containing components involved in innate immunity. This is a paradigm shift in our understanding of viral replication and the interaction between viruses and innate cellular immunity.

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1. Introduction

Membraneless organelles ranging in size from 100 nm to a few μm contribute to the compartmentalization of the interior of eukaryotic cells and are involved in a wide range of biological processes [1]. Often called liquid organelles, biomolecular condensates (BMCs) or simply biocondensates, they can either be located in the cytosol (e.g. stress granules -SG- and P-bodies) or in the nucleus (e.g. Cajal bodies, nucleoli and nuclear speckles). Typically, such organelles contain dozens or hundreds of different macromolecular species (proteins or nucleic acids) interacting inside. They are dynamic structures, very sensitive to their environment. In particular, they assemble and disassemble much more quickly than membrane-bounded organelles.

Several viruses have been shown to establish specialized intracellular compartments, called viral factories, viral inclusions, or viroplasms, which lack membranes and concentrate viral proteins, nucleic acids and some cellular factors. These compartments host essential steps in the viral cycle, provide a platform facilitating viral replication and assembly, and often protect the viral genome from

cellular defenses. In several cases, these compartments, which may be cytosolic or nuclear, have been shown to have liquid-like properties [2].

The discovery that many viral factories are membraneless and have liquid properties is a paradigm shift in the field of viral replication: the proteins involved in replication work in an environment with distinct properties allowing fine regulation of exchanges with the cell. This invites us in particular to reconsider the interactions between viral factories and the components of the cellular innate immunity. On the one hand, the sequestration of viral-associated molecular patterns (VAMPs) in liquid compartments raises the question of their accessibility to cytosolic pattern recognition receptors (PRRs). On the other hand, the viral factories can sequester cellular proteins involved in innate immunity and keep them away from their functional signaling pathway. It is also interesting to note that many proteins associated with antiviral innate immunity form or are recruited into BMCs upon exposure of the cell to several distinct stresses [3,4], making interactions between viruses and the host cell even more complex.

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2. General considerations about BMCs

2.1. Liquid–liquid phase separation

There is a fairly general consensus that BMCs are formed by liquid–liquid phase separation (LLPS) [1–3,5] - See however [6,7] -. LLPS is a phenomenon well-known to physicists. This is a reversible process consisting of de-mixing into two distinct liquid phases, with different solute concentrations. In the cell, LLPS results in the formation of BMCs enriched in certain proteins or nucleic acids (a so-called dense phase) which will coexist with a dilute or light phase.

The transition between mixing and de-mixing depends on numerous physical parameters such as the concentration of constituents, temperature, pH, salt concentrations, molecular crowding of the medium, etc. Varying these parameters in a systematic manner makes it possible to construct a phase diagram (Fig. 1A and B). The phase diagram is characterized by a coexistence (or binodal) line, which delimits the region where phase separation is thermodynamically accessible. Within this coexistence region, there is a second line, the spinodal that encloses an inner region where the single-phase mixture is unstable and phase separation spontaneously occurs. In the region located between the binodal and the spinodal, the system may remain in a metastable single-phase because phase separation requires a rate-limiting nucleation step [5] (Fig. 1B).

In simple model systems, when the concentration of the solute is below the saturation concentration (C_{sat}), the system is in a one-phase regime (Fig. 1A). Above C_{sat} , the system demixes into the light and the dense phases. At equilibrium, along a horizontal line, the respective concentrations of the light- and dense-phases (C_L and C_D) are fixed and only the volume fractions of the two phases are changing relatively to each other [5] (Fig. 1A). Within living cells, which operate out of equilibrium, such behavior is observed using artificial simplified systems (using proteins that phase separate upon light-activation) but, in general, heterotypic multicomponent

interactions give rise to condensates that do not exhibit a fixed C_{sat} [8].

2.2. Properties of BMCs

In biological systems, the condensates are highly enriched in some macromolecules (proteins or nucleic acids) which are much more concentrated in these structures than in the light phase, due to LLPS, while excluding some others. This allows a spatial regulation of cellular processes (Fig. 2). A distinction is frequently made between core (or scaffold) and client proteins found in the BMCs. A core protein is a protein necessary for BMC formation while a client protein is recruited inside the BMCs thanks either to its ability to bind another constituent protein, or to a specific physico-chemical property making it more soluble in the dense phase than in the light one. Core and client proteins are often subject to post-translational modifications (PTMs), such as phosphorylation, that affect their ability to undergo phase separation or be recruited into formed BMCs [9].

BMCs often contain multivalent proteins [1] forming homo or hetero-oligomers, RNA-binding proteins, and proteins having intrinsically disordered domains (IDD) consisting of low complexity amino acid sequences (enriched in glycines, prolines, polar and charged amino acids, sometimes containing aromatic residues, but largely depleted in hydrophobic amino acids) and, therefore, lacking a fixed or ordered 3D structure [1–3,5]. The weak interactions underlying LLPS are still poorly understood. In some systems, π - π stacking between aromatic residues and π -cationic interactions involving aromatic and positively charged residues, have been shown to be essential [10]. However, the lack of specificity of this type of interactions within the cell does not explain the formation of distinct and immiscible BMCs [6]. Finally, BMCs can undergo gel-transition that may further irreversibly evolve into hydrogels formed by amyloid-like fibers [5].

Another important point is that the order of magnitude of the number of macromolecules (either proteins or nucleic acids) in a

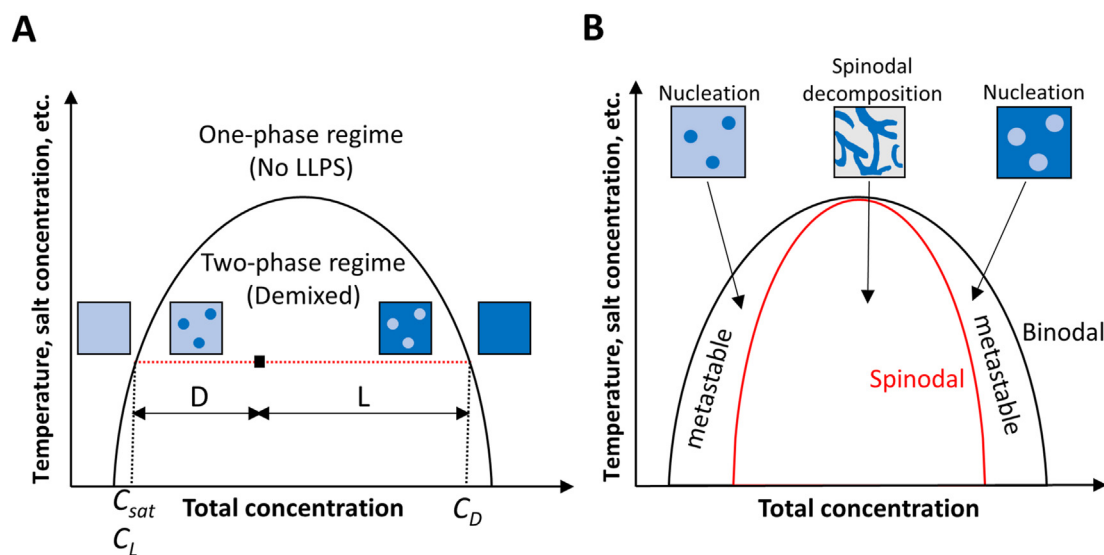


Fig. 1. Phase diagrams of LLPS for a binary system (adapted from Ref. [5]). A) Below C_{sat} , the system is in a one-phase regime. In the two-phase regime, the system demixes into a light phase and a dense phase with respective concentrations C_L and C_D . At equilibrium, $C_{sat} = C_L$. Under any condition (indicated by a black square) on the dotted line (in red), the volume fraction of the light phase $F_L = L/(D + L)$ and that of the dense phase is $F_D = D/(D + L)$. B) The binodal delimits the region where phase separation is thermodynamically accessible. The spinodal encloses an area where the single-phase is unstable and where phase separation spontaneously occurs. In the area located between the binodal and the spinodal, the system may remain in a metastable single-phase and phase separation requires a nucleation step.

