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# Hidden Aspects of Valency in Immune System Regulation

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## Abstract

Valency can be defined as the number of discrete interactions a biomolecule can engage in. Valency can be critical for function, such as determining whether a molecule acts as a scaffold for assembling large supramolecular complexes or it forms a functional dimer. Here, we highlight the importance of the role of valency in regulating immune responses with a focus on innate immunity. We discuss some of the ways in which valency itself is regulated through transcriptional, post-transcriptional, and post-translational modifications. Finally, we propose that the valency model can be applied at the whole cell level to study differences in individual cell responses with relevance to putative therapeutic applications.

### Keywords

valency; NLRP3; inflammasome; pro-inflammatory cytokines; IL-1β; IL-1α; AIM2; cGAS; myddosome; MYD88; IRAK4; IL-18; pyroptosis; ASC; CASP1; DNA sensing; stress granules

## Valency dictates physical interactions between molecules

The question "Can 1000 reviews be wrong?" was part of the title of a preview accompanying two articles published in *Cell* in 2005 [1]. The articles exposed an important limitation in the consensus model of the adherens junction-actin filament connection; it had been assumed that α-catenin, a molecule that can bind to both actin filaments and β-catenin, acted as a bridge between the adherens junction complex and actin filaments [1]. However, studies showed that α-catenin cannot bind to β-catenin and actin filaments simultaneously because the molecules shared the same binding site on α-catenin [2,3]. Essentially, α-catenin had a **valency** (see Glossary) of one, for physical interactions in this scenario. This was an important fact that, mainly due to the inadequacy of structural and biochemical data available at the time, was not considered in the original model. This highlights the need to consider the number of independent binding sites available when building models of biological processes that require physical interactions.

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The physical interaction between biomolecules is a fundamental property for the function of all biological systems. These interactions can occur between molecules of the same type (homotypic interaction) or different types (heterotypic interaction), leading to the assembly of biomolecular complexes that are often the functional units controlling processes such as biochemical reactions or information transfer. Assembly of biomolecular complexes is regulated fundamentally in two ways, by the spatiotemporal localization of the interactors and by the availability of the interaction sites within them. Biochemists have borrowed the concept of valency from chemistry to model and explain the role of binding site availability in the regulation of biomolecular complex assembly [4]. Valency in this context can be defined as the number of independent binding sites on a molecule that allow homotypic or heterotypic physical interactions [4]. In this opinion, we focus on the regulation mediated by valency, mainly in the context of immune system regulation. Signaling downstream of innate immune sensors is regulated by biomolecular complexes that require homotypic and heterotypic interactions for their assembly. We propose that assembly of these biomolecular complexes is critically regulated by multivalent interactions. We further propose that the idea of valency can be extended to the whole cell level and define the concept of cellular valency. Additionally, we discuss examples of cells exploiting cellular valency to regulate their response.

## Valency regulates the assembly of supramolecular structures and phase separation

The idea that the number of binding sites, or valency, plays a central role in the assembly of supramolecular structures such as **stress granules** and **P-bodies** has been extensively studied and tested [4–7]. The basic premise is that a valency of at least two is required to assemble a large complex: the valency of one would mean that complex formation would be limited to a dimer, with the valency of each molecule being satisfied by binding to the other (Fig. 1A). Moreover, the efficiency of assembly of large supramolecular structures has been shown to increase with an increase in valency, resulting in a higher probability of assembly (or **phase separation**) at lower concentrations of the components, as evidenced from *in vitro* experiments with the SRC homology 3 (SH3) domain and its proline-rich motif (PRM) ligand [4]. Phase separation is inhibited in the presence of high concentrations of monovalent molecules that can compete for binding sites on the multivalent scaffold molecules, further corroborating the important role valency plays in these processes [4]. Thus, multivalency is a fundamental property required for phase transitions of biomolecules.

One of the earliest studies to use the concept of valency involved antibody-antigen binding [8]. Different antibody classes have different valency for their antigen based on the number of immunoglobulin molecules in the functional unit of the antibody class. Multivalency of antibodies is the fundamental property that gives rise to a property known as **avidity**, where higher valency leads to higher avidity, if the binding affinity remains the same. Another well-studied example is that of lectin-carbohydrate interactions [9]. If both the lectin and its ligand carbohydrate are bivalent, formation of the linear chain lectin-carbohydrate complex can occur (Fig. 1B). However, if they have a valency greater than two, formation of ordered

**lattice-like structures** is possible (Fig. 1C) [9]. Taken together, this evidence shows that the valency of biomolecules can determine the size and topology of biomolecular complexes.

