REVIEW

Phase separation in immune regulation and immune-related diseases

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Abstract
Phase separation is an emerging paradigm for understanding the biochemical interactions between proteins, DNA, and RNA. Research over the past decade has provided mounting evidence that phase separation modulates a great variety of cellular activities. Particularly, phase separation is directly relevant to immune signaling, immune cells, and immune-related diseases like cancer, neurodegenerative diseases, and even SARS-CoV-2. In this review, we summarized current knowledge of phase separation in immunology and emerging findings related to immune responses as they enable possible treatment approaches.

Keywords Phase separation · Immunity · Inflammation · Autoimmunity · Cancer

Introduction
Phase separation refers to the phenomenon that when the concentration of solutes exceeds a certain threshold, known as the saturation concentration, the intermolecular force between biomacromolecules is bigger than that between biomacromolecules and the surrounding substances [1]. Above this threshold, constituents separate spontaneously into two phases yet with different concentrations, one dilute and one condensed. These spatiotemporally regulated subcellular compartments form the basis of biochemical reactions. Phase separation allows certain proteins to condensate while excluding undesired proteins outside and then favors consequent biochemical reactions (Fig. 1). Phase separation well answered how membrane-less organelles in eukaryotes manage to assemble and how this process is modulated. For instance, P granules were membrane-less organelles and were demonstrated to have a spherical shape, and their components were capable of simultaneous dynamic exchange [2]. Stress granules (SGs) are typical examples of membrane-less organelles which are found to have unstable liquid shells yet stable non-liquid cores [3]. Unlike protein aggregation where substrates lose biochemical activity due to misfolding or loss of mobility, phase-separated substrates often retain their bioactivity and conformation [4]. Also, due to the lack of membranal structures, phase separation enables organelles to exchange much more rapidly without the transportation across layers of membranes [5]. Liquid–liquid phase separation (LLPS) is a process in which liquid phases form liquid droplets with higher viscosity and show traits like a spherical shape; live image tracing shows rapid protein diffusion within the condensates as well as exchange with the surrounding environment as measured by fluorescence recovery after photobleaching (FRAP) [1, 6].

Liquid condensates can exchange substances with dilute phases, and liquid condensates themselves also commonly undergo reversible fusion and fission. However, some droplets can abnormally transition to solid aggregates and may lead to cellular dysfunction and the development of some diseases. For example, mutations in SG proteins, such as fused with sarcoma (FUS) and hnRNPA1, are confirmed to form fibrillar aggregates associated with neurodegenerative diseases [7]. In addition, the state of phase separation is affected by a number of environmental factors that include, but not limited to, temperature, pH, and salinity [8]. Meanwhile, phase separation can also be modulated by cytomembrane structures, post transcriptional modifications (PTMs) like phosphorylation/methylation of amino acids, the concentration of proteins, the availability of RNA, chaperones, RNA helicases, and so forth [9, 10]. But there are still gaps in our knowledge about the exact mechanism.

In recent years, phase separation has shown its importance in numerous events such as chromatin organization [11].
The proteasome foci formed by phase separation ensures a tight-controlled equilibrium between protein synthesis and degradation [13]. Mounting evidence has also highlighted that phase separation is implicated in immune cell maturation and activation, immune signaling, immune modulation, and in tumorigenesis. Here, we underscore the pleotropic effect of phase separation in immune regulation and discuss their potential in the pathogenesis of several immune-related diseases such as cancer, Aicardi-Goutières syndrome (AGS), and neurodegenerative diseases. The phase separate-competent proteins already identified might only be the tip of the iceberg, with potentially more novel and fundamental events to be revealed.