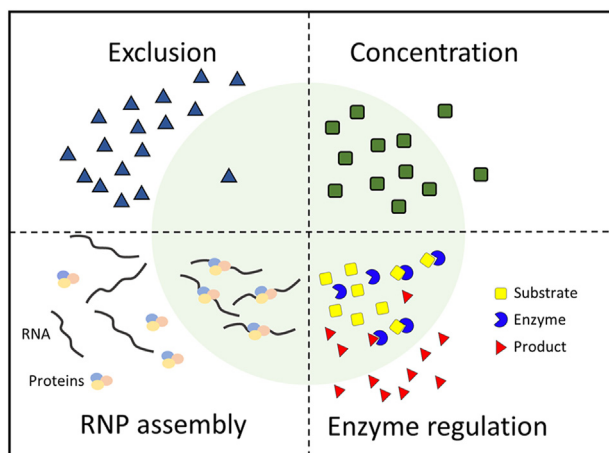


Fig. 2. Properties and functions of BMCs. BMCs can either concentrate or exclude some macromolecules (proteins or RNA). As examples, macromolecule concentration can promote complexes assembly, e.g. ribonucleoproteins (RNP), or can enhance enzyme-catalyzed reaction, e.g. by concentrating enzyme and substrate and excluding the reaction product.

100 nm BMC is a few thousand whereas it is a few million when its diameter is 1 μm . It is entirely possible that the properties of these BMCs of different sizes as well as the rules governing their assembly are distinct.

2.3. Experimental characterization of BMCs

A major experimental challenge is to demonstrate unambiguously that a specific structure is indeed a phase-separated liquid body in the cellular context. *Bona fide* phase separated compartments are expected to exhibit differences in molecular crowding and/or intermolecular interactions that affect free diffusion, resulting in significantly different diffusion coefficients of macromolecules in the dense and light phases. Therefore, one of the most valuable experiments to confirm whether these compartments arise from LLPS is to determine these coefficients in the BMC and in the light phase. These quantitative experiments require sophisticated equipment which is not always available in laboratories. Thus, currently, common criteria for defining such structures are that they are spherical, can fuse together (and then relax into a larger spherical structure), and recover from photobleaching. In addition, upon contact with a physical obstacle, the BMC can split into smaller condensates that quickly relax towards spherical shapes or deform before subsequently returning to its initial spherical shape. Among the other criteria, we can also cite the rapid disappearance of these structures when the cell undergoes a hypotonic shock and their return when the cell regains its homeostasis as well as their sensitivity to 1,6-hexanediol (1,6-HD) which disrupts weak hydrophobic interactions between IDD that drive LLPS [5].

On the one hand, it is not always simple to verify all these properties experimentally. For example, sphericity and deformability are difficult to evaluate for structures smaller than 200 nm. Likewise, fluorescence recovery after photobleaching (FRAP) is not necessarily easy to implement if the structures are very mobile in the cell. On the other hand, several of these experimental approaches are not devoid of artifacts. Prolonged exposure of yeast and mammalian cells to 1,6-HD is cytotoxic and induces changes in cell morphology, associated with the formation of aberrant assemblies [11]. Even FRAP experiments, often considered the gold standard in the field, do not unequivocally demonstrate that BMCs have liquid properties because fluorescence recovery is not unique to freely diffusing molecules in solution [7]. Furthermore, the

interpretation of FRAP data largely depends on the model used to analyze the experimental curves [12].

Finally, cell fixation is known to induce artifacts that confuse data interpretation [13]. Beyond these artifacts, in immunofluorescence, some BMCs either appear in the form of an annular structure (due to peripheral labeling by the antibody that cannot penetrate the structure) [14] or are simply not recognized by the antibody [15].

Many conclusions are also drawn from *in vitro* experiments carried out on purified proteins. In this type of approach, molecular crowding agents (PEG, Ficoll, etc.) are often used which can also be responsible of artifacts. Moreover, the concentration of proteins used in those experiments is sometimes one or two order of magnitudes higher than their cellular concentration. Here again, these experiments must be interpreted with great caution. They nevertheless remain essential because they allow a fine adjustment of different physical or chemical parameters (which is not possible in cells) and to evaluate their influence on LLPS. They also make it possible to use technical approaches (e.g. NMR) to finely characterize interactions between the BMCs components [16].

3. Virus-induced BMCs

3.1. Membraneless viral factories and replication centers

The first cytoplasmic membraneless viral factories characterized in detail were those of large DNA viruses such as the *Poxviridae*, the *Iridoviridae* and the *Asfaviridae* [17–19] (Fig. 3). These cytoplasmic factories exhibit several characteristics reminiscent of those of the cellular aggregates which concentrate misfolded proteins in the cell [20]. They are located near the microtubule organizing center (MTOC), recruit mitochondria, contain molecular chaperones, and are surrounded by a vimentin cage. For other double-stranded DNA (dsDNA) viruses such as adenoviruses and herpesviruses that replicate in the nucleus, once the genomes have been released into the nucleoplasm, they associate with viral proteins in membraneless nuclear inclusions called replication centers (RCs) [21] (Fig. 3).

Regarding RNA viruses, it has been known for years that rotaviruses, that are members of the *Reoviridae* family with 11 segments of double-stranded genomic RNA (dsRNA), form cytoplasmic inclusions, called viroplasms, during cellular infection. These viroplasms were later shown to constitute the site for transcription and replication of the viral genome as well as the packaging site of newly synthesized pregenomic RNA segments into the viral cores [22] (Fig. 3).

