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Phase separation in immune signaling

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Abstract

Immune signaling pathways convert pathogenic stimuli into cytosolic events that lead to the resolution of infection. Upon ligand engagement, immune receptors together with their downstream adaptors and effectors undergo substantial conformational changes and spatial reorganization. During this process, nanometer-to-micrometer-sized signaling clusters have been commonly observed that are believed to be hotspots for signal transduction. Because of their large size and heterogeneous composition, it remains a challenge to fully understand the mechanisms by which these signaling clusters form and their functional consequences. Recently, phase separation has emerged as a new biophysical principle in organizing biomolecules into large clusters with fluidic properties. Although the field is still in its infancy, studies of phase separation in immunology are expected to provide new perspectives for understanding immune responses. Here, we present an up-to-date view of how liquid–liquid phase separation drives the formation of signaling condensates and regulates immune signaling pathways including those downstream of T cell receptor, B cell receptor and the innate immune receptors cGAS–STING and RIG-I. We conclude with a summary of the current challenges the field is facing and outstanding questions for future studies.

Introduction

Phase separation is a well-understood phenomenon in physical chemistry, but a relatively new concept for most biologists, including immunologists. It describes the segregation of biomolecules from a homogeneous environment into two distinct phases (the condensed phase and the dilute phase), of which the concentration and mobility of solutes differ significantly from each other. Because of the aqueous and fluidic environment of the intracellular space, liquid–liquid phase separation (LLPS) has been frequently observed in cells. LLPS is a concentration- and environment-dependent condensation process driven by solute–solute interactions that energetically overcome solute–solvent interactions (in cells

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the solute can be protein, nucleic acid, lipid, sugar, or other metabolites). In a liquid-like state, the condensed phase frequently exchanges materials with the dilute phase, and this liquid-like property has an important role in defining the composition and biochemical activity of molecules in the condensed phase. Sometimes, liquid condensates can 'age' and transition to a gel-like or solid state in which biomolecules are highly crosslinked and statically reside in the condensed phase. Because the cellular condensates are commonly formed through multiple types of interactions, a spectrum of intermediate states between the liquid and solid states could be observed. Interestingly, abnormal or irregular transition of condensates to a solid state is associated with certain neurodegenerative diseases. Examples include FUS (fused in sarcoma) condensates in frontotemporal lobar degeneration and TDP43 (TAR DNA-binding protein 43) condensates in amyotrophic lateral sclerosis[1].

In immunology, LLPS was characterized in the context of both the cell surface receptor pathways including the T cell receptor (TCR)[2, 3] and B cell receptor (BCR) pathway[4, 5], and the cytosolic signaling pathways including the cGAS-STING[6, 7], RIG-I[8], and NF-κB pathway[9]. As compared to other fields of study, including stress responses and transcriptional regulation, where phase separation has been well documented, research into the relevance of phase separation to immune signaling is still limited. In this article, we discuss the possible ways in which phase separation can bring new mechanistic insights into understanding immune responses. We start by introducing the basic features of phase separation as relevant to immunologists, followed by discussion of cell-surface signaling cascades (phase separation on membranes) and intracellular signaling pathways (phase separation inside cells). We conclude with a discussion of several outstanding questions for future studies.

Phase separation in 3D vs 2D

Cellular condensates formed through LLPS can be generally categorized into two types (FIG. 1): 3D structures formed in the cytoplasm and nucleus that do not have membrane wrapped around them; and 2D or near-2D structures that are formed along the cell membrane. These 3D and 2D condensates have similarities in terms of their large size (up to micrometers in magnitude), complexity in chemical composition, and dynamic exchange of materials with the environment. Indeed, a recent preprint (non-peer-reviewed data) describes that many 2D condensates can be viewed as 3D condensates that are 'wetted' (cover the surface with a low contact angle) on fluidic membranes, forming a prewet phase[10]. Although 3D condensates are the most commonly studied entities in the field of phase separation, 2D membrane-associated condensates (clusters) are well represented in immune signaling because cell-surface immune receptors frequently form micron or sub-micron sized clusters upon ligand engagement. In this section, we introduce the properties of both 3D and 2D phase-separated structures and discuss the physical mechanisms, physiological triggers, and signaling functions of these condensates.

Phase separation in 3D: membrane-less organelles.

One of the major motivations for studying phase separation is to understand how biomolecules self-assemble in space and time to form subcellular structures. The cytoplasm

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is filled with organelles such as mitochondria, the endoplasmic reticulum and the nucleus, which are topologically separated from the cytoplasmic environment by lipid bilayers. However, other cellular compartments exist that are not encased in membranes. How these structures are maintained and dynamically reorganized remain crucial questions for cell biologists. One of the earliest studied examples of these membrane-less organelles is that of P granules in *Caenhorhabditis elegans* embryos, which have liquid-like properties: they undergo spontaneous fusion, fall apart under shear force, wet on hard surfaces, and rapidly dissolve or assemble, reflecting a LLPS process[11] (BOX 1). A large number of other membrane-less organelles have since been identified as structures formed through phase separation, including stress granules, P bodies, nucleoli, Cajal bodies, promyelocytic leukemia (PML) bodies, inclusion bodies, polycomb bodies and Negri bodies (reviewed in REFS[12, 13]). In addition, a few new membrane-less compartments have recently been identified, including splicing condensates, autophagosome cargo condensates, and endoplasmic reticulum-associated TIS granules[14-16]. These examples have expanded the traditional view of membrane-based compartmentalization of the cytoplasm and revealed another level of complexity of the intracellular space.

Phase separation in 2D: membrane-associated condensates.

In addition to 3D condensates formed in the cytoplasm or nucleus, phase separation can also occur on 2D surfaces (FIG. 1)[2, 17-24]. Near-micron-scale signaling domains enriched in receptors, adaptors, effectors and particular lipids are frequently observed on the plasma membrane[25-28]. In the late 1990s, a lipid raft model was formally proposed to explain these signaling domains, whereby lateral interactions between lipids drive the formation of a liquid-ordered phase on the plasma membrane that organizes membrane proteins into domains[29]. However, there is a lack of direct evidence supporting the presence of large lipid domains in live cells at physiological temperatures and so whether the lipid raft model explains signaling domain formation remains controversial[30, 31]. Alternatively, recent studies have shown that multivalent interactions between membrane-proximal proteins can drive the formation of liquid-like large signaling clusters (phase-separated condensates) both *in vitro* and in live cells[2, 19, 32]. Of note, although the evidence that lipids form giant domains on the native plasma membrane is lacking, they can modulate membrane-associated protein condensates; for example, cholesterol enhances TCR clustering[33], potentially through the cholesterol-interacting transmembrane domain of the TCR complex. Conversely, protein condensates can influence phase separation of lipids; for example, condensates of the linker for activation of T cells (LAT), downstream of TCR triggering, promote phase separation of cholesterol on model membranes[34]. Together, the current evidence supports the notion that combined interactions between proteins and lipids shape phase separation on 2D membranes.