### Phase separation in immune regulation

#### TCR signaling

Upon detecting the antigens presented by MHC molecules, T cell receptor (TCR) activates T cells and initiates downstream immune responses. In this process, downstream effector proteins coalesce into dynamic clusters by spontaneous phase separation to facilitate signaling [14] (Fig. 2). The phosphorylation of TCR complex by lck recruits and activates zeta-associated protein of 70 kDa (ZAP-70) [15]. Then, ZAP70 phosphorylates LAT, which provides docking sites for SH2/SH3 domain protein such as Grb2, Gads, and phospholipase Cγ (PLCγ) [16, 17]. The multivalent interactions between the above proteins drive LAT cluster formation. The LAT clusters colocalize with ZAP70 but exclude phosphatase CD45 [14]. They also promote TCR signal transduction by promoting MAPK phosphorylation and actin remodeling [18].
PLCγ1 is often viewed as a hydrolytic enzyme that is recruited to the LAT cluster, activated downstream of TCR activation [19]. PLCγ1 produces inositol trisphosphate (IP3) and diacylglycerol (DAG) by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2) to activate calcium and PKC pathways respectively [20]. However, a recent study delineated a novel role of PLCγ1 in potentiating LAT cluster formation. PLCγ1 crosslinks LAT through its SH2 domains and promotes the phase separation of LAT and LAT-dependent ERK activation [21]. PLCγ1 also inhibits the dephosphorylation of LAT by CD45, which is achieved by excluding CD45 from the cluster [21]. As natural killer cells and mast cells share the same LAT cluster with T cell, Zeng et al. anticipate that PLCγ1 may play a similar role in other immune cells [21].

Aberrant TCR function could lead to T cell immunodeficiency or T cell–mediated autoimmune diseases. For example, dysfunction of ZAP-70 could lead to T cell–mediated autoimmune arthritis [22]. Also, TCR is vital to the differentiation of Foxp3(+) regulatory T cells (Treg), the malfunction of which causes lethal multiorgan immune responses [23].

**BCR signaling**

Effector B cell activation is essential in the generation of antibodies and adaptive immune responses. Phase separation confers B cell antigen receptor (BCR) with higher sensitivity to pathogens. BCR is temporally and spatially regulated through adaptor proteins like SH2 domain-containing leukocyte protein of 65 kDa (SLP65) and Cbl-interacting protein of 85 kDa (CIN85) [24]. Although SLP65 and CIN85 are adaptor proteins that regulate B cell signaling; they are insufficient to trigger BCR activation. Upon cognate antigen ligation, spleen tyrosine kinase (Syk) phosphorylates SLP65 in a SLP65/CIN85 complex-dependent manner [25]. Phosphorylated SLP65 then leads to mobilization of Ca²⁺ or the translocation of nuclear factor kappa-beta (NF-κB) to initiate immune responses [26].

Previously shown, the binding of N-terminal amino acid residues in SLP65 to lipid components on the cellular surface forms a macromolecular assembly which is required for BCR activation [27]. Also, the coiled-coil domain in CIN85 drives CIN85 trimerization and further recruits CIN85 trimers [28]. These studies hint that the oligomerizations of adaptor proteins may contribute to signal amplification, but the exact mechanism was unknown.

A recent in vitro study showed that the activation of BCR is a tripartite course, requiring the phase separation of SLP65, CIN85, and lipid vesicles into droplets [29] (Fig. 3). The study utilized small unilamellar vesicles (SUVs) to recapitulate psychological phase separation threshold in vivo. SLP65 uses its N-terminal lipid binding to detect and anchor small vesicles on curved cellular surfaces [29]. The interaction between SH3 domain in CIN85 and proline-rich motifs (PRMs) in SLP65 facilitates the transformation to droplets [29]. This in vitro study confirmed the requisite role of phase

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**Fig. 3** Phase separation in BCR signaling. Upon antigen ligation, SLP65 is phosphorylated by Syk. Then, the tripartite phase separation of SLP65, trimer CIN 85, and lipid vesicles into droplets leads to BCR activation. SLP-65 is anchored to the lipid vesicles through its N-terminal. The PRMs in SLP-65 and SH3 domain in CIN85 form a positive loop that promotes phase separation. (Created with Biorender.com)
separation. But it still warrants in vivo investigation into the ultrastructure of this droplet and the existence of other vesicular modulators of BCR.

**cGAS-STING pathway**

Cyclic GMP-AMP synthase (cGAS) is a cytosolic immune stimulatory DNA sensor that detects pathogenic and intracellular endogenous DNA and leads to immune activation [30]. cGAS possesses positively charged residues on its N-terminal, as well as 3 DNA binding domains (DBDs) on its C-terminal. Both terminals contribute to the cGAS-DNA phase separation, thus triggering the formation of liquid droplets which enables cGAS and DNA within to interact at a higher concentration [31, 32]. DNA exonuclease TREX1 degrades cytosolic DNA and inhibits consequent immune activation, preventing autoimmune diseases like AGS and systemic lupus erythematosus (SLE) [33–35]. Recent studies confirmed that the LLPS between cGAS and DNA also suppresses immune inhibitor BAF, TREX1 and restricts self-DNA degradation [36].