Similarly, it was shown that the cytoplasmic inclusions formed in the cells infected by viruses of the *Mononegavirales* (MNV) order (i.e. viruses having a genome made of a single strand RNA molecule of negative polarity), which had been known for years but little studied, harbored several viral replication stages (Fig. 3). A well-known example are the Negri bodies (NBs), which are cytoplasmic inclusion bodies with a diameter up to a few μm , formed during rabies virus (RABV) infection. They were described by Adelchi Negri in 1903 and are easily observed using Seller's staining (a mixture of saturated solutions of methylene blue and basic fuchsin dissolved in methanol). These structures are typical of RABV infection of the brain and, consequently, have been used as histological evidence of infection. Similar structures, which are also called NBs, are present in the cytoplasm of infected cell cultures of both neuronal and non-neuronal origin [23]. Confocal microscopy has revealed that proteins involved in viral transcription and replication are selectively concentrated in NBs [23,24]. Thus, the RABV RNA-dependent RNA polymerase (L), its cofactor phosphoprotein (P), and nucleoprotein (N, that is tightly associated with the viral genomic RNA) accumulate in NBs. Fluorescence *in situ*

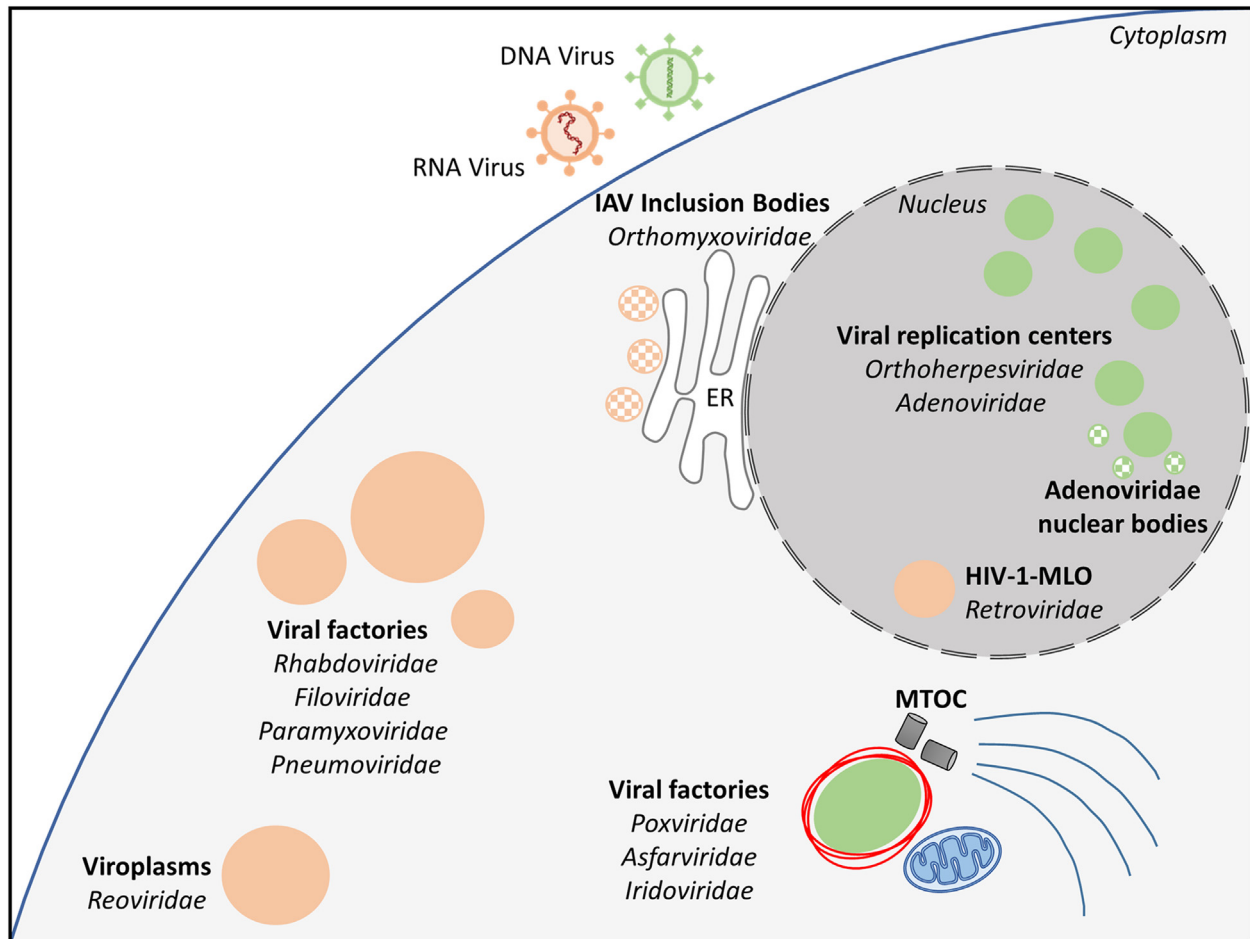


Fig. 3. Virus-induced BMCs RNA viruses (orange) and DNA viruses (green) induce the formation of viral condensates. These virus-induced condensates can host different stages of viral cycles. Condensates involved in the replication/transcription of viral genomes are in color, condensates facilitating certain assembly stage are hatched. The MTOC is represented as well as the vimentin cage (in red) around DNA cytosolic viral factories.

hybridization (FISH) experiments indicated that viral mRNAs as well as antigenomic and genomic RNAs are also concentrated in NBs [23]. Finally, using RNA pulse labeling with BrUTP and immunofluorescence staining using anti-BrdU, it was demonstrated that viral RNAs (either messenger, genomic, or antigenomic) are synthesized within the NBs [23] demonstrating that NBs are viral factories.

Shortly thereafter, cytoplasmic inclusions formed by other MNV including vesicular stomatitis virus (VSV, a member of the *Rhabdoviridae* family as RABV) [25], Ebola virus (EBOV, a *Filoviridae*) [26], respiratory syncytial virus (RSV, a *Pneumoviridae*) [27], human metapneumovirus (HMPV, a *Pneumoviridae*) [28], and measles virus (MeV, a *Paramyxoviridae*) [29] have been shown to host several viral replication stages.

3.2. Liquid properties of RNA viruses factories

The first demonstration that viral factories have liquid properties and were formed by LLPS was performed on RABV NBs [30] (Table 1). This was suspected due to their spherical aspect and the fact that in electron microscopy, they appear as dense and homogeneous structures devoid of membranes. The construction of a recombinant RABV expressing a fluorescent version of RABV P made it possible to perform live imaging on infected cells and to characterize the dynamics of NBs. It has been shown that, in

infected cells, NBs can fuse and reversibly deform when they encounter a physical barrier. They also quickly disappear in response to a hypotonic shock and reform within 10–15 min when the cell returns to homeostasis. Finally, when entire NBs were photobleached, the rapid fluorescence recovery was also consistent with a liquid nature and indicated that RABV P was able to shuttle between the NBs and the cytoplasmic pool [30].

Similar conclusions were then drawn about the liquid nature of other MNV factories including those formed by MeV [31], mumps virus (MuV) [32], VSV [33], RSV [27,34], HMPV [35], EBOV [36,37] and even a plant rhabdovirus, Barley yellow striate mosaic virus (BYSMV) [38] (Table 1). Beyond their liquid nature, MNV factories share several properties: their size increases over time [30,31,35] which is gradually accompanied by a loss of their spherical shape due to membrane association for RABV [30] or to a hardening/gelation process for MeV [31]. In terms of proteins involved in the LLPS process, the phosphoprotein P (or its equivalent, VP35, in EBOV) and the nucleoprotein (N) play a key role thanks to their IDD and their ability to form multimers [14,16,30–32,34,36]. Very generally, the transient expression of P and N in cells, alone or in combination, is sufficient to induce the formation of cytoplasmic inclusions having the same liquid properties as the viral factory [30,31,34–36]. In a certain number of cases, it has even been shown that these proteins expressed in heterologous and purified systems were capable of inducing phase separations *in vitro*

Table 1
Summary of the viral BMCs mentioned in this review.