The valency of nucleic acids plays an important role in cellular homeostasis. Phase transitions involving repetitive DNA (for example, ribosomal DNA [rDNA] and telomeres) also exploit the multivalent nature of rDNA, telomeres, and their constituent proteins [10,11]. These phase transitions are critical for maintaining genome organization and regulating transcription [12–16]. Increases in valency can promote phase transitions that can have pathophysiological consequences [17]. In **repeat expansion disorders**, such as amyotrophic lateral sclerosis, myotonic dystrophy, and Huntington's disease, the valency of repetitive DNA, or transcribed RNA, increases with the increasing number of repeats [18–20]. Working with chemically synthesized RNA with a defined number of repeats, the valency of the RNA has been shown to determine its phase separation kinetics [17]. Taken together, emerging evidence suggests that the valency of nucleic acids may play important roles in certain human diseases.

## Valency regulates innate immune sensing and signaling

Innate immune sensors need to have at least two functionalities – sensing and signal transduction. This requires a valency of at least two, with distinct binding sites for the ligand that is sensed and the downstream adaptor/signal transducer. As a general principle, ligand binding activates or uninhibits the adaptor/transducer to increase its valency, allowing the sensor to activate downstream signaling. Assembly of an immune signaling complex called the **myddosome** depends on the valency of its component proteins (Fig. 2A) [21]. The complex is nucleated by activation of the sensor, Toll-like receptors (**TLRs**), through mechanisms such as the lipopolysaccharide (LPS)-mediated activation of TLR4 [21]. Working with the human myddosome complex, different types of homotypic interactions between the death domains (DD) of the MYD88 transducer have been found to allow assembly of the coordinating TLR sensor-mediated inflammatory signaling (Fig. 2B) [22]. The DD shows bivalency in the myddosome with one of the interaction interfaces in MYD88 stretching into its intermediate domain (ID) [22]. Disruption of the homotypic interactions through mutagenesis of critical amino acid residues in the transducer MYD88, which decreases the valency, disrupts the assembly of the myddosome [22]. Conversely, increasing the valency of the MYD88 transducer by adding a new interaction motif through genetic engineering allows novel signaling through the myddosome complex [23]. For example, using murine immortalized bone marrow-derived macrophages (BMDM), one study showed that genetically engineered MYD88 containing the pLxIS motif (p, hydrophilic residue; x, any residue; S, phosphorylation site) was able to activate interferon (IFN) signaling, while wild type MYD88 could not [23].

In addition to the role of valency in the adaptor/transducer, valency within the ligand is also important. A recent study suggested that the innate immune sensor of cytosolic DNA, cyclic GMP-AMP synthase (**cGAS**), phase transitions into a liquid-like state upon ligand binding, forming a large supramolecular structure that acts as a signaling platform to activate downstream molecules; this phase transition is dependent on the length of the DNA molecule and greatly enhances the innate immune response to cytosolic DNA relative to

controls, by activating cGAS [24]. Since cGAS does not bind in a sequence-dependent manner, increases in the length of the ligand DNA can be interpreted as an increase in the valency of the ligand. Additionally, Influenza A virus (IAV) infection sensing by Z-DNA–binding protein 1 (ZBP1) also suggests that the multivalency of the ligand is important [25–27]. Specifically, studies in mouse models have shown that ZBP1 is a sensor of IAV infection [26,27]. In a recent study, super-resolution microscopy of the ZBP1-IAV complex indicated that multiple ZBP1 molecules were bound to the IAV ribonucleoprotein complex, suggesting that IAV ribonucleoprotein complexes are multivalent for ZBP1 binding [25]. These examples suggest that valency of these ligands is important in innate immune sensing.

Some cytokines such as IL-12 and IL-23 have a valency greater than one that allows them to bring together co-receptors for signaling [28]. The functional significance of multivalency of some cytokines remains a question that needs to be explored. IL-1 cytokines acting as ligands induce potent pro-inflammatory signaling upon binding to their receptor. IL-1β becomes activated after proteolytic processing and binds to its receptor (IL-1R) [29]. A great mystery in innate immunity is the function of another IL-1 cytokine, IL-1α [30,31]. IL-1α does not require proteolytic processing and has another domain that is not required for binding to its shared receptor IL-1R [29,32]. This suggests a hitherto undiscovered function of the second valency provided by the IL-1α N-terminal domain. Thus, these studies indicate that the valency of sensors, adaptors/transducers, and ligands can all contribute to regulation of innate immune sensing and signaling.