The activation of cGAS results in an increase in cGAMP which is a secondary messenger in innate immune responses [31, 37]. cGAMP activates STING which then recruits TBK1 and leads to the oligomerization and translocation of interferon (IFN) regulatory factor 3 (IRF3) [32]. The translocation of IRF3 promotes type-I IFN production along with other inflammatory cytokines thereby triggers innate immune signaling [30]. This process is also known as IFN gene–dependent IFN stimulatory DNA (ISD) pathway [38]. The study by Qin et al. disclose that IRF3, IFN-stimulated response element (ISRE) DNA, and compartmentalized IRF7 together undergo LLPS in which deacetylation by sirtuin (Sirt1) is identified as an essential step [39]. The liquid droplet formed by IRF3 and ISRE DNA facilitates type-I IFN production and IFN-stimulated genes. Senescent cells and aged mice with Sirt1 deficiency showed hyperacetylation at their DBDs accompanied by diminished innate immune response, which can be rescued by Sirt1 agonist [40].

Latest research has revealed a negative role of LLPS in this pathway. In the milieu of translocated STING activated by cGAMP, the remaining STING resident in the endoplasmic reticulum undergoes LLPS condensation and forms a spherical biocondensate with puzzle-like structures, termed “STING-TBK1-cGAMP sponge.” This sponge prevents STING and TBK1 from overreaction and suppresses immune responses [41]. Besides signal transduction, cGAS is also capable of identifying pathogen-associated molecular pattern (PAMP) in which phase separation helps aggregate exogenous pathogens [42].

Interestingly, a recent study revealed that phase separation could be an evolutionarily conserved way of viral immune evasion. Viral tegument proteins like UL37 from herpes simplex virus (HSV) [43], VP22 from HSV1 [44], and pUL83 from human cytomegalovirus [45] were identified as cGAS antagonists that are responsible for viral immune resistance in an unknown way. Kaposi sarcoma-associated herpesvirus (KSHV) inhibitor of cGAS (KicGAS) or ORF52 forms liquid droplets upon DNA binding [46]. The oligomerization of KicGAS not only inhibits phase separation between cGAS and DNA but also impedes cGAS activation. Another study by Xu et al. showed that tegument protein ORF52/VP22 from herpesvirus diffuses into the cGAS-DNA condensates. ORF52/VP22 replaces cGAS and phase separates with DNA instead [47]. Similarly, another tegument protein ORF9 from Varicella-Zoster virus (VZV) is also identified as an antagonist of cGAS. The homolog of ORF52/VP22, ORF9, is capable of coalescing into a spherical liquid droplet displaying LLPS traits [47]. Also, the phase separation between cGAS and DNA is abrogated upon ORF9. Another study by Herzog et al. utilized confocal microscopy to show that ORF9 not only colocalizes with cGAS but also interacts and phase separates with cGAS and DNA [47, 48]. It is possible that phase separation is a common mechanism through which viral tegument protein interrupts with cGAS-STING signaling.

To conclude, LLPS (1) promotes the interaction between cGAS and DNA, (2) activates cGAS through inhibition of BAF1 and TREX1, (3) forms the STING Phase-Separator to limit overreaction; (4) helps cGAS identify PAMP, and (5) enables viral immune evasion. Thus, we conclude that phase separation displays pleiotropic effects in cGAS-STING signaling.

**MAVS-induced IFN production**

Severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) is an urgent threat to global public health worldwide [49]. Coronaviruses possess nucleocapsid proteins (NPs) that interact with the viral RNA genome and the M protein to assemble into virion, a complex vital for viral replication [50]. Upon immune recognition of the viral RNA, adaptor protein mitochondrial antiviral signaling protein (MAVS) gathers to activate NF-κB and IRF3 that instigates type I IFN pathway [51–53].

SARS-CoV-2 utilizes a highly efficient immune evasion mechanism as exemplified by low IFN level in human lung tissue ex vivo and the difficulty to detect IFN expression in the patients’ serum [54, 55]. The SARS2-NPs encompass intrinsically disordered regions (IDRs) that are prone to phase separate. Indeed, Wang and colleagues observed that SARS2-NPs coalesce into dynamic droplets but intriguingly independent of the IDRs [56]. Instead, the dimerization domains on their C-terminals drive LLPS, thereby blunting MAVS aggregation and proper ubiquitination.
Iseman et al. report that the LLPS of SARS2-NP is most efficient at 33 to 37° and reduces as the temperature decreases [57]. Hampered MAVS aggregation would dampen innate antiviral defense and lead to immune evasion due to weakened IFN production and related genes. Savastano and colleagues observed that the LLPS of SARS2-NP attracts RNA-dependent RNA polymerase complex which promotes viral replication [58].