As compared to 3D phase separation forming membrane-less organelles in the cytosol, there are several unique physical and chemical features of 2D phase separation on membranes. First, membranes restrict the motion of proteins, which increases their effective macromolecular concentrations and accelerates biochemical reactions by orders of magnitude as compared to a 3D environment[35]. Indeed, the protein concentration threshold to induce condensates on the 2D membrane is much lower than in 3D solution[19, 20, 36].

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Second, lipids are much smaller in size, are higher in density and are more mobile than membrane-bound proteins. These properties can lead to unique mechanisms that modulate liquid-like condensate formation. Third, cellular membranes contain numerous protrusions or invaginations with convex or concave shapes. The local membrane curvature may affect the distribution and diffusion of lipids and membrane-bound proteins and thus the formation of condensates[37].

Mechanisms of phase separation.

Two major mechanisms have been proposed to drive liquid-like condensate formation, involving folded domains (secondary structures such as α helix or β strand) and/or intrinsically disordered regions (IDRs; unfolded regions that do not contain secondary structures). In the first mechanism, proteins containing tandem folded domains can be crosslinked through well-defined protein–protein interactions. A seminal example of this is the formation of nephrin clusters by multivalent interactions between phosphotyrosines and Src homology 2 (SH2) domains, and between proline-rich motifs and SH3 domains[18]; this was the first example of successful reconstitution of phase-separated droplets *in vitro*. A similar mechanism involving crosslinking applies when one of the protein components is replaced by DNA or RNA molecules. In this scenario, the length of the nucleic acid, which determines the binding valency (the number of interacting sites), has a crucial role in setting the threshold for phase separation[38-40].

The second major mechanism involves IDRs[41, 42], protein regions that usually do not contain folded domains and are generally unstructured. A similar term that is frequently used in the literature is low-complexity domains (LCDs). By definition, LCDs contain over-represented amino acids and are usually unstructured. IDRs and LCDs are largely overlapping, but IDRs are defined by their structure whereas LCDs are defined by their sequence. IDRs (and LCDs) can sometimes self-assemble to form homotypic or heterotypic higher-order structures in a concentration-dependent manner. For a list of databases that summarize candidate proteins for LLPS based on either experimental results or sequence predictions, see Supplementary Table 1. It should be noted that although the presence of IDRs suggests the ability of a protein to phase separate, this always requires experimental verification.

Although the protein sequences and mechanisms driving phase separation vary, one common feature present in almost all cases is that phase separation is driven by multivalent interactions. As the binding valency (number of binding sites per protein) increases, the threshold for forming phase-separated domains decreases, in a non-linear manner. For example, increasing the binding valency from 3 to 4 reduces the threshold to form condensates between proline-rich motifs and SH3 domains by about 10-fold[18]. Consistent with this correlation between valency and tendency to phase separation, dimerization or oligomerization domains (for example, coiled-coil domains), when coexisting with IDRs on the same polypeptide, can promote phase separation[43]. Of note, the interactions driving phase separation are not limited to the strong interactions that hold conventional macromolecular complexes together. Weak interactions, including cation-pi interactions and Van der Waals interactions, could also provide the driving force for LLPS because a high

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avidity can be achieved when combining low-affinity interactions with multivalency. The low affinity of individual interactions also enables flexibility in reorganizing structures and is crucial to the liquid-like property of condensates.

Physiologically relevant triggers.

As more examples of LLPS are discovered, we view phase separation as a basic property of biomolecules (similar to their size, charge and hydrophobicity) rather than as a special feature of certain molecules. Instead of asking which proteins can phase separate, it is more important to address under what physiological conditions phase separation occurs. Indeed, accumulating evidence suggests that liquid-like condensates are regulated by multiple physiological triggers. These regulatory processes have been extensively studied in yeasts that are directly exposed to various environmental challenges. For example, in response to high temperature or low pH, the poly(A)-binding protein Pab1 forms condensates, which increases cell survival[44]. It has been noted that condensates could form either above or below a threshold temperature, depending on the specific interactions mediating LLPS[45]. Redox metabolism is also an important factor in regulating LLPS; for example, the yeast RNA-binding protein Pbp1 phase separates when the cell is in a reduced state, which allows for the partitioning of the target of rapamycin complex 1 (TORC1) and the modulation of TOR signal transduction[46]. Physiological triggers are also frequently converted into post-translational modifications such as phosphorylation that either promote or inhibit phase separation. For example, antigenic stimuli to TCR trigger the downstream phosphorylation of LAT, which drives liquid-like microcluster formation to promote T cell activation[2]; and cell cycle-regulated, DYRK3-mediated phosphorylation of SC35 dissolves splicing speckles (nuclear domains enriched in pre-mRNA splicing factors) and prevents the trapping of mitotic regulators[47]. Post-translational modifications can also modulate phase separation during pathological processes. Acetylation inhibits the phase separation of the neurodegenerative disease-associated Tau protein *in vitro*[48]. Hypomethylation of FUS promotes phase separation and transition to a gel-like state, which disrupts the formation of ribonucleoprotein granules and reduces protein synthesis in neuron terminals[49]. Studying phase separation in the context of these physiologically or pathologically relevant triggers is important because only then can the functional, biological consequences of phase separation be revealed. This applies not only to *in vivo* studies but also to *in vitro* assays, in which individual components of phase-separated condensates are purified to reconstitute condensate formation. *In vitro* reconstitution can be a powerful way to dissect the mechanisms of phase separation, but it is important that the buffer conditions of *in vitro* assays, including salt, pH and temperature, reflect intracellular conditions. The use of crowding reagents, including polyethylene glycol (PEG) or dextran, needs to be cautiously evaluated and justified.

Functional consequences of condensation.

The extensively characterized mechanisms of condensate formation provide the foundation to address the functions of condensate assembly. These functions include effects on biochemical activities of individual molecules, intracellular signaling and metabolic pathways, and organism-level phenotypes. In the field of cell signaling research, the idea that clusters or ‘signalosomes’ of signaling molecules promote signal transduction has been

prevalent and there is much evidence to support this concept. What new properties or perspectives could liquid-like condensates bring to our understanding of cell signaling? We view liquid-like condensates as one type of signaling platforms. The traditionally defined signaling clusters share some features with phase-separated condensates. For example, they both concentrate molecules. However, phase-separated condensates are usually within the upper size range (micrometer scale) of signaling clusters and thus have features, such as exclusion, that do not typically exist in the nanometer-scale clusters. Moreover, liquid-like condensates are usually formed through weak intermolecular interactions, which results in a high rate of material exchange with the environment that could potentially allow for a continues flow of substrates into the condensate to promote enzymatic reactions. Moreover, the heterogeneous composition of liquid-like condensates could enable them to interact with a wide range of effectors, having a broad spectrum of activities that might favor responses to different environmental stimuli. On a purely speculative note, as liquid-like condensates mature into gel-like structures they might transduce force more efficiently. Many immune receptors (such as TCR, BCR and the low-affinity IgG receptor FcγRIIA) are force sensitive[50-52], such that a change in the material property of condensates might regulate receptor activity. We elaborate on some of these points in the following sections but many areas still require investigation.