## The valency of sensors, adaptors, and ligands can regulate inflammasome activation

**Inflammasomes** are cytoplasmic complexes that drive a pro-inflammatory programmed cell death process termed **pyroptosis**. Mammalian inflammasomes consist of three components: a sensor protein that senses pathogen- or damage-associated molecular patterns (**PAMPs** and **DAMPs**, respectively), a catalytic actuator caspase (caspase-1, CASP1) that processes the effector cytokines and pore-forming protein gasdermin D (GSDMD), and often an adaptor that brings the sensor and caspase together [33]. The adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (**ASC**) contains a pyrin domain (PYD) and a caspase activation and recruitment domain (CARD) that allow assembly of the inflammasome complex through homotypic interactions with the PYD of the sensor and CARD of CASP1 [34]. Hypothetically, ASC seems to have a valency of two, which would limit the number of molecules in the inflammasome complex to three – one each for the sensor, CASP1, and ASC (Fig. 3A) because ASC needs to simultaneously interact with both the sensor and the catalytic actuator CASP1 [34]. However, the CARD domain of ASC is capable of multiple simultaneous homotypic interactions with other CARDs [35,36]. This multivalency in the adaptor molecule allows assembly of a larger supramolecular complex termed the ASC speck [37–39]. In addition, the ligand itself can act as a multivalent platform for assembly of the inflammasome in some cases [40,41]. For example, absent in melanoma 2 (**AIM2**) inflammasome activation requires 80 base pairs of double-stranded DNA [40,41]. However, the crystal structure of the AIM2 HIN domain in complex with double-stranded DNA has revealed that the ligand binding interface only

spans 7–8 base pairs [40]. The size discrepancy between the length of double-stranded DNA required for inflammasome activation and the significantly shorter length of the ligand binding interface for AIM2 suggests that multiple AIM2 molecules need to bind to a ligand DNA molecule for inflammasome assembly, with the DNA molecule providing the required multivalency [40]. Additionally, the valency of the sensor molecule can play a role in **NLRP3** and **NLRC4** inflammasome formation. Specifically, the NLRP3 PYD alone appears to be a poor nucleator of ASC polymerization, but a truncated NLRP3 protein containing both the PYD and nucleotide binding domain (NBD) greatly enhanced ASC polymerization in mammalian cells relative to controls [35,42]. This suggested that the NBD domain could provide additional valency for homotypic interactions, thus leading to more ASC polymerization for efficient inflammasome assembly (Fig. 3B) [35].

The NLRC4 inflammasome presents an interesting case where the adaptor ASC is recruited to the inflammasome through a CARD-CARD interaction between the NLRC4 CARD and ASC CARD domains [43]. This raises a question about the stoichiometry and structure of the NLRC4 inflammasome. If the ASC CARD is bound to the NLRC4 CARD, how does it recruit CASP1? We propose that two models are possible (Fig. 3C): In one model, the ASC CARD has a valency of two and contains distinct binding sites for the NLRC4 CARD and CASP1 CARD. In the second model, NLRC4 CARD-mediated recruitment of ASC can lead to ASC PYD-mediated polymerization of ASC filaments. The ASC CARD from another ASC molecule in the ASC filament is then free to recruit CASP1. It is interesting that ASC is dispensable for NLRC4 inflammasome activation because the NLRC4 CARD can directly bind to the CASP1 CARD, suggesting that the CARD domains of ASC and CASP1 might share a binding site on NLRC4 [44,45]. Although high-resolution cryo-electron microscopy (cryo-EM) structures are available for the NLRC4 inflammasome, these questions have not been resolved because the structures either lack the CARD or contain only the CARD domains [46–48]. Taken together, the evidence points towards multivalency of the adaptor ASC, ligands, and sensors playing critical roles in the assembly of a functional inflammasome complex, although further work is necessary to elucidate these roles and the putative mechanistic models that drive such functions. Understanding the role valency plays in inflammasome assembly is not just for academic interest; it can also help guide development of more potent drugs that target specific valency sites to modulate inflammasome activation. For example, targeting the ASC-NLRP3 valency for inhibition might be useful for treating patients with cryopyrin-associated periodic syndrome while leaving the NLRC4 and NLRP1 inflammasome functions intact to avoid systemic immunosuppression.