Research has shown that successful induction of IFN, especially in the early stage, prevents severe clinical manifestations [59]. Given that SARS2-NPs phase separation plays a crucial role in immunosuppression, targeting SARS2-NP phase separation might elevate IFN production and improve clinical outcomes with lower morbidity.

NF-κB signaling pathway

NF-κB signaling pathway is an omnipresent and tightly regulated signaling pathway crucial to cell fate and immune modulation. Briefly, NF-κB signaling pathway can be initiated either canonically or non-canonically. The canonical pathway exerts rapid but transient effects while the non-canonical pathway exerts slow yet persistent effects [60].

Transcription factors of the NF-κB family include p50, p52, p65 (RelA), c-Rel, and RelB, which stay quiescent in the cytoplasm due to the restriction of inhibitor of κB (IκB) [61]. Inducers of the canonical pathway such as inflammatory cytokines, extrinsic pathogens, and external antigens lead to the ubiquitilation or phosphorylation of IκB kinase (IKK) subunits that in turn phosphorylate IκB and beget its demolition [62]. The proteasomal degradation of IκB lets go of NF-κB so NF-κB dimers translocate from the cytoplasm into the nucleus where they bind to specific DNA sequences and regulate gene expression. The precise signal amplification of NF-κB signaling pathway modulates a great variety of inflammatory mediators, chemokines, and cytokines, which fuels up immune cells and activates immune responses [61, 63]. Therefore, aberrant induction, transmission, or resolution of kinases and adaptors in this pathway is disease causative.

Respiratory syncytial virus (RSV) promotes p65 subunit, a member of the NF-κB family, to form liquid-like condensate in the vicinity of the nucleus, which abrogates the translocation of p65 [64]. This evidence has delineated another role of phase separation in viral intrusion. Phase separation may be a generalized mechanism utilized by other immune-antagonistic viruses.

The NF-κB essential modulator (NEMO) is a subunit of IKK complex that phosphorylates IκB, which activates the NF-κB signaling pathway [65]. Patients bearing NEMO mutations show impaired NF-κB signaling and immune disorders [66]. A recent study unveiled that NEMO activates NF-κB signaling by LLPS. NEMO binds to K63-linked or linear polyubiquitin chains through NEMO ubiquitin-binding (NUB) domain and zinc finger [67]. Such multivalent interactions lead to LLPS and form a liquid droplet where IKK is activated through phosphorylation by TAK1. The authors argue that the contents within the liquid droplet formed by NEMO LLPS require further investigation, and it would be promising to manipulate NEMO if possible [67].

Fig. 4 Phase separation in cGAS-STING signaling pathway. cGAS phase separates with DNA to form a liquid droplet that excludes inhibitory mediators like BAF and TREX1 outside. Then, secondary messenger 2′,3′-cGAMP is produced, and its translocation activates STING and TBK1, eventually upregulating type-1 IFN secretion that is essential for innate immune responses. Meanwhile, after cGAS stimulation, the remaining STING forms a puzzle-like structure that avoids excessive activation of STING and TBK1. Tegument protein ORF52/VP22 from herpesvirus replaces cGAS and then phase separates with DNA to impede 2′,3′-cGAMP production, which causes viral immune evasion.
**NLRP6 inflammasome**

Inflammasomes are cytosolic protein complexes regulating cytokine secretion and pyroptosis in response to viral intrusion [68]. A canonical inflammasome is composed of sensor proteins, apoptosis-associated speck-like protein containing a CARD (ASC), and effector molecule Caspase 1. Through adaptor proteins, sensor proteins recruit and activate caspase 1 which then proteolytically cleaves and endows bioactivity to pro-IL-1β and pro-IL-18 [69]. Caspase 1 also cleaves gasdermin D (GSDMD) whose N-terminal assembles into pores across the cell membrane leading to apoptosis [70]. NLR and pyrin domain (PYD)-containing protein (NLRP)6 inflammasome is responsible for antiviral defense [71], IFN production [72], and inhibition of NF-κB signaling pathway [73].