It should be noted that data addressing the functional importance of condensation need to be carefully interpreted, with some studies showing an association between function and condensate formation rather than a causal relationship. In most cases, proteins, instead of condensates per se, are manipulated, which does not perfectly address the functional consequences of condensate formation. Fairly speaking, this caveat applies equally to any newly identified cellular structures in history, including many membrane-bound organelles. We suggest several ways in which a cause-and-effect relationship between condensate formation and function could be established. These include independent means to manipulate condensate formation (for example, generating point mutations, chemical perturbations and physical manipulation by optical trap), carefully characterizing the physical properties (Table 1) and chemical composition of condensates and quantitatively correlating them with functional outcomes, reconstituting condensates in vitro or in different cellular settings, and engineering condensates by swapping IDRs to show that the functional outcome is related to condensation rather than a specific protein sequence. As the field moves forward, more tools and techniques are expected to become available to comprehensively determine the functions of condensates.

Phase separation on the immune cell surface

Cell surface receptors on immune cells, together with ligand and/or downstream binding partners, can form nanometer- to micrometer-sized clusters on the plasma membrane[53-55]. In this section, we discuss how phase separation regulates the formation of these membrane clusters and hence immune signaling cascades. We focus on activating signaling cascades because of the available examples though there is evidence suggesting that the inhibitory receptor PD1 forms microclusters when engaged with PD-L1[56]. However, it remained to be determined whether PD1 microclusters are formed through phase separation.

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TCR signaling.

A prominent feature of the TCR signaling pathway is that major components in the pathway have been reported to form discrete micrometer- or submicrometer-sized clusters, known as T cell microclusters, on the plasma membrane. T cell microclusters were initially described in the late 1990s[57]. Since then, continuous efforts from multiple groups have built up a comprehensive list of the proteins that form microclusters, which includes the transmembrane receptors TCR, CD28 and PD1; the kinases LCK and ZAP70; the adaptor proteins LAT, GRB2, GADS (also known as GRAP2), SLP76 (also known as LCP2) and NCK1; and the enzymes SOS1, PLCγ1 and CBL[25, 56, 58] (FIG. 2). The formation of T cell microclusters depends on ligand binding and phosphorylation, and the composition of clusters is heterogeneous and dynamic. Components of the clusters usually have a higher density and lower mobility compared with the surrounding environment, as revealed by single-molecule imaging[59]. The high density can increase the likelihood of molecular interactions within clusters and the low mobility may facilitate reaching the minimal binding time that is required for interactions to occur. While investigating the key players in forming T cell microclusters, it was shown that three proteins — LAT, GRB2 and SOS1 — can form oligomers in solution through multivalent interactions[60]. To determine the mechanism of microcluster formation in the context of the plasma membrane, a supported lipid bilayer-based reconstitution system was established[61]. It was shown that LAT microclusters have liquid-like properties and are formed through LLPS of LAT and its binding partners[2]. Interestingly, two enzymes, SOS1 and PLCγ1, also have an enzyme-independent, scaffolding role in promoting LAT cluster formation[62, 63], and the composition of LAT clusters affects whether they undergo a smooth centripetal movement, which is driven by the retrograde flow of actin, at the immunological synapse[64].

What are the biochemical functions of LAT condensates? LAT condensates promote tyrosine phosphorylation, which is a key activation marker for the TCR signaling pathway. This is achieved by the enrichment of kinases in clusters, but exclusion of phosphatases. Interestingly, the exclusion properties of LAT condensates are dependent on charge: LAT condensates are negatively charged and therefore partially exclude negatively charged phosphatases such as CD45[2]. In addition to charge, the exclusion can also be mediated by other factors including protein size[65]. Moreover, LAT condensates promote downstream signaling pathways by increasing the membrane dwell time of SOS1 and N-WASp, which activate the RAS signaling pathway and actin remodeling, respectively[3, 66].

Although the assembly of LAT condensates is well studied, the disassembly process is less well understood. Endocytosis and ubiquitylation have been proposed to decrease the concentration of LAT on the plasma membrane and promote cluster disassembly[67]. Also, the cytosolic phosphatases SHP1 and SHP2, in complex with THEMIS, can be recruited to LAT clusters and potentially dephosphorylate LAT to disassemble the cluster[68]. This forms a potential negative feedback loop to regulate LAT cluster formation, which could be important for resetting TCR signaling to a baseline level after activation. Interestingly, although LAT is the key to driving microcluster formation in the TCR signaling pathway, it is dispensable for microcluster formation of chimeric antigen receptors (CARs)[69]. It has been proposed that the cytosolic domain of a CAR, once phosphorylated, can

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establish multivalent interactions with the LAT binding partners GADS and SLP76 to form microclusters in the absence of LAT, although the specific mechanisms of CAR microcluster formation and whether these microclusters have liquid-like features need to be determined[69]. It also remains an open question how the co-signaling domains of CARs, including CD28 and 4–1BB domains, modulate CAR microcluster composition and downstream signaling outcomes.

In contrast to LAT microclusters, which are relatively well characterized, the mechanism of transmembrane receptor clustering (for example, of TCR, CD28 or PD1) remains much less clear. There are still many open questions about the nature of receptor microclusters in T cells and the extent to which LLPS drives their formation. On one hand, these receptors contain multiple phosphorylable tyrosines, share certain cytosolic binding partners with LAT, and partially overlap with LAT microclusters, suggesting a potential role of LLPS. On the other hand, the mobility and oligomerization states of these receptors are determined not only by their binding partners in T cells, but also by the ligands with which they interact, which are located on another cell membrane. The local membrane geometry in the immunological synapse could also affect receptor organization[70]. Attempts to deconstruct these individual elements and reconstitute back, although technically challenging, will be crucial to reveal the mechanism of the spatial organization of signaling receptors on the T cell surface.

The physiological function of phase separation in the TCR signaling pathway is another intriguing question to explore. One of the remarkable features of the TCR signaling machinery is the ability to differentiate between self and non-self antigens, the mechanisms of which are still not fully understood[71, 72]. The affinity of TCRs for self antigens compared with non-self antigens is only several-fold different, but the signaling output is all or nothing. Phase separation could provide an appealing explanation for this behavior because phase separation is a highly coordinated and collective process that results in a binary outcome. A small change in input such as a slight increase in the affinity of the antigen–TCR interaction, could trigger phase separation and activate the downstream signaling cascade. A careful titration of antigen density and affinity will be needed to test how phase separation might play a role in self versus non-self discrimination.