## Mechanisms for regulating the valency of a biomolecule

### Transcriptional regulation

Since valency may play an important role in innate immune signaling, it is not surprising that several mechanisms have evolved to regulate it. Valency can be controlled during gene expression as well as post-translationally. Expression-based regulation results in the synthesis of molecules that differ in their number or types of binding sites, and mechanisms of regulation include alternative transcription start site selection, alternative splicing,

alternative polyadenylation, and alternative translation initiation [49–54]. MYD88, the central component of the myddosome [21], has two isoforms that are generated by alternative splicing [55,56]. The longer isoform forms the myddosome complex to activate NF-kB signaling [56]. The shorter isoform ($MYD88_s$) lacks the intermediate domain that is required to recruit another component of the myddosome, interleukin-1 receptor-associated kinase 4 (**IRAK4**) [55,56]. Due to the loss of the binding capacity in the intermediate domain, $MYD88_s$ has a lower valency than the longer isoform and acts as a negative regulator of myddosome signaling in human and murine cell lines [56]. $MYD88_s$ is induced in response to LPS-mediated signaling, desensitizing HEK293T and Mf4/4 cells to LPS; it might perhaps act as a mechanism to control runaway inflammation [55,56]. Another myddosome component, IRAK4, also has two isoforms, $IRAK4_S$ and $IRAK4_L$, which are regulated by alternative splicing and alternative translation initiation. Differential expression of these isoforms has been associated with disease; specifically, exon usage analysis of samples from The Cancer Genome Atlas found that human acute myeloid leukemia cells express IRAK4-L, while normal bone marrow-derived $CD34^+$ hematopoietic cells and mononuclear cells express $IRAK4_S$. $IRAK4_L$ contains the three domains required for myddosome assembly and signaling, while $IRAK4_S$ lacks the N-terminal death domain [57]. Studies with MYD88, IRAK4, and other molecules suggest that the cells utilize expression-based mechanisms for determining the valency of molecules to regulate biological processes. Cells can therefore exploit multivalency, or the lack thereof, to mount optimal responses that can maintain cellular and organismal homeostasis.

## Post-transcriptional regulation

Molecular modifications of transcripts can also influence binding abilities and valency. Post-transcriptional modification of RNAs alters their binding specificity to proteins involved in epitranscriptomic regulation [58]. Similarly, DNA modifications play critical roles in gene expression regulation by altering the binding specificity of DNA for transcription factors and DNA-modifying enzymes [59]. Moreover, chromosomal translocations can lead to gene fusions that alter the regulation of gene expression or which generate chimeric proteins. Chimeric proteins might have additional valencies that can have profound effects on cell physiology and host homeostasis. Specifically, the Philadelphia chromosome generated by a chromosomal translocation of human chromosomes 9 and 22 is a common feature of chronic myelogenous leukemia [60]. This chromosomal translocation generates a gene fusion in which the oncogene *ABL* is fused to the (B-cell receptor) *BCR* gene [61]. The fused gene produces the BCR-ABL protein that has gained a valency for SH2 domain binding, which contributes to its oncogenicity in B cells [62]. This additional SH2 binding valency in the BCR-ABL chimeric protein then allows it to form a complex with the adaptor protein GRB-2 that leads to activation of Ras signaling [62]. A number of similar cases of gain of valency as well as loss of valency through gene truncations have been described [63].

## Post-translational regulation

In addition to the impact of transcript expression and modifications on the valency of the resulting protein, the valency of protein molecules can also be regulated post-translationally. Proteolytic processing provides one post-translational mechanism to regulate valency in the innate immune system. For example, the mammalian inflammasome-dependent cytokines

IL-1β and IL-18 are expressed as inactive monomers that cannot bind to their receptors [64,65]. Essentially, they have a valency of zero for their receptors. Removal of their autoinhibitory domains by inflammasome activation-dependent proteolytic cleavage unmasks the hidden valency increasing it to one and allowing them to bind to their receptors [34,66–68]. Another example of proteolysis-dependent regulation of valency is provided by a versatile kinase receptor-interacting serine/threonine kinase 1 (RIPK1). In its initial state, RIPK1 is fully functional; however, CASP8 proteolytically cleaves RIPK1, bifurcating its valency onto two fragments and inhibiting RIPK1 scaffold function-dependent processes [69,70]. Recent studies have shown that the valency of the inflammasome sensor **NLRP1B** is also regulated by proteolytic cleavage, with the proteasome removing the N-terminal inhibitory domain to control activation [71–74].