A recent study unveiled the phase separation potential of NLRP6 inflammasome and hypothesized that phase separation acts as the driver in NLRP6 inflammasome activation (Fig. 6). Briefly, in response to double stranded (ds)-RNA, NLRP6 inflammasome phase separates to facilitate the recruitment of ASC and caspase 1 [74]. Shen et al. also identified lipoteichoic acid (LTA) as a potent initiator of NLRP6 inflammasome phase separation [74]. NLRP6 inflammasome might be a pattern sensor that interacts with certain structure instead of certain molecule. With this in mind, it would be essential to unravel the mystery of its ultrastructure and molecular pattern. Shen et al. proposed that it is phase separation that confers the possibility of diverse cellular effects to NLRP6 inflammasome [74]. Indeed, upon interaction with ASC, the dynamic phase separated NLRP6 inflammasome solidifies to contribute to inflammasome maturation. Phase separation presents a mechanism utilized by NLRP6 inflammasome that directs to pro-/anti-inflammatory consequences, and hopefully, this will enable treatment options to be developed.

**Phase separation in cancer**

**Tumorigenesis**

Known as “the guardian of the genome,” p53 is a pivotal tumor suppressor protein that regulates multiple cellular events including cell cycle arrest, genomic stability, apoptosis, and senescence [75, 76]. In response to DNA lesion recognition, ATM/ATR kinases phosphorylate p53 and its
repressor MDM2 to stabilize p53 [77]. The activated p53 attenuates cyclin/CDK signaling pathway and initiates G1/S cell cycle arrest to ensure genomic stability. As one of the main mediators of DNA damage response and an interactor of p53, p53-binding protein 1 (53BP1) recruits other DNA damage repair proteins, drives p53 target gene transcription, and dictates the oncogenicity of cells [78, 79]. Research has shown that 53BP1 phase separates to form a droplet-like DNA damage response compartment in the vicinity of DNA breaks (Fig. 7). The phase-separated 53BP1 then serves as a scaffold for p53 molecules and p53-activatory molecule recruitment, thereby amplifying signals and preserving genomic integrity [80]. Disruption of 53BP1 phase separation abrogates p53 enrichment and impairs p53 genomic stability.

A recent study has unveiled that AHNAK, a G1-enriched interactor of 53BP1, binds to the oligomerization domain in 53BP1 to curb phase separation [81]. It also dampens downstream interaction with p53, whereas depletion of AHNAK leads to apoptosis in cancer cells and senescence in normal cells [81]. The counterbalance between 53BP1 and AHNAK prevents excessive cell proliferation and might provide mechanistic insight into novel anticancer treatments. Recently, it has been substantiated that 53BP1 also preserves heterochromatin integrity through partitioning with heterochromatin protein (HP)1α [82]. Therefore, the phase separation property of 53BP1 might be involved with other biological processes meriting further explanation.

cGAS-STING pathway plays a dichotomous role in antitumor immunity [83]. Neurofibromin 2 (NF2) is a tumor suppressor upstream of Hippo pathway that controls tissue growth [84]. Although wildtype NF2 promotes DNA nucleic acid sensing, missense mutations or deletions in the N-terminal FERM domain (NF2m) are causative in numerous cancer types including mesothelioma, melanoma, and breast cancer [85]. The tumorigenicity and dysregulated antitumor immunity of NF2m is proposed due to the inhibition of cGAS-STING pathway. Meng et al. substantiated that activated IRF3 triggers mutant NF2m to sequester into condensates in which RACK1-PP2A complex deactivates TBK1 and the downstream antitumor immunity [86]. It would be of great potential to screen out similar signaling structures, for example R-Smad, to innovate new therapeutic targets targeting NF2 phase separation and the cGAS-STING pathway.

Glycogen is the principle storage form of glucose, primarily stored in the liver and muscle cells. Cancer cells have shown to alter this well-tuned metabolism so the surrounding microenvironment becomes aggressive and hostile [87]. Hippo pathway is a conserved pathway that regulates
tissue differentiation across all animal species. Phosphorylated nuclear factor Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are restricted in the cytoplasm and undergo proteasomal degradation. Through dephosphorylation by MST1/2 kinases, YAP and TAZ accumulate in the nucleus and modulate gene expression through interaction with DNA-binding transcription factors such as transcriptional enhanced associate domain (TEAD) [88–90].