BCR signaling.

Similar to the role of LAT in the TCR signaling pathway, the scaffold protein SLP65 (also known as BLNK) drives LLPS in the BCR signaling pathway. It has been shown that SLP65 forms liquid-like condensates with its binding partner CIN85 (also known as SH3KBP1) through a classical multivalent interaction between the SH3 domains of trimeric CIN85 and the proline-rich motifs of SLP65 (FIG. 3)[4, 73]. In contrast to the LAT condensates that are formed on the plasma membrane following TCR activation, SLP65 condensates are pre-formed in the cytoplasm of resting B cells and associate with the plasma membrane following BCR activation[74]. Although SLP65 and CIN85 are sufficient to form condensates at high concentrations, liposomes (spherical vesicles) have a crucial role in promoting condensate formation at physiological cellular concentrations of SLP65 and CIN85. SLP65 contains an amino-terminal lipid-binding domain that recognizes small

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unilamellar vesicles with radii of 20 nm, but not large unilamellar vesicles with radii of 60 nm[4]. Preferential binding of SLP65 to small, highly curved vesicles *in vitro* parallels findings that SLP65 localizes to intracellular vesicles enriched with VAMP7, a protein that is known to contribute to the trafficking of TCR pathway components. This co-localization is selective, as SLP65 does not colocalize with RAB8-positive or RAB27-positive vesicles[73]. Cryo-electron tomography (Cryo-ET) imaging has shown that liposomes in the *in vitro*-reconstituted condensates are spaced on average 5 nm apart. This fits a model in which CIN85 molecules are sandwiched between SLP65 molecules that are, in turn, bound to adjacent vesicles[4]. The Cryo-ET approach revealed the internal organization of the tripartite SLP65–CIN85–vesicle condensates, and a similar approach could potentially be applied to other studies of LLPS.

What is the function of SLP65 condensates? SLP65 mutants lacking the N-terminal vesicle-binding domain cannot form condensates either in the cytoplasm or on the plasma membrane, which is accompanied by defects in BCR signaling (for example, calcium influx). Importantly, these defects can be rescued by fusing another vesicle-recognizing domain (N-BAR) to the N-terminal truncation mutant of SLP65[4]. Together, these data support the function of SLP65 condensates in promoting calcium signaling and potentially other downstream signaling pathways. The functional roles of vesicles in SLP65 condensates, beyond serving as a structural component, remain to be determined, although it is proposed that SLP65 condensates could exploit the trafficking pathway of VAMP7-positive vesicles to quickly deliver a large number of BCR signaling components to the plasma membrane for efficient signal transduction. There are also a few other questions worthy of further investigation: do SLP65 condensates recruit other BCR signaling components? Do SLP65 condensates have a role in sorting vesicles and regulating vesicular trafficking? Do the condensates disassemble or degrade and, if so, how?

The lipid bilayer (vesicle)-facilitated phase separation of SLP65 concurs with other works showing that BCRs form nanoclusters and microclusters when engaged with soluble antigens and APC-presented antigens, respectively[75, 76]. BCRs are selectively enriched in liquid-ordered phase and BCR nanoclusters overlap with specific lipid and protein probes on the B cell membrane[77]. Increasing cholesterol levels promoted BCR signaling by stimulating calcium influx, whereas decreasing cholesterol levels resulted in reduced calcium influx. The liquid-ordered phase is also enriched in the Src kinase LYN and depleted of the phosphatase CD45, which thereby promoted signaling propagation[5]. Whether the BCR itself forms clusters through LLPS remains to be determined, and further work is also needed to analyze the condensation states of downstream BCR effectors.

Innate immune receptors.

There are a large number of innate immune receptors that cluster at the cell surface upon ligand binding, including phagocytic receptors such as *Drosophila melanogaster* Draper[78] and dectin 1[79], as well as the mast cell receptor FcεRI[80-82]. These receptor clusters are similar to TCR and BCR signaling clusters in that they contain immunoreceptor tyrosine-based activation motifs (ITAMs) or ITAM-related sequences; are nanometer to micrometer in size; include multivalent interacting proteins that enhance signal transduction; spatially

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exclude inhibitors of signal propagation; and are affected by lipid composition (for example, cholesterol levels). Moving forward, it will be interesting to evaluate the mechanism by which these receptors form clusters and to confirm whether LLPS plays a role.

Liquid-like condensates in immune cells

In addition to signal transduction on the plasma membrane, phase separation also regulates intracellular immune signaling events, including the cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway, which responds to double-stranded DNA (dsDNA), and the retinoic acid-inducible gene I (RIG-I) pathway, which responds to single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). Moreover, viral proteins can hijack immune pathways in host cells by trapping key signaling components in liquid-like condensates, as illustrated by the nuclear factor-κB (NF-κB) pathway.

cGAS liquid-like condensates.

cGAS senses abnormal cytosolic dsDNA derived from pathogens or from nuclear or mitochondrial damage[83]. dsDNA binds and activates cGAS, resulting in the synthesis of 2′3′-cyclic GMP–AMP (cGAMP). cGAMP, in turn, activates STING, leading to downstream signaling that induces the expression of type I interferons and other proinflammatory cytokines[84]. Recently, it has been shown that DNA binding to cGAS induces the formation of liquid-like condensates (FIG. 4a). Several essential multivalent elements are involved in the formation of cGAS–dsDNA condensates, including the positively charged N-terminal domain of cGAS, long DNA strands (>100 base pairs) and free zinc ions. A newly identified DNA-binding domain in the catalytic core of cGAS also enhances condensate formation[85]. Functionally, the formation of condensates can promote cGAS activity by protecting DNA from degradation by the exonuclease TREX1[86]. In addition to DNA, cGAS also forms liquid-like condensates with dsRNA though dsRNA does not activate cGAS to produce cGAMP[6]. Interestingly, recent data from a preprint (not yet peer reviewed) indicate that high concentrations of dsRNA compete with dsDNA for cGAS binding and inhibit cGAS activity, whereas dsRNA at low concentrations promotes phase separation and the production of cGAMP[87]. The differential effect of dsDNA versus dsRNA on cGAS activation raises an intriguing question how RNA virus infection might influence the response of host cells to a DNA virus at the level of cGAS regulation.

Two tumor-associated mutations, G303E and K432T, at one of the DNA-binding sites of cGAS result in a reduced ability to form cGAS condensates and decreased cGAMP production[85]. However, these mutations may also affect DNA binding and enzymatic activity, so it is not known whether the reduced ability to form condensates explains the decreased cGAMP production. Further investigations are needed to understand how the formation of cGAS condensates affects downstream pathways in both immune cells and tumor cells and how these cGAS mutants contribute to altered immune responses during tumor progression. Manipulating cGAS condensate formation may provide a new way in which to modulate the antitumor immune response.