Post-translational modifications (**PTMs**) of proteins can also regulate valency. PTMs can induce conformational changes that expose or occlude binding sites, leading to an increase or decrease in valency, respectively. They can also change the surface properties of the protein, making it more or less prone to interact with its ligand. Intrinsically disordered regions (**IDR**) in proteins are promiscuous binding motifs that can drive the assembly of supramolecular structures in cells through IDR-IDR binding [75,76]. Phosphorylation in the IDRs can regulate their valency. For example, phosphorylation in the IDR of fused in sarcoma (**FUS**) inhibits assembly of FUS granules, likely by making IDR-IDR interactions energetically unfavorable due to electrostatic repulsion and decreasing valency [77]. Similarly, phosphorylation of the IDR of human GLE1, a stress granule component, triggers disassembly of stress granules in HeLa cells [78]. PTMs themselves can also act as binding sites, thereby increasing the valency of a protein [79]. K27 ubiquitination of stimulator of interferon genes (**STING**) increases its valency and allows it to bind the serine/threonine-protein kinase **TBK1** and activate downstream signaling upon cytosolic DNA sensing [80]. M1-K63 hybrid ubiquitination of myddosome components can also increase valency, allowing recruitment of the transforming growth factor β-activating kinase 1 (TAK1) and IκB kinase (IKK) complex for their activation [81]. In another example, K63 ubiquitin chains can allow proteins to bind DNA, thereby modifying the valency of the K63-ubiquitinated proteins and facilitating the repair of damaged DNA [82]. Prenylation is another PTM that can expand the valency of proteins [83]. For example, prenylation of a protein can allow it to bind to a lipid membrane, increasing its valency for lipid membranes from zero to one [84–87]. Thus, post-translational modifications of proteins can increase or decrease their valencies.

Collectively, these examples illustrate different ways in which the valency of a diverse array of biomolecules can be regulated, and some cases in which such dysregulated valency status might contribute to, or result in, disease.

## The valency of a cell might determine its response to external stimuli

We have described many examples of the valency of a biomolecule determining its function and regulation. We propose that the idea of valency can also be applied at the whole cell level. For example, similarly to a biomolecule that has a defined number of binding sites for other molecules, or a valency, a cell also has an exact number of sensor molecules on its

surface to recognize a particular PAMP; we can define this number as the “cell’s valency” for that PAMP (Fig. 4A). The same idea can be applied to any ligand-receptor combination, the number of adaptor and signal transducer molecules, the number of **tRNA** or mRNA molecules, the number of specialized ribosomes, gene copy numbers, or even the number of organelles, such as mitochondria and micronuclei (Fig. 4B) (See Outstanding Questions). Cells have evolved to utilize their valency in creative ways to regulate their functions. For example, 61 codon-tRNA pairs are used to code for 20 amino acids, meaning that some amino acids are coded by multiple redundant codon-tRNA pairs. The set of tRNAs possessed by a cell might be considered the tRNA valency of the cell. The tRNAs for certain codons are present in higher numbers than other redundant codons are, and the codons corresponding to those more abundant tRNAs are utilized more often by the cell [88–90]. These codons are considered ‘**optimal codons’** because they allow the fastest possible translation rate [91,92]. However, cells might use suboptimal codons to regulate co-translational protein folding and even protein stability, modulating the speed of translation by utilizing the tRNA valency of a cell [93,94]. As an example, the use of a suboptimal codon in the γ-actin open reading frame exposes a lysine residue ($K^{18}$) for ubiquitination and subsequent proteasome-mediated co-translational degradation in HEK293T cells [94].

The set of ribosomes present constitutes another type of valency in cells. A number of studies over the last decade have suggested that all ribosomes are not created equal [95,96]. Ribosomes differing in composition have different translational efficiency for specific mRNAs and act as a ribosome filter to fine-tune gene expression programs [95]. For instance, mice lacking one of the ribosomal proteins, RPL38, exhibit developmental defects linked to inefficient translation of certain *Hox* mRNAs [97]. Moreover, working with *Saccharomyces cerevisiae*, researchers have shown that cells can regulate the ribosome composition based on external stimuli, thereby regulating their ribosomal valency [98,99].