Clinically, glycogen accumulation in pre-malignant liver tissue is linked to worse clinical prognosis and survival rates [91]. A recent study discovered that both in human and mice pre-malignant liver cells, the enrichment of glycogen is attributed to diminished glucose-6-phosphatase (G6PC) which dephosphorylates G6P into free glucose [92]. Glycogen forms dynamic droplets through spontaneous phase separation in which retained Laforin-MST1/2 complex perturbs the interaction between WW45 and MST1/2 [92]. This activates YAP and concomitant tumor progression. Reversely, abrogation of the glycogen phase separation lowers liver tumor incidence. Liu et al. believe that glycogen might serve as a biomarker in early liver cancer, but there remains much to be done regarding the molecular basis [92]. For instance, whether accumulated glycogen in cancer tissues that did not store glycogen undergoes phase separation as well. The aforementioned study targets tumor initiation, but whether this principle is applicable during tumor progression needs further investigation.

Non-POU domain-containing octamer-binding protein (NONO) is a paraspeckle protein that promotes the phase separation of TAZ and the following oncogenic gene expression [93]. Li et al. observed elevated levels of TAZ LLPS and NONO in glioblastoma, while depletion of NONO reduces TAZ LLPS [93]. Taken together, digging the inverse correlation between phase separation and cancer might provide new framework for early detection and treating approaches.

**Leukosomal transformation**

Chromosomal translocation generated by the fusion between IDR-containing segment in nucleoporins (NUPs) and chromatin/DNA-binding factor is central to the pathology of numerous leukemia [94]. NUP98-HOXA9 is a pathological transcription factor chimera containing homeodomain IDR. Previously shown, NUP-HOXA9 interacts with mixed lineage leukemia 1 (MLL1) and the non-specific lethal (NSL) histone-modifying complexes to drive hematopoietic malignancies [95]. NUP98-HOXA9 undergoes phase separation through IDR and forms leukemogenic puncta. The NUP98-HOXA9 condensate promotes the availability of chromatin to transcription factor and forms super enhancer–like long looping between enhancers and oncogene promoters [96]. Chandra et al. discovered that apart from the most studied NUP98-HOXA9, NUP98-PRRX1, NUP98-KDM5A, and NUP98-LNP1 also phase separate both homotypically and heterotypically to drive leukemogenesis [97]. Therefore, this mechanism is applicable to a wide range of NUP98 fusion oncoproteins (FOs) sharing similar structure.

**Anti-PD-1 therapy resistance**

Anti-PD-1 therapy has received durable effects across diverse tumor indications [98]. However, some patients showed poor treating efficacy due to IFN-γ-induced immunosuppression [99]. A recent study revealed that IFN-γ promoted YAP phase separation which causes resistance against anti-PD-1 therapy [100]. Activated downstream of the hippo pathway, YAP and its transcription partner TEAD bind to promoters or enhancers that modulate gene transcription [101]. YAP harbors IDR on its N- and C-terminal that are essential to phase separation as discussed before. Anti-PD-1 treatment initiates YAP phase separation by its coiled-coil and formed by its C-terminal IDRs [100]. This course is modulated by the IFN-γ signaling. YAP condensates partition with TEAD4, histone acetyltransferase EP300, and Mediator 1, and together, they serve as target gene transcription hubs that promote the transcription of CD155 (Fig. 8) [100]. CD155 is a cell adhesion molecule that binds to tigit on T cells and promotes tumor metastasis [102]. The overexpression of CD155 resists anti-PD-1 therapy [103]. Phase separation of YAP is targetable due to its essential role in anti-PD-1 therapy resistance. But there are still opening questions regarding the molecular mechanism of YAP interaction. Also, the infrastructure and the dispersion of scaffold proteins within the condensate remain unclear.

**Phase separation in AGS**

Aicardi-Goutières syndrome (AGS) is an autoimmune encephalopathy caused by genetic mutations in nucleic acid metabolism or sensing in TREX1, RNase H2B, and RNase H2C [104, 105]. AGS patients display different clinical manifestations including an aberrant rise of type I IFN in cerebrospinal fluid and serum, intracranial calcification etc. Type I IFN-induced nucleic acid signaling is central to the etiopathogenesis of AGS [106].

As an important DNA sensor, cGAS is implicated in AGS as well as the downstream cGAS-STING pathway. Drug-targeting STING palmitoylation has shown efficacy in ameliorating inflammation in AGS mice model [107]. GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) directly binds to cGAS and enlarges its size, which facilitates the binding between cGAS and DNA [108]. Epigallocatechin gallate (EGCG) abrogates the interaction between G3BP1 and cGAS and terminates the downstream synthesis of IFN and inflammation [108]. Drugs targeting
cGAS-DNA phase separation might be an effective solution for treating AGS and other cGAS-STING pathway–related autoimmune diseases although this postulation requires further investigation.