Moreover, pathogen proteins can also regulate the phase separation of cGAS. Streptavidin, a secreted protein from the bacterium *Streptomyces avidinii*, binds to cGAS to enhance

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cGAS–DNA interactions and promote LLPS of this complex. This results in enhanced cGAS activation and interferon-β production[88]. Whether or how this mechanism benefits *S. avidinii* remains unclear. On the other hand, herpesvirus protein ORF52 and VP22, which inhibit cGAS activity, disrupt cGAS-DNA droplet formation. This disruption depends on the formation of new droplets composed of viral proteins and DNA. Interestingly, DNA preferentially forms droplets with ORF52 other than cGAS, even though the DNA-binding affinity of ORF52 is lower than the one of cGAS[89]. This suggests that droplet formation can influentially alter the way that biomolecules interact with each other.

G3BP1, which is a key player in the formation of stress granules (cytosolic phase-separated structures composed of RNA and various proteins that arise under conditions of cellular stress)[90], is also crucial for DNA sensing and efficient activation of cGAS. The percentage of cells containing cGAS condensates was significantly reduced in G3BP1-deficient cells. Interestingly, the regulatory function of G3BP1 on cGAS phase separation does not depend on stress granules[91]. It is unclear how the partitioning of G3BP1 in cGAS droplets versus stress granules is regulated.

cGAMP, once produced by cGAS, binds and activates STING, which is accompanied by polymerization of STING[92]. A recent study showed that STING forms condensates with stacked ER membrane in the presence of excess amount of cGAMP. The STING condensates recruit the downstream signaling kinase TBK1 but exclude the transcription factor IRF3, and thereby lose the ability to trigger robust interferon production[7]. Interestingly, another recent study showed that a multivalent STING agonist PC7A triggers STING condensate formation and stimulates the prolonged production of pro-inflammatory cytokines[93]. These examples illustrate an emerging notion that condensation could have both positive and negative roles in regulating signaling. Future studies are required to determine the structural and compositional differences between the two aforementioned STING condensates to understand their opposite signaling outcomes.

RIG-I and stress granules.

RIG-I triggers the innate immune response against ssRNA viruses or dsRNA viruses[94]. Tripartite motif-containing protein 25 (TRIM25), an E3 ubiquitin ligase, catalyzes the K63-linked ubiquitylation of RIG-I on its two caspase-recruitment domains (CARDs), which is required for its interaction with mitochondrial antiviral signaling protein (MAVS) and its ability to induce antiviral signal transduction and interferon production[95]. A recent preprint (not yet peer-reviewed) showed that RNA binding triggers LLPS of TRIM25, which recruits RIG-I to condensates and increases its ubiquitylation by TRIM25[8] (FIG. 4b). By contrast, RNF125, another E3 ubiquitin ligase, has been shown to negatively regulate the RIG-I pathway by catalyzing K48-linked ubiquitylation of RIG-I that leads to its proteasomal degradation[96]. G3BP1, the core component of phase-separated stress granules, directly interacts with RNF125 in virus-induced stress granules to promote its auto-ubiquitylation and degradation. G3BP1 also interacts with RIG-I to enhance its binding to dsRNA and downstream signaling pathways[94, 97]. Together, these results suggest that G3BP1 in stress granules functions as a positive regulator of RIG-I signaling. Further investigation will be

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needed to address how TRIM25-induced condensates and G3BP1-mediated stress granules coordinately regulate the partitioning and activity of RIG-I.

Besides RIG-I, virus-induced stress granules also colocalize with several other innate immune proteins, including melanoma differentiation-associated gene 5 (MDA5), protein kinase R (PKR), 2′-5′ oligoadenylate synthase (OAS) and ribonuclease L (RNase L)[98, 99]. However, the consequences of localization of these proteins to stress granules in the context of innate immune responses remain to be explored. In addition to halting protein translation to block viral replication, virus-induced stress granules[98, 100] could have an important role in integrating multiple immune signaling pathways through phase separation to confer effective anti-viral responses.

NF-κB pathway.

Upon infection, some viral proteins form membrane-less liquid-like condensates known as inclusion bodies to promote viral genome replication and alter antiviral immune responses to escape host immune surveillance[101-103]. One example is the inhibition of the NF-κB pathway by respiratory syncytial virus (RSV). In RSV-infected cells, the NF-κB subunit p65 is rapidly sequestered into perinuclear intracytoplasmic puncta that are synonymous with inclusion bodies (FIG. 4c). The trapped p65 cannot translocate into the nucleus to activate the downstream transcription of pro-inflammatory cytokine genes and other antiviral genes[9]. Similarly, MAVS and MDA5, two upstream regulators of the NF-κB pathway, are recruited into RSV-induced inclusion bodies as a mechanism to inhibit the expression of interferon-β[104]. In another example, the sequestration of phosphorylated p38 mitogen-activated protein kinase (MAPK) and O-linked N-acetylglucosamine transferase into RSV-induced inclusion bodies can suppress MAPK-activated protein kinase 2 signaling and stress granule formation, respectively, both of which would otherwise inhibit RSV replication[105]. Whether other viruses that generate inclusion bodies, such as Rabies, Ebola or Nipah viruses, use similar mechanisms of immunomodulation requires further elucidation.

Conclusions and outstanding questions

Phase separation offers several new perspectives for understanding biological systems such as immune responses. At the molecular level, phase separation highlights the importance of weak interactions and unstructured protein domains, which have frequently been ignored in studying protein–protein interactions in the past but seem to have crucial functions in driving LLPS. At the subcellular level, studies of phase separation reveal new membrane-less organelles or intracellular compartments for signal transduction. At the physiological level, phase separation provides mechanistic insights for understanding cellular decision-making processes in the immune response. The role of phase separation in immune signaling has so far been shown in a few pathways, including TCR, BCR, cGAS, RIG-I, and NF-κB signaling. However, the field is still in its infancy and further investigations, both in terms of technological developments and physiological exploration, are required to comprehensively test the functions of phase separation in immunity. Looking forwards, we envision that the following areas will be exciting to explore.

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Condensate structure at atomic resolution.

One challenging area in the LLPS field is to determine the internal organization and structure of the condensate components at atomic resolution. This becomes even more complicated in the presence of biomembranes. Although electron microscopy and crystallography have been used to answer such structural questions in other contexts, their application to liquid-like objects remains limited. Advances in nuclear magnetic resonance imaging and computational simulations may provide new avenues for approaching this question. Understanding the internal organization of condensates will benefit the design of agonists and antagonists for perturbing condensates and the associated immune responses. For example, STING condensates could be an interesting therapeutic target because of an important role of STING in anti-cancer immunity and because different STING condensates seem to have opposite effects on IFN production, as discussed above.