The valency of membrane-bound organelles can also play important roles in regulating cellular processes. In a recent study, mitochondrial replication increased mitochondrial valency and was found to be important for NLRP3 inflammasome activation [100]. In another report, NLRP3 binding to a dispersed trans-Golgi network was required for the activation of the NLRP3 inflammasome [101]. Furthermore, enzymatic activity potential can also be considered a type of valency. This can account for redundancies between different enzymes that act on the same substrate. For example, CASP1 and CASP8 can both process IL-1β and have been shown to play a role in a mouse model of osteomyelitis [102]. In this murine model, IL-1β–driven autoinflammatory osteomyelitis was inhibited with the loss of both *Casp1* and *Casp8*. Mice lacking only one of these caspases had increased susceptibility to disease compared with the double knockout animals [102]. Overall, we propose that the number of cellular components or the activity potential of a cell can determine cellular valency with respect to that component or activity.

Using this concept, cellular valency might determine the cell’s response to external stimuli (See Outstanding Questions). For example, in the innate immune system, LPS stimulation leads to strong concentration-dependent kinetics for myddosome assembly, with higher concentrations of LPS leading to a more rapid assembly of the myddosome relative to stimulation with lower concentrations [103]. According to our model, this concentration

dependence might be explained by the fraction of TLR4 molecules on the cell surface that are LPS-bound at a given concentration. At the highest LPS concentration tested in the study, most if not all of the TLR4 molecules on the cell surface might be expected to be LPS-bound, allowing maximal signaling and faster myddosome assembly. Similarly, under lower LPS concentrations, a fraction of TLR4 molecules would be expected to be free, potentially leading to lower downstream signal strength and slower kinetics of myddosome assembly relative to the signal strength and kinetics observed at higher concentrations [103]. We hypothesize that this dose-dependence could be generalized to explain the observed differences in the responses of individual cells to stimuli. Such differences might be expected to be greater under conditions when the components of a cell involved in responding to a stimulus or a ligand are present in limiting amounts (Fig. 4C). Since ligand-receptor binding is an equilibrium process, it is reasonable to suppose that the strength of an interaction might also be a critical parameter in determining the fraction of satisfied receptor valency. For example, when a population of cells is stimulated with less than the saturating concentration of a ligand, there might be differences in the fractions of ligand-bound and free receptors between cells. This might in turn lead to differential responses in individual cells.

Cells can exploit their finite valencies with respect to their components to make cell fate decisions. If there is a common essential factor required for two different cellular processes, there might be a competition between the two processes for this factor. For example, when stress granule and NLRP3 inflammasome assembly are competing for the same common essential factor – e.g. DDX3X protein molecules-- the DDX3X valency might allow the cell to make a ‘live-or-die’ cell fate decision [104]. In murine BMDM, induction of stress granules inhibited NLRP3 inflammasome activation by sequestering DDX3X, preventing the cells from undergoing pyroptosis [104]. The concept of valency at the whole-cell level might also have important pathophysiological consequences and might lead to interesting future applications. An example would be assessing the optimal dose of a drug. Indeed different cell types might have different valencies for a drug. Hypothetically, the dose might be tailored based on the valency of the cell type being targeted. Valency could also be an important consideration in mathematical modeling of single-cell responses. Accordingly, studies of single-cell response have revealed differences in the responses of individual cells, even though all cells in a given population might be expected to be of the same genotype [105–107]. Presumably, such differences might be due to stochastic differences in the valencies of various types of cells. Furthermore, cells in a population can communicate with each other through cell-cell contacts and secreted signaling molecules, such as cytokines and chemokines. Thus, the amounts of cytokines and chemokines in the extracellular space might be expected to be lower than the amount needed to ensure that all the receptors are bound by their corresponding ligands. In such cases and as mentioned above, these differences in the fractions of ligand-bound receptors might also be expected to contribute to the differences in the responses of individual cells. Overall, such examples suggest that the concept of valency might be helpful in the study of immune responses.

## Concluding remarks

In summary, we propose that valency is a fundamental property of biomolecules and cells. The valency of biomolecules is critical for the assembly of functional biomolecular complexes. Cells have evolved multiple mechanisms to regulate the valency of biomolecules to ensure homeostasis. We extend the concept of valency to whole cells and propose that it might be used to explain variations in the responses of individual cells. Testing the valency hypothesis to explain the responses of individual cells would require the development of more accurate and precise quantification techniques that