**Phase separation in neurodegenerative diseases**

Neurodegenerative diseases are characterized by reduction in cognition or motor functions especially among the aging population. Accumulating studies have indicated that autoimmune responses are linked with amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Parkinson’s disease (PKD), both epidemiologically and experimentally [109–112]. The essential role of phase separation underlying the etiopathogenesis of neurodegenerative diseases has been highlighted by several notable data. This change in viewpoint might hold promise for future treatment against neurodegenerative diseases.

The reason why neurons are sensitive to deviant phase separation is probably that neurons are unable to eliminate intracellular protein aggregates through cell division [42]. To start with, LLPS of FUS in SGs are associated with ALS and FTD. FUS, residing RNA binding proteins (RBPs), is usually located in the nucleus. However, in the case of patients with ALS and FTD, FUS aggregates in the cytoplasm, particularly in ribonucleoprotein and SGs [113, 114]. Because of the increased concentration in cytoplasm, FUS first undergoes reversible LLPS and then irreversible liquid–solid transition with hnRNP-A1 [113]. Solid aggregates of FUS thereafter undermine the neural system.

In most cases, protein separation is driven by low-complexity domains (LCDs) [9]. Likewise, in the case of FUS, it is motivated by the prion–like LCD on its C-terminal [115, 116]. On its N-terminal is the nuclear localization signal (NLS) which contains an arginine- and glycine-rich (RGG/RG) domain [113]. Arginine on its C-terminal and tyrosine on its N-terminal can trigger phase separation through multivalent cation–π interactions, thereby forming stable hydrogel and intervenes the functions of ribonucleoproteins in nerve endings [117].

Phase separation of FUS is modulated by nucleus transportin1 (TNPO1)/Karyopherin-β2 and the methylation status of arginine in the RGG/RG domain on its N-terminal [113, 117]. TNPO1 translocates FUS into the nucleus to prevent excessive accumulation of FUS. Meanwhile, TNPO1 can interact with the arginine residue in RGG3-PY domain on the N-terminal of FUS to produce weak intermolecular forces that weaken interactions among FUS [113, 114]. TNPO1 also functions as a chaperone that suppresses the intermolecular force between arginine and other residues, which hinders phase separation [113]. Aside from all this, TNPO1 directly inhibits the aggregation of FUS in SGs, thereby preventing subsequent LLPS and liquid–solid transition [113]. TNPO1 not only inhibits and reverses aberrant aggregation of FUS but also impacts other RBPs like TAF15, EWSR1, hnRNPA1, and hnRNPA2 [115].

Normally, arginines in the RGG/RG domain on the N-terminal of FUS should be demethylated by protein arginine methyltransferases (PRMTs). As for FUS-related FTD patients, their arginines in the RGG/RG domain are mono-methylated or simply unmethylated [113]. In normal conditions, the demethylated arginines interfere with cation–π interactions by affecting hydrogen bonds or hydrophobicity of their own, thereby inhibiting the phase separation of FUS and its aggregation in SGs [113]. Aberrant methylation of FUS in FTD patients promotes phase separation and triggers disease.

The same principle also applies for tau protein in Alzheimer’s disease. At physiological concentration, LLPS of tau is catalyzed by the electrostatic force of its N-terminal and the
hydrophobic force of MTB domain on its C-terminal [116]. This process is affected by its phosphorylation status or related gene silencing. Similarly, huntingtin protein and α-synuclein causes Huntington’s disease and PKD, respectively [118].

Discussion and perspective

Phase separation refers to the process in which biomolecules condense and form different liquid droplets thus regulating various physiological processes at the protein level. In general, phase separation facilitates numerous cellular events by aggregating essential proteins. In this review, we introduced the basic knowledge of phase separation and its role in the immune system. We emphasized immune signaling and related diseases such as cancer, AGS, and neurodegenerative diseases. Studies in immune cell signaling have proved that phase separation serves as a basic scaffold for understanding the assembly and modulation of signaling complexes. But phase separation is also a highly efficient immune evasion mechanism utilized by viruses. We also concluded the role of phase separation in tumorigenesis, leukemogenesis, and resistance against anti-PD-1 treatment. Lastly, we connected phase separation to AGS and neurodegenerative disorders, and we believe that this fundamental biological process could be a future therapeutic target.