Phase separation across the immunological synapse.

So far, most studies of phase separation have focused on a single environment, either in the cytoplasm (for example, cGAS or RIG-I signaling) or on the plasma membrane (for example, TCR or BCR signaling). However, the immunological synapse is a complicated sandwich-like structure composed of five environments: the cytosol and plasma membrane of an immune cell; the intermembrane space; and the plasma membrane and cytosol of an antigen-presenting cell. These different environments are coupled through multiple ligand–receptor pairs and may influence their assembly structures as well as confer two-way signaling. A multiple-membrane reconstitution system, together with light-sheet microscopy on live-cell conjugates, will benefit a comprehensive understanding of the phase separation behavior at immunological synapses.

Engineering immune signaling by targeting phase separation.

Given the role of phase separation in promoting TCR signaling, phase separation might also be targeted to engineer T cells for cancer immunotherapy. A large number of methods have been developed to control phase separation (BOX 2). These could be exploited to engineer key signaling molecules in T cells to boost their antitumor activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 |

Defining liquid–liquid phase separation

The liquid-like properties of phase-separated proteins have classically been described as the ability of protein concentrates to form round droplets that undergo fusion and fission, exchange materials with the environment, and exhibit fluorescence recovery after photobleaching (FRAP)[12, 123, 124]. FRAP is a commonly used technique to assess the fluidity of biomolecular condensates involving bleaching of fluorescently tagged molecules and observing recovery of the fluorescent signal in the bleached area over time, which is typically owing to molecular diffusion of fluorescent molecules from outside the bleached area. However, there is an increasing need for these parameters to be clarified, particularly as some condensates are known to mature and harden as revealed by decreased recovery over time, becoming 'gel-like or solid'[124]. This introduces a temporal component to phase-separated structures that requires further elucidation with regards to functional consequences. Because of heterogenous compositions and multiple types of interactions driving condensate formation, there could be a spectrum of states rather than defined boundaries between liquid and solid states and some condensates might be a mixture of liquid- and solid-like structures. Concerningly, liquid–liquid phase separation (LLPS) has also been used loosely to describe condensates that are actually generated through other processes, such as scaffold-driven assembly, the mechanism of which is distinguished from phase separation[125]. Ectopic overexpression systems are commonly used to investigate LLPS but may result in artificial phenotypes that would not occur under physiological conditions. Furthermore, although FRAP is insightful, it is also heavily exploited as the ultimate, defining experiment for LLPS. This can be fallacious as fluorescence recovery cannot always be attributed to diffusion; rather, recovery may also be a consequence of strong, high-affinity interactions between unbleached probes and macromolecules that do not undergo LLPS[124, 125]. The aforementioned liquid-like criteria concerning morphology and kinetics, although important, are not the only relevant features of LLPS; organization, surface tension, viscosity and turbidity are other characteristics that should be measured on nascent condensates. Therefore, multiple techniques should be used to determine liquid-like behaviors, many of which are summarized in Table 1 and in more detail in other reviews[124, 126].

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Box 2 |

Methods to manipulate phase separation

Both chemical and optogenetic tools are available to artificially induce or promote phase separation. For example, the iPOLYMER system uses heterodimerization of FKBP (FK506 binding protein) and FRB (FKBP rapamycin binding domain) fusion proteins; using this system, artificial RNA granules can be formed upon treatment with rapamycin[127]. A large number of optogenetic approaches have also been developed to control phase separation. In the OptoDroplets system, a light-inducible cryptochrome 2 (CRY2) domain is fused to a protein that has the potential to phase separate (for example, containing an intrinsically disordered region). Blue light induces oligomerization of CRY2, which triggers condensate formation in a reversible manner in live cells. This method has been used to induce the phase separation of ribonucleoproteins[128]. Other optogenetic tools include Corelet (which promotes phase separation using ferritin spheres[129]), PixELL (which induces condensate disassembly[130]) and DropletTF (which promotes gene transcription both *in vitro* and in mice[131]). One challenge of applying optogenetic approaches to *in vivo* studies is the low tissue penetrance and high phototoxicity of blue or other visible light. This technical hurdle could potentially be circumvented by using near-infrared nanoparticles that convert infrared light, which has deep tissue penetrance and low phototoxicity, to visible light that is compatible with current optogenetic tools[132].

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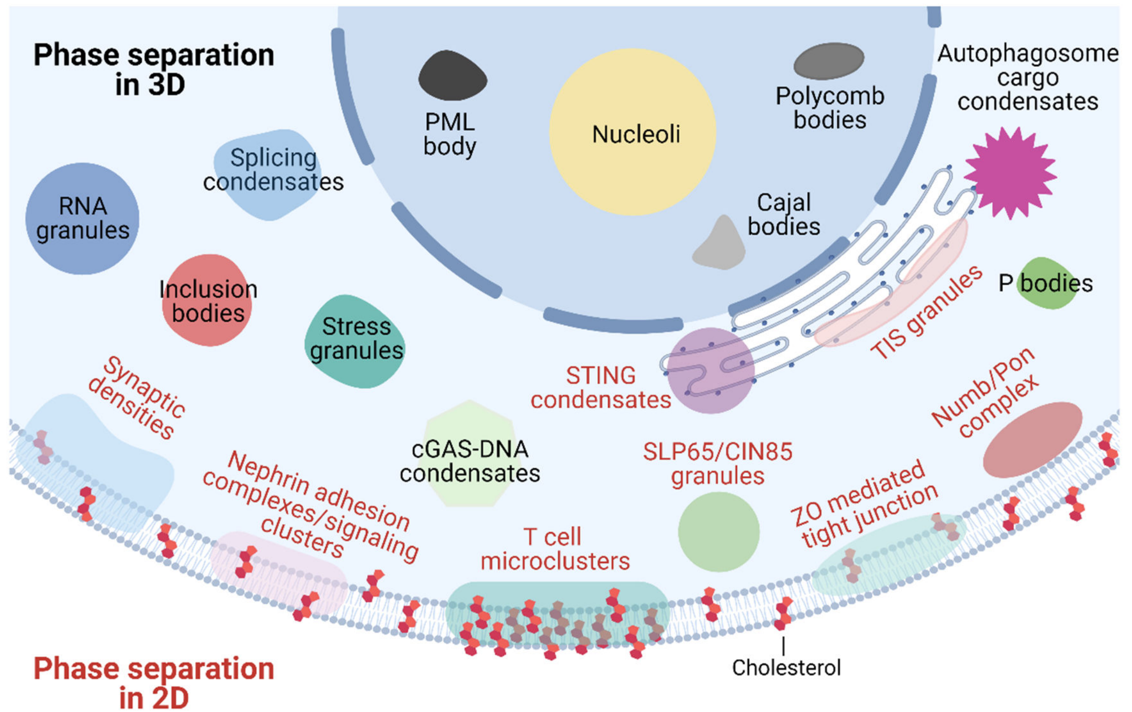

Figure 1 |. Phase separation in 3D and 2D.