Genome organization	Order	Family	Examples	Function of the BMCs	Viral components	Ref.
Viral condensates with liquid-like properties						
Non segmented ssRNA	Mononegavirales	Rhabdoviridae	RABV, VSV, BYSMV	RNA synthesis (genome transcription and replication)	P, N, L	[30,33,38]
		Filoviridae	EBOV	RNA synthesis (genome transcription and replication)	Vp35, N, L	[36,37]
		Paramyxoviridae	MeV, MuV	RNA synthesis (genome transcription and replication)	P, N, L	[31,32]
		Pneumoviridae	RSV, HMPV	RNA synthesis (genome transcription and replication), sequestration of NF- κ B subunit for RSV	N, P, L, M2-1	[27,34,35,60]
		Nidovirales	Coronaviridae	SARS-CoV2	Unclear although several functions have been proposed	NC
Ortervirales	Retroviridae	HIV	Nuclear reverse transcription centers	Viral capsid		[40]
		Rous sarcoma virus	Unknown	NC, CA, RT, IN		[50]
Segmented ssRNA	Articulavirales	Orthomyxoviridae	IAV	Viral assembly	vRNPs	[49]
	Bunyavirales	Phenuiviridae	SFTSV	Inhibition of IFN responses	NSs	[59]
Segmented dsRNA	Reovirales	Reoviridae	Rotavirus	Viral genome transcription and replication, packaging of the pregenomic RNA segments into the viral cores	NSP2, NSP5	[39]
dsDNA	Rowavirales	Adenoviridae	AdV	Replication centers	DBP	[41]
				Capsid assembly and genome encapsidation	52K	[47]
	Herpesvirales	Orthoherpesviridae	HSV-1 HCMV EBV KSHV	Replication centers	ICP4	[44]
				Replication centers	UL112-113	[42]
				Genome transcription	EBNA2, EBNA-LP	[43]
Latency regulation	LANA	[45]				
Amyloid Structures						
Segmented ssRNA	Bunyavirales	Phenuiviridae	RVFV	Role in pathology	NSs	[61]

[14,16,34,35,38]. This suggests that the principle of a viral factory formed by LLPS already existed in ancestral viruses of this order. However, over the course of evolution, each family, each genus has developed its own factory properties and organization. As an example, RSV factories contain highly dynamic subcompartments that concentrate newly synthesized viral mRNA and the viral transcription anti-terminator M2-1 while excluding viral genomic RNA and RNA polymerase complexes [27]. On the other hand, the EBOV polymerase is not homogeneously distributed inside the viral factories formed by the viral nucleoprotein (NP) and VP35 and instead clusters into foci [37].

Beyond MNV, rotavirus viroplasm have also been shown to be spherical and fuse [39]. Their formation requires the proteins NSP2 (an RNA chaperone) and NSP5 (that contains IDD) [39]. Upon mixing, purified NSP2 and NSP5 readily form BMCs at physiologically relevant micromolar concentrations. These condensates (cellular or acellular) were sensitive to several alcohols including 1,6-HD, 1,3-propanediol, and propylene glycol [39]. Taken together, all of these experiments indicated that rotavirus viroplasm form via LLPS. Again, at late stages of infection, the viroplasm exhibit altered material properties, losing their sphericity and becoming resistant to propylene glycol, suggesting that they are undergoing a gelation process [39].

Finally, once released into the nucleus, HIV-1 capsids associate with IDD-containing cleavage and polyadenylation specificity factor subunit 6 (CPSF6) proteins that form virus-induced BMCs, called HIV-1-MLO (for HIV-induced membraneless organelles) (Fig. 3 and Table 1). HIV-1 MLOs constitute nuclear reverse transcription centers, while the viral dsDNA, once synthesized, rapidly migrates outside these structures. In this case, it appears that HIV-1 hijacks a

cellular factor prone to LLPS to establish an environment favorable to viral replication [40].

3.3. Liquid properties of replication centers of DNA viruses

LLPS is not exclusive to the replication cycle of RNA viruses, since several studies showed that nuclear RCs of DNA viruses also have liquid properties (Fig. 3 and Table 1). The adenovirus RCs were demonstrated to coalesce, fuse and divide, indicating that they have properties of liquid condensates [41]. One of their major component, the viral ssDNA-binding protein (DBP), which contains IDDs and low complexity proline-rich regions, recapitulates their assembly and properties when transiently expressed. At later stages of infection, RCs eventually form an intranuclear mesh with less fluid-like features [41].

Similarly, herpesviruses RCs coalesce as infection progresses and can fuse together in a liquid-like manner [42–44]. In the case of herpes simplex virus type 1 (HSV-1), the key player in this process is apparently the viral transcription factor ICP4. This protein is an intrinsically disordered protein and a major component of the RCs, that is capable of driving protein condensation in the nucleus of transfected cells [44]. In the case of human cytomegalovirus (HCMV), the UL112-113 proteins, that are core components of RCs, undergo LLPS to form RCs in the nucleus, which is necessary to recruit the viral DNA polymerase for viral genome replication. Here again, purified UL112-113 proteins undergo LLPS at low salt concentration and their oligomerization domains and IDDs are required for LLPS in the cell [42]. Finally, EBNA2 and EBNA-LP proteins of Epstein–Barr virus (EBV) form BMCs in the nucleus having liquid properties and enriched in superenhancer sites

recognized by MYC and Runx3. Their mixing *in vitro* is also sufficient to induce LLPS. This phase separation of EBNA2 and EBNA-LP was proposed to be important for their transcription factor activity [43].

Condensate formation seems also to play an essential role during herpesvirus latency. This is exemplified by Kaposi's sarcoma-associated herpesvirus (KSHV) which induces 1,6-HD sensitive nuclear BMCs of its latency-associated nuclear antigen (LANA) to tether viral genomes to centrosomes during cell division [45]. LANA contains IDD and binds to the terminal repeats of KSHV genomes. Its oligomerization is required for the formation of the BMCs and its colocalization with cellular factors, such as DAXX, and the origin recognition complex. However, upon lytic reactivation, LANA BMCs increase in number and size and adopt a distinct morphology forming ring-like structures associated with KSHV DNA, from which DAXX is evicted [45].

Overall, the behavior of adenovirus and herpesvirus nuclear RCs is reminiscent of that of cytoplasmic MNV viral factories: at early stages of infection, they are spherical and behave like homogeneous BMCs and, as the infection progresses, their morphology and properties evolve. Eventually, it is also worth mentioning that neither HSV-1 [46] nor AdV RCs [47] are disrupted by 1,6-HD treatment. Furthermore, for HSV-1-RCs, although Pol II recruitment to RCs exhibits characteristics of liquid–liquid demixing, the polymerase diffuses freely across RC boundaries, which is inconsistent with an LLPS model. Consequently, an alternative model of HSV-1 RCs formation, not based on LLPS, has been proposed [46].