Our understanding of phase separation in immune system is still at its infancy. For example, a recent study indicated that autoantigen Microrchidia 3 (MORC3) interacts with dsDNA to form liquid droplets [119]. Given that MORC3 is involved in idiopathic inflammatory myopathies and dermatomyositis sine dermatitis [120, 121], it would be interesting to investigate into cellular activities following MORC3 phase separation as well as its pathogenicity.

It is of great value to dig further into the ultrastructure and functional consequence of unknown proteins with the help of databases such as PhaSePro, PhaSepDB, and LLPSDB that target LLPS theoretically [122]. Latest discoveries on artificial membraneless organelles and TFs have shown that our understanding of phase separation has moved on to the next stage where we manipulate LLPS and observe the consequences of such modifications [123, 124]. But so far, most studies are accomplished through a monocellular view that restricts reactants to a narrow range. The limitation of these studies is the neglect of complex, intercellular interactions, not to mention the anfractuous immune system. We need to move from in vitro to in vivo, from cellular to animal models to better elucidate the role of phase separation in biochemical reactions, which may contribute to drugs targeting phase separation.

Abbreviations AGS: Aicardi-Goutières syndrome; ALS: Amyotrophic lateral sclerosis; ASC: Apoptosis-associated speck-like protein containing a CARD; RGG/RG: Arginine- and glycine-rich; BCR: B cell antigen receptor; CIN 85: Cbl-interacting protein of 85 kDa; CGAS: Cyclic GMP-AMP synthase; DAG: Diacylglycerol; DBDs: DNA binding domains; Ds: Double stranded; EGCG: Epigallocatechin gallate; FRAP: Fluorescence recovery after photobleaching; FTD: Frontotemporal dementia; FUS: Fused with sarcoma; FOs: Fusion oncoproteins; GSDMD: Gasdermin D; G6PC: Glucose-6-phosphatase; G3BP1: G3PAse-activating protein SH3 domain-binding protein 1; HSV: Herpes simplex virus; HP: Heterochromatin protein; IRF3: IFN regulatory factor 3; ISD: IFN stimulatory DNA; ISRE: IFN-stimulated response element; kxB: Inhibitor of kxB; IFN: Interferon; IRF3: IFN regulatory factor 3; IDRs: Intrinsically disordered regions; IKK: IkB kinase; KSHV: Kaposi sarcoma-associated herpesvirus; KiCGAS: KSHV inhibitor of CGAS; LAT: Linker for the activation of T cells; LTA: Lipoteichoic acid; LLPS: Liquid-liquid phase separation; LCDs: Low-complexity domains; MORC3: Microrchidia 3; MAVS: Mitochondrial antiviral signaling protein; MAPK: Mitogen-activated protein kinases; MLL1: Mixed lineage leukemia 1; NUB: NEMO ubiquitin-binding; NF2: Neurofibromin 2; NLRP: NLR and pyrin domain (PYD)-containing protein; NONO: Non-POU domain-containing octamer-binding protein; NSL: Non-specific lethal; NF2m: r-Terminal FERM domain; NF-kB: Nuclear factor kappa-beta; NLS: Nuclear localization signal; NPs: Nucleocapsid proteins; NUPs: Nucleoporins; TNPO1: Nucleus transportin1; 53BP1: P53-binding protein 1; PKD: Parkinson’s disease; PAMP: Pathogen-associated molecular pattern; PI2P: Phosphatidylinositol 4,5-bisphosphate; PLCγ: Phospholipase Cγ; PPRMs: Proline-rich motifs; PRMTs: Protein arginine methyltransferases; PKC: Protein kinase C; T: T cells; RSV: Respiratory syncytial virus; RBPs: RNA binding proteins; SARS-CoV-2: SARS coronavirus 2; SARS: Severe acute respiratory syndrome; SLPS65: SH2 domain-containing leukocyte protein of 65 kDa; Sirt: Sirtuin; SUVs: Small unilamellar vesicles; Syk: Spleen tyrosine kinase; SH2: Src homology 2; SGs: Stress granules; SLE: Systemic lupus erythema; TCR: T cell receptor; NEMO: The NF-kB essential modulator; TAZ: Transcriptional coactivator with PDZ-binding motif; TEAD: Transcriptional enhanced associate domain; IP3: Triphosphate; VZV: Varicella-Zoster virus; YAP: Yes-associated protein; ZAP-70: Zeta-associated protein of 70 kDa.

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