The intracellular space is filled with biomolecular condensates formed through phase separation. These include 3D membrane-less organelles such as nucleoli, Cajal bodies, promyelocytic leukemia (PML) bodies and polycomb bodies in the nucleus; and stress granules, cGAS–DNA condensates, inclusion bodies, P bodies, splicing condensates, RNA granules, autophagosome cargo condensates, SLP65–CIN85 granules and endoplasmic reticulum-associated TIS granules and STING condensates in the cytoplasm. 2D condensates associated with the plasma membrane include T cell microclusters, nephrin-containing adhesion complexes, pre- or post-synaptic densities, NUMB–PON complexes and zonula occludens (ZO)-mediated tight junctions.

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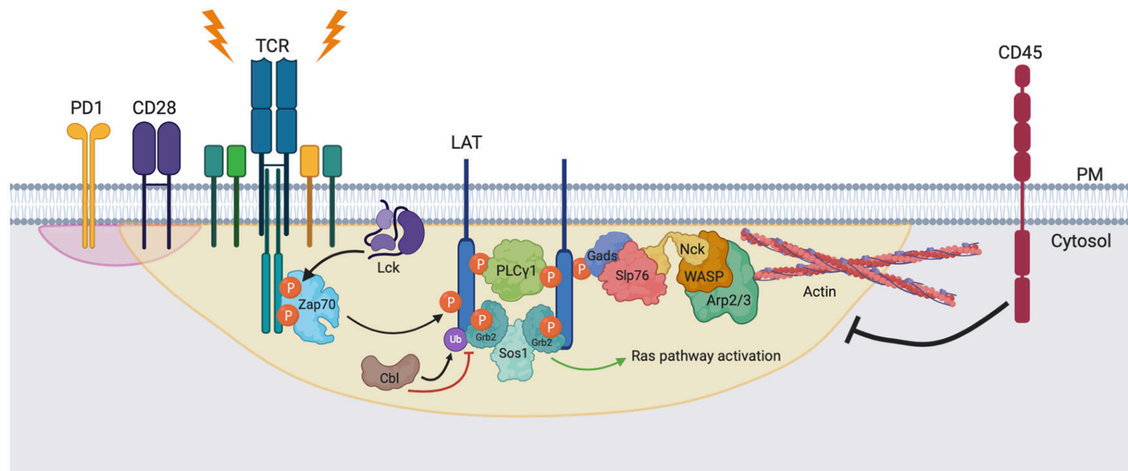

Figure 2 |. A microcluster view of T cell receptor signaling.

Upon antigen engagement, the T cell receptor (TCR) complex is phosphorylated by LCK on the immunoreceptor tyrosine-based activation motifs (ITAMs) of its CD3 chains. The phosphorylated TCR complex recruits the kinase ZAP70, which then phosphorylates LAT, resulting in the formation of liquid-like condensates of LAT. These LAT microclusters are enriched with adaptor proteins (such as GRB2, GADS, SLP76 and NCK1) and effector proteins (such as SOS1, PLCγ1, WASp and ARP2/3) to trigger the activation of downstream pathways, including RAS signaling, calcium influx (not shown) and actin remodeling. LAT microclusters exclude the phosphatase CD45 to protect phosphotyrosines, which are an activation marker of TCR signaling. CBL, an E3 ubiquitin ligase, is recruited to LAT microclusters to attenuate clustering and hence TCR signal transduction. The TCR co-receptors CD28 and PD1 overlap with LAT microclusters when engaging their own ligands.

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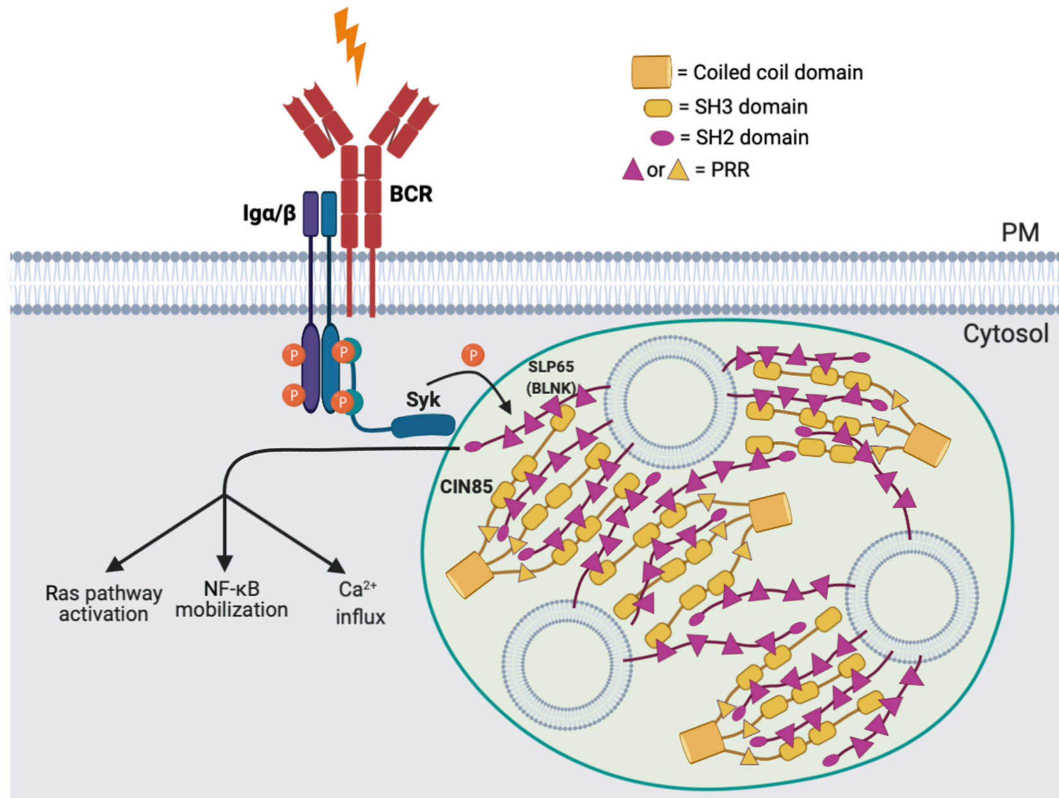

Figure 3 |. Signaling condensates in the B cell receptor pathway.