To date, there are few data (if any) concerning the physicochemical nature and molecular bases underlying the morphogenesis of viral factories of large DNA viruses whose replication is cytoplasmic. This is thus a field of investigation that remains completely open.

3.4. Other viral BMCs

Other viral BMCs that are not directly involved in the replication/transcription of viral genomes but are associated with other stages of the viral cycle, very often an assembly stage, have also been described (Table 1). Indeed, by increasing the local concentration of viral components and excluding cellular proteins, which ensures specific interactions, viral BMCs facilitate virion assembly (Fig. 3).

In the case of AdV, at the periphery of the RCs, there are other nuclear BMCs which bring together the viral proteins involved in the assembly of the capsid and the encapsidation of the genome [47] (Fig. 3). The phase separation is driven by a 52-kDa protein (52K) that plays an essential role in the coordinated assembly of infectious progeny particles. The BMCs it forms coordinate the assembly of capsid with the delivery of viral genomes necessary for the production of complete packaged particles. Failure to form BMCs or to internally recruit viral factors critical for assembly results in a lack of genome packaging and the assembly of only non-infectious particles [47].

Regarding herpesviruses, it has been suggested that the tegument, an amorphous multiprotein layer located between the nucleocapsid and lipid envelope of the virion is itself a BMC, made of a heterogeneous mixture of intrinsically disordered tegument proteins. Indeed, UL11, the smallest tegument protein of HSV-1 was shown to undergo LLPS *in vitro* [48].

Influenza A virus (IAV) is a segmented negative-strand RNA virus with each of the eight segments encapsidated as an individual viral ribonucleoprotein (vRNP) complex. For an IAV particle to be

infectious, all eight vRNPs must be packaged into a virion. Virions rarely contain more than eight segments and each segment is present as a single copy per virion. It has been shown that IAV-infected cells contain inclusion bodies located near the endoplasmic reticulum (ER) exit site (Fig. 3). These inclusions have liquid properties and contain vRNPs and GTPase Rab11 that is required for their formation. Installation of these structures precedes inter-vRNPs interactions suggesting that these inclusions facilitate these interactions [49].

Retrovirus assembly is mediated by the Gag polyprotein through its oligomerization and its ability to recruit the viral genomic RNA and several host factors. Gag is proteolytically cleaved after viral budding into the mature matrix, capsid (CA), and nucleocapsid (NC) proteins (that are common to all retroviruses) as well as small virus specific peptides. The Gag polyprotein of Rous Sarcoma virus, which is enriched in IDDs, particularly in its NC domain, was demonstrated to form condensate-like structures exhibiting fluid-like behavior both in transfected cells and *in vitro* [50]. Similarly, purified HIV-1 NC and CA as well as the reverse transcriptase (RT) and the integrase (IN) were shown to individually form condensates *in vitro* in buffers containing Ficoll as a crowding agent [47]. When the proteins were mixed, they were shown to co-condensate [51]. Using a HEPES-based fluorescently-labeled protein transfection method, the authors also visualized NC, IN, and RT co-condensates with liquid behavior in live cells. NC appears to play a key role in the co-condensation process acting as a scaffold on which client CA, RT, and IN condensates assemble [51].

Finally, in the case of positive-strand RNA viruses, viral factories are mainly associated with membranes. Nevertheless, several teams have independently shown that the SARS-CoV-2 NC protein is capable of phase separation in cell-free systems or when ectopically expressed [52]. Similarly, the primer-independent RNA polymerase (nsp8) of SARS-CoV-2 has been reported to form BMCs [53]. To date, all these inclusions have not been observed in the infected cell, which calls into question all the functions attributed to them based on experiments carried out *in vitro* or in transfected cells.

4. Cellular antiviral innate immunity and BMCs

4.1. Antiviral innate immunity pathways

The host interferon (IFN) response represents one of the first barriers that viruses must overcome in order to establish a productive infection. The IFNs are expressed and secreted following viral components recognition by dedicated receptors able to bind to VAMPs and known as pattern recognition receptors (PRRs). Subsequent binding of IFNs to their receptor, present at cell surface, leads to the up-regulation of hundreds of IFN-stimulated genes (ISGs) that establish an antiviral state to limit further viral replication and spread [54].

Two subsets of PRRs, the Toll-like receptors (TLRs) and the RIG-I like receptors (RLRs), are involved in the recognition of RNA viruses [55,56]. TLRs are transmembrane proteins that reside either at the cell surface or in endosomal compartments [55]. TLR3, which recognizes double-stranded RNA (dsRNA), and TLR7, which recognizes single-stranded RNA (ssRNA) molecules, are key players in the detection of RNA viruses. RNA binding by those TLRs activates a signaling cascade that leads to the phosphorylation of transcription factors IRF3 and/or IRF7 by IKK-related kinases, TBK1 and IKKε. The phosphorylation of IRF3 and IRF7 results in their homo- or heterodimerization, which induces type I IFN-related genes [55].