The scaffold protein SLP65, its binding partner CIN85 and liposomes (spherical vesicles) form liquid-like signaling condensates in the cytosol of resting B cells. Condensate formation is mediated through multivalent interactions between the proline-rich motifs of SLP65 and the SH3 domains of CIN85, and between the amino-terminal lipid-binding domain of SLP65 and vesicles. CIN85 is trimerized by its coiled-coil domain, which further increases its interaction valency. Upon B cell receptor (BCR) stimulation, the kinase SYK is recruited and activated at the BCR, which phosphorylates SLP65 as the condensates approach the plasma membrane. Downstream pathways are further triggered including RAS activation, NF-κB mobilization and calcium influx.

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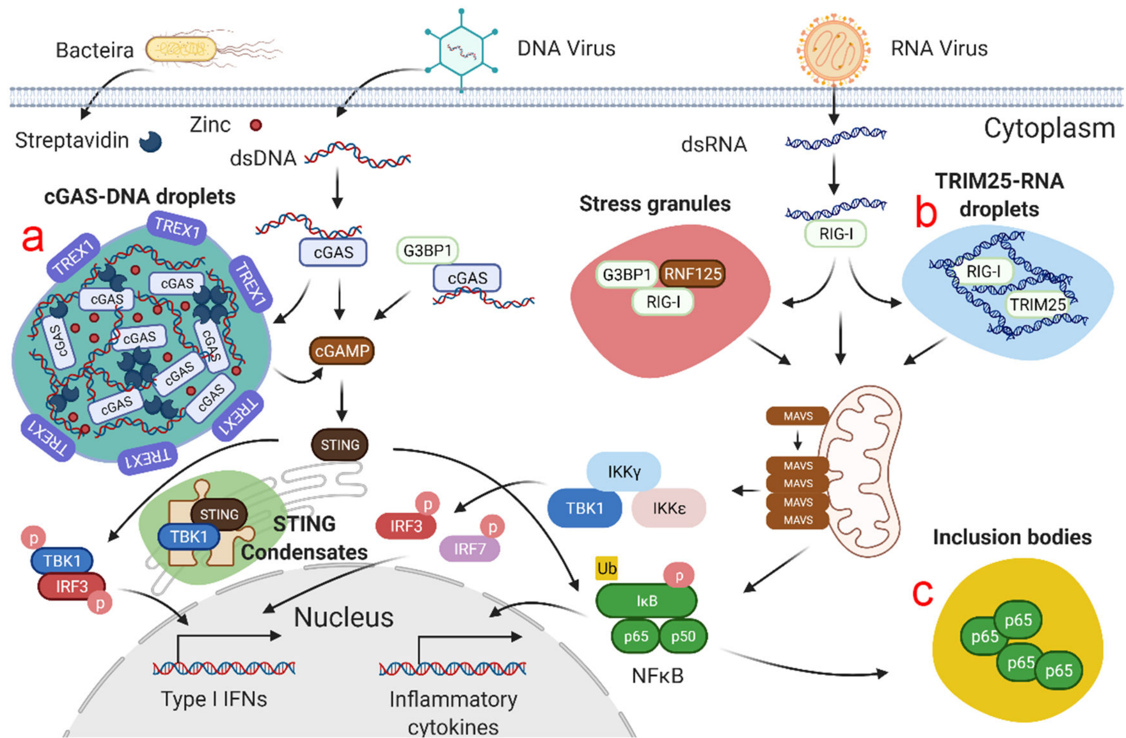

Figure 4 |. Phase separation of intracellular innate immune signaling pathways.

a | Cyclic GMP–AMP synthase (cGAS) forms liquid-like condensates with double-stranded DNA (dsDNA) to enhance the production of 2′3′-cyclic GMP–AMP (cGAMP) by protecting DNA from degradation by the exonuclease TREX1. Bacteria-derived streptavidin, free zinc ions, RNA and the stress granule protein G3BP1 regulate the formation of cGAS–dsDNA condensates and production of cGAMP. cGAMP, in turn, activates STING, leading to downstream signaling through TBK1 and IRF3 that induces the expression of type I interferons and other proinflammatory cytokines However, overproduction of cGAMP induces the formation of STING condensates on the endoplasmic reticulum (ER), which recruit TBK1 but exclude IRF3 and thereby prevent overactivation of the innate immune response by limiting interferon production. **b** | The E3 ubiquitin ligase TRIM25 forms liquid-like condensates with RNA (preprint data; not yet peer reviewed), which recruit the RNA sensor retinoic acid-inducible gene I (RIG-I) and promote RIG-I activation through K63-linked ubiquitylation and downstream signaling through mitochondrial antiviral signaling protein (MAVS). In parallel, G3BP1 recruits the E3 ubiquitin ligase RNF125 into stress granules and destabilizes RNF125, which inhibits the K48-linked ubiquitylation of RIG-I that would otherwise lead to its proteasomal degradation. G3BP1 also promotes RNA binding to RIG-I to trigger RIG-I activation. **c** | Nuclear factor-κB (NF-κB) functions downstream of RIG-I–MAVS signaling. The p65 subunit of NF-κB is trapped in the inclusion bodies that are formed by phase separation of the viral replication machinery. Trapped p65 is unable to translocate into the nucleus to induce the expression of pro-inflammatory cytokines.

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Table 1 |

Methods to characterize liquid-like condensates

Method	Condensate properties studied	Notes	References*
Differential interference contrast (DIC) microscopy	Morphology and dynamics	–	106, 107
Regular fluorescence microscopy	Morphology, fusion kinetics, surface tension and composition	Small-sized, monomeric fluorescent tags are preferred; use low ratio of labelled to unlabeled components to avoid optical-artifact	108, 109
Fluorescence recovery after photobleaching (FRAP)	Diffusivity	Can measure recovery and diffusion coefficients, from which viscosity can be calculated	11, 108
Fluorescence correlation spectroscopy (FCS)	Diffusivity	Can calculate diffusion coefficients and size of particles in live cells	65, 108
Distributed amphifluoric Förster resonance energy transfer (DAmFRET)	Density and degree of order/ disorder	High throughput	110, 111
Photoluminescence lifetime imaging	Viscosity and internal organization	–	112
Particle tracking	Viscosity, elasticity and mesh size	Uses bead injection or expression of genetically encoded nanoparticles	113, 114
Light scattering	Morphology and structure	Includes dynamic, static, multi-angle, small-angle, small-angle x-ray and small-angle neutron scattering techniques	18, 44
Correlated electron and light microscopy (CLEM)	Organization	–	115, 116
Cryo-electron tomography (Cryo-ET)	Morphology and organization	Restricted by sample thickness	4, 117
Optical tweezers	Surface tension and viscosity	–	118, 119
Atomic force microscopy (AFM)	Stiffness and structure	–	49, 120
Nuclear magnetic resonance (NMR) imaging	High-resolution structure and chemical environment	Low complexity sequences, high droplet viscosity and droplet heterogeneity may confound resonance	121, 122

*Owing to reference limits, two examples are provided for each technique.