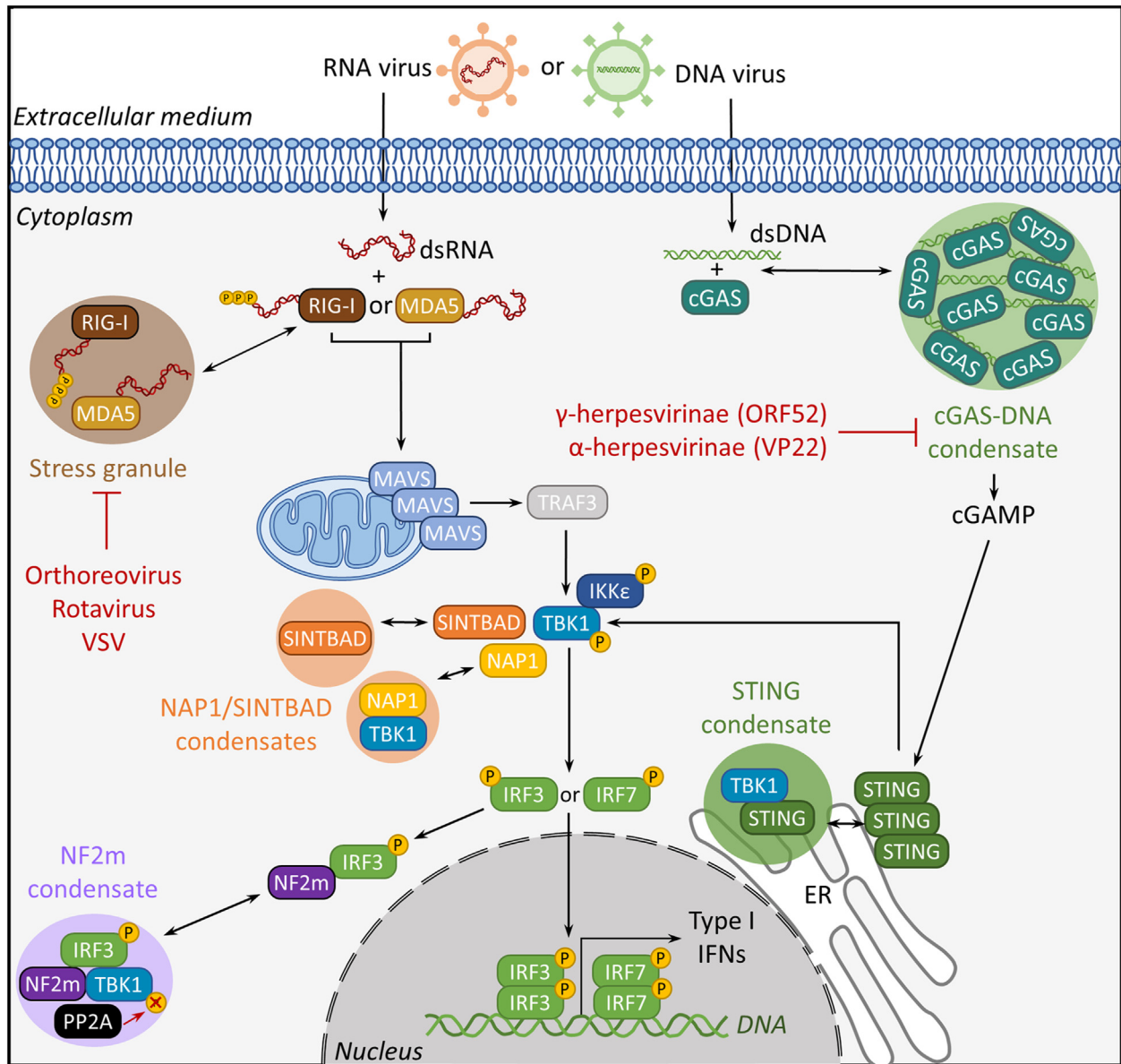


Fig. 4. BMCs formed along IFN induction pathway Schematic representation of the different condensates formed along IFN induction pathways. RIG-I and MDA-5 are cytosolic PRRs recognizing non-self RNAs in the cell cytoplasm and thus RNA virus infection. Upon activation, they associate with the mitochondrial antiviral signaling protein (MAVS) at the outer mitochondrial membrane. This interaction induces MAVS oligomerization and TBK1-activating complexes resulting in phosphorylation, dimerization, and nuclear import of IRF3 and/or IRF7, and consequently the expression of type I IFNs. Upon detection of cytosolic dsDNA, cGAS forms liquid condensates with DNA, which stimulates its enzymatic activity. The generated 2',3'-cGAMP binds and activates STING, which itself activates TBK1. NAP1 and SINTBAD are TBK1 adaptors that also form condensates. Steps neutralized by viruses are indicated by red lines. For more details, see text.

RIG-I and MDA-5 are cytosolic receptors recognizing non-self RNAs in the cytoplasm (Fig. 4) [56]. RIG-I is activated by short stretches of 5'ppp or 5'pp dsRNA while MDA-5 is activated by longer stretches of dsRNA in a 5' phosphate-independent manner. They both contain two caspase activation and recruitment domains (CARDs) at their N-terminus. RNA binding triggers a structural transition resulting in CARDs exposition. Downstream signal transduction occurs by the CARD-dependent association of RIG-I/MDA-5 with the mitochondrial antiviral signaling protein (MAVS) at the outer mitochondrial membrane. This interaction induces MAVS oligomerization and assembly of TBK1- and/or IKKε

containing complexes (involving adaptor proteins such as NAP1/AZI2, SINTBAD/TBKBP1 and TANK) that activate TBK1 and IKKε kinase activity. Once again, this results in IRF3 and/or IRF7 phosphorylation, followed by their homo- or heterodimerization, nuclear translocation, and consequently the expression of type I IFNs (Fig. 4) [56].

Regarding dsDNA viruses, the sensing of cytosolic dsDNA by cyclic GMP-AMP synthase (cGAS) is also a danger signal. DNA binding stimulates cGAS enzymatic activity [57]. The generated cyclic dinucleotide 2',3'-cGAMP then acts as second messenger by binding and activating the stimulator of IFN genes (STING) (Fig. 4).

As a result, STING translocates from the ER to the Golgi complex and activates TBK1 and/or IKK ϵ , leading once again to the expression of type I IFNs (Fig. 4).

Finally, in addition to their central role in type I IFN expression, activated TBK1 and IKK ϵ also induce the non-canonical NF- κ B pathway leading to the expression of several genes involved in inflammatory and apoptotic responses.

4.2. Interplay between antiviral innate immunity and viral factories

Beyond the fact that they promote the replication of viral genomes, as well as the assembly of virions by concentrating the proteins and macromolecules necessary for these stages, viral factories also make it possible to conceal the infection from the actors of cellular innate immunity. This is particularly the case for MNV: dsRNAs, which trigger IFN responses, are located inside the viral factories. Thus the phase separation may prevent the detection of these VAMPs by RIG-I and MDA-5 [58].

On the other hand, the viral factories or other virus-induced inclusion bodies may sequester cellular proteins involved in innate immunity and keep them away from their functional signaling pathway. For example, the nonstructural protein NSs of the severe fever with thrombocytopenia syndrome virus (SFTSV), a *Phenuiviridae* having a segmented negative strand RNA genome) relocalizes RIG-I, TBK1, IKK ϵ , and IRF3, into NSs-induced cytoplasmic spherical inclusion bodies. This results in a strong inhibition of IFN responses [59]. Similarly, RSV is able to sequester NF- κ B subunit p65 into its viral factories to inhibit innate immune signaling [60]. Note that the inclusions that sequester innate immunity proteins do not all have liquid properties. Thus, several viral proteins involved in virulence, such as the NSs protein of Rift Valley fever virus (RVFV), have been described to form amyloid structures which are apparently capable of preventing the antiviral cellular response, most probably by sequestering key players in this response [61] (Table 1).

4.3. The induction of IFN pathway by viral infections involves the formation of BMCs

It now appears that phase-separated BMCs play a role throughout the IFN-induction pathway (Fig. 4) [4]. Thus, RIG-I and MDA-5 are often detected in SGs induced by viral infection [62,63] (Fig. 4) and it has been proposed that SGs can serve as platforms for the activation of several PRRs [63]. As a result, there is a subtle interplay between SGs and viral factories.

In general, inhibition of SGs formation is advantageous for viral replication since the presence of SGs inhibits the translation of mRNAs they sequester [64]. Indeed, several dsRNA viruses, such as rotaviruses, have been shown to disrupt SGs or sequester their components in the viroplasm [65]. In VSV-infected cells, it has been shown that several proteins such as the cellular poly(C) binding protein 2 (PCBP2), the T-cell-restricted intracellular antigen 1 (TIA1), and the TIA1-related protein (TIAR), that are normally associated with SGs, are found associated within the viral factories [66]. However, other SG markers, such as eukaryotic initiation factor 3 (eIF3) or eIF4A are not present in the viral factories [66]. Finally, in the case of RABV infection, SGs come into close contact with NBs but do not fuse with them indicating that their liquid phases are not miscible [30]. However, RABV mRNAs are transported from NBs (their site of synthesis) into SGs via an unknown mechanism suggesting an antiviral role for SGs [62]. Consistent with this idea, depletion of protein kinase R (PKR), which prevents RABV-induced SGs formation, promotes viral multiplication [62].

Regarding DNA viruses, binding of cytosolic dsDNA to cGAS also induced the formation of BMCs in which cGAS is activated [67].

Downstream of the pathway, upon 2',3'-cGAMP-induced activation, STING itself polymerizes [68] and forms condensates with stacked ER membrane [69] (Fig. 4). Again, DNA viruses are able to thwart this process. Thus, gamma- and alpha-herpesvirinae genomes encode a tegument protein (ORF52 for gamma-herpesvirinae and VP22 for alpha-herpesvirinae) that uses a conserved mechanism to restrict cGAS-DNA phase separation [70] (Fig. 4). These proteins accumulate into, and disrupt, the pre-formed cGAS-DNA BMCs. The viral proteins do not appear to interact directly with cGAS and the inhibition process rather depends on DNA-induced LLPS separation by the viral protein. Finally, the number of ORF52 proteins present in viral particles is sufficient to target cGAS-DNA BMCs in early infection stages [70].

Beyond the initiation of the cascade leading to IFN induction, other BMCs are involved downstream in the signaling pathway. Thus, during RABV infection or upon dsRNA detection by innate immunity sensors, TBK1 and its adaptor proteins NAP1/AZI2 and SINTBAD/TBKBP1 are found in BMCs having liquid properties [15] whose role remains to be elucidated (Fig. 4).

Finally, it is worth noting that inappropriate BMCs formation may impact cellular immunity. As an example, mutants of the tumor suppressor Neurofibromin 2 (NF2) have recently been shown to form liquid BMCs [71]. These BMCs are induced by activated IRF3, following nucleic acid sensing by both the RLR-MAVS pathway and the cGAS-STING pathway. They contain both TBK1 and the phosphatase PP2A which inactivates the kinase by dephosphorylating it [71] (Fig. 4). As these BMCs have not been detected with wild-type NF2, it remains unclear whether such a platform, containing IRF3 and numerous enzymes, participates in other critical cellular processes.

One may wonder about the evolutionary reasons which made BMCs omnipresent in the field of IFN pathway induction. First, by concentrating proteins, the formation of BMCs facilitates proximity-induced enzyme activation. Second, the dynamics of liquid BMCs as well as their sensitivity to their physicochemical environment enable the formation of biological switches which can be further regulated by PTMs. Indeed, LLPS is consistent with a threshold-type digital signaling response allowing a fast reaction to a danger signal that is an all-or-nothing process.

4.4. ISG expression is also associated with BMCs formation

Several ISG products also form BMCs (Fig. 5). This is the case of different Mx proteins, which are large GTPases of the dynamin family, induced in cells exposed to type I and type III IFNs and presenting a broad spectrum of antiviral activities [72]. There are two human Mx proteins (MxA and MxB) and two murine Mx proteins (Mx1 and Mx2) [72]. Human MxA forms cytoplasmic BMCs of variable size, some of which are attached to intermediate filaments [73], while murine Mx1 instead forms nuclear bodies [74] (Fig. 5). In both cases, the BMCs are disassembled by 1,6-HD and upon exposure to hypotonic buffer suggesting that they are formed by LLPS [73]. However, FRAP was limited, indicating only ~20% internal mobility [73]. It should be noted that, on the cytoplasmic side of nuclear pores, human MxB also forms membraneless structures that are also suggested to be phase-separated BMCs [72]. Although the mechanisms of action of Mx proteins are not fully understood, their antiviral activity is directly linked to the localization of the BMCs. For example, human MxA has an antiviral activity against IAV and VSV while the nuclear murine Mx1 has antiviral activity against IAV (which replicates in the nucleus of the infected cell) but not against VSV (whose entire cycle is cytoplasmic) [72] (Fig. 5). Moreover, in VSV-infected Huh7 cells, previously transfected with a plasmid expressing GFP-MxA, the viral N protein was found associated with GFP-MxA BMCs [73].

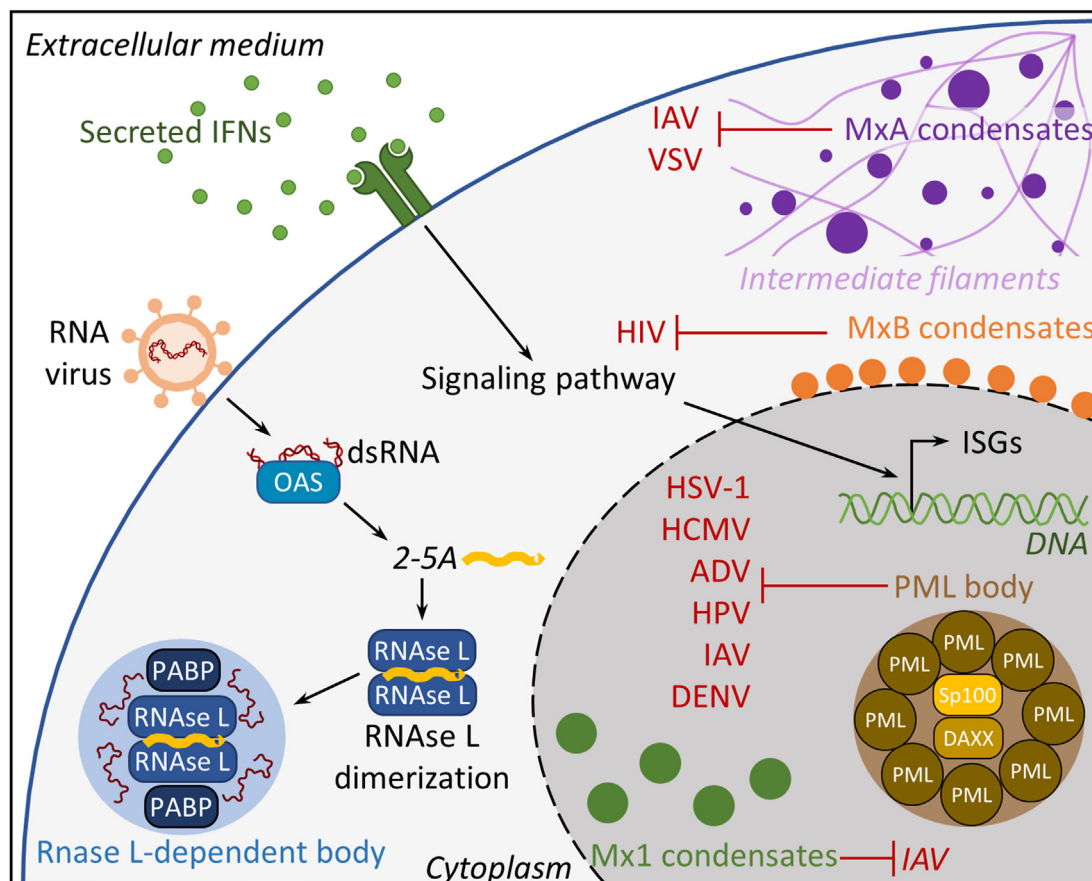


Fig. 5. BMCs formed by ISGs products. Some ISGs products can form condensates that interfere with different viral cycles. The name of the viruses that are concerned are indicated in red. For more details, see text.

IFN treatment also induces the upregulation of several proteins constituting the promyelocytic leukemia protein (PML) nuclear bodies, including PML itself and the speckled protein of 100 kDa (Sp100) [75]. PML bodies exhibit liquid-like properties [76] and have a wide range of antiviral activity. Indeed, PML depletion and PML nuclear bodies disassembly reduce the capacity of IFNs to protect from viral infections [75]. The PML protein has opposing effects at distinct stages of infection. On the one hand, human papillomavirus (HPV) infection requires the presence of PML protein suggesting that PML bodies are essential for establishing infection [77]. On the other hand, Sp100 and death domain-associated protein 6 (DAXX, another resident protein of the PML bodies), which act as transcriptional repressors, limit viral gene expression of several DNA viruses including HCMV [78], AdV [79], and HPV [80] (Fig. 5). As a well-characterized example, PML bodies, which initially surround incoming HSV-1 genomes, are disrupted by newly synthesized viral ICPO proteins [81]. More surprisingly, PML nuclear bodies also exert antiviral activity on some RNA viruses including dengue virus [82] and rhabdoviruses [83] although their cycle is strictly cytoplasmic. Again, RNA viruses have evolved mechanisms that counteract PML function. As an example, RABV P interacts with and retains PML in the cytoplasm to reorganize PML bodies [84] and is therefore thought to counteract the antiviral effect of the PML IV isoform against RABV infection [83].

Finally, the activation of RNase L upon RNA virus infection results in the formation of the so-called RNase L-dependent bodies (RLBs) that are highly dynamic and contain polyadenylated RNA and polyA binding protein (PABP) [85] (Fig. 5). RNase L is not an ISG as it is present in a latent state in all cells. However, it is activated by

2',5'-oligoadenylates (2-5A) synthesized by 2-5A synthetases (OAS, which are ISGs), that are themselves activated by binding to dsRNA. Once activated, the endonucleolytic cleavage of mRNAs induces the formation of RLBs [86].

The activity of the ISG products - often toxic to the cell - must be tightly regulated. In particular, although these proteins are synthesized when the cell is exposed to IFN, they should only be active when the cell is actually infected. Here again, the formation of BMCs with regulatory activity, which can be positive or negative, certainly plays a primordial role in these processes.

5. Target for antiviral therapies

The fact that BMCs play a central role in the replication of several viruses and in innate immunity makes them an attractive target for antiviral treatment. Indeed, molecules that either dissolve or harden BMCs should have an impact on their functions.

Thus, two molecules capable of hardening viral BMCs have been identified and demonstrated to have an antiviral action. First, cyclopamine has been shown to inhibit RSV replication, both *in vitro* and in mouse models, due to its ability to harden viral factories which lose their liquid properties and acquire gel properties [87]. Second, nucleozin has been shown to exert its antiviral activity against IAV by hardening the viral BMCs containing vRNPs and Rab11 both in cells and in infected mice [88]. In this work, it was demonstrated that the hardening effect of nucleozin was directly related to its ability to stick together all forms of IAV nucleoprotein (either free or in vRNPs) [88]. This indicates that targeting the multivalency and/or the interaction strength between

condensate components could be a general way to inhibit functions associated with viral BMCs. Indeed, the viscosity of the viral BMCs must be finely tuned for the proper functioning of the viral polymerases/enzymes and the correct assembly of virions.

For the moment, no molecule dissolving viral BMCs has been shown to have an impact on viral replication. There is no doubt, however, that this is an interesting approach worth exploring.

Finally, modulating the partition coefficient of drugs targeting the replication complex so that they accumulate in viral factories could increase their effectiveness as well as their specificity and thus limit their toxicity.

6. Conclusions and perspectives

Numerous recent articles have underlined the importance of BMCs at the stages of replication of viral genomes and virion assembly as well as in the innate immune response (both in signaling pathways and in the regulation of the activity of ISG products). However, this field is still in its infancy and should hold many surprises as it changes our way of understanding viral replication and the regulation of innate immunity.

An interesting aspect regarding the interactions between viral factories and innate immunity that, to our knowledge, has not yet been raised is that the viral factory is a foreign body in the cell. Thus, the presence of viral factories having liquid properties is a signature of viral infection. In one way or another, they can also be considered VAMPs. Some cellular proteins may be capable of recognizing the interfaces between the cytoplasm and the viral factory, thus triggering a signaling cascade. It is also possible that some other proteins, potentially products of ISGs, can modify the factory properties by solubilizing or hardening it. These are open questions that remain to be explored further.

Little is known about the proteome of these compartments. Regarding viral factories, to date, it is essentially cellular proteins that stably bind viral proteins that have been identified. Clearly, the proteome of the viral factories is much larger. However, their liquid nature as well as the fast exchange between them and the rest of the cell (the half-time residence of the proteins is in the order of a few seconds) preclude their purification in their integrity. Proximity labeling is an alternative promising approach that has been already used to identify the proteome of SGs [89]. Likewise, the presence of specific RNAs of cellular origin (that are much less abundant than the viral ones) in the MNV factories remains to be investigated. It should be noted, that the proteome and RNome of viral factories as well as other cellular BMCs are probably not uniform and likely vary over time making the overall analysis all the more complex [89].

Interactions between BMCs (from cellular or viral origin) and the cytoskeleton [25,28,30,31,69] as well as cellular membrane compartments [14,30,69] have been documented. However, their role and the molecular bases underlying these processes remain to be finely characterized. In the case of viral factories, understanding these processes may contribute to the development of novel antiviral strategies.

As mentioned above, we have only a very general understanding of the molecular basis behind LLPS and their regulation. A more detailed understanding is absolutely required to rationally develop molecules interfering with these processes. These molecules must also specifically target the compartments of interest and not have a general effect on others. Minimal acellular or cellular systems, recapitulating the properties of natural BMCs, are tools for characterizing the weak interactions that allow the formation of these compartments and carrying out initial molecule screens.

It should be noted that in this review, we focused on virus-induced organelles with liquid properties. As mentioned above,

recent studies have shown that viral proteins involved in virulence often form amyloid-like fibrils [90]. The molecular and cellular mechanisms underlying their formation, their functions, as well as their relationships with other cellular or viral BMCs remain to be explored.

Finally, if there is a justified enthusiasm around membraneless organelles, which results in a large number of new cellular BMCs identified, it is clear that we often still remain at a descriptive level. A significant effort will have to be made to fully understand the function of these condensates, particularly with regard to the regulation of innate immunity and, beyond that, more generally of cellular signaling pathways.

CRediT authorship contribution statement

Damien Glon: Conceptualization, Validation, Writing – original draft, Writing – review & editing. **Benjamin Léonardon:** Writing – review & editing. **Ariane Guillemot:** Writing – review & editing. **Aurélien Albertini:** Writing – review & editing. **Cécile Lagaudrière-Gesbert:** Conceptualization, Writing – original draft, Writing – review & editing. **Yves Gaudin:** Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that they have no conflicts of interest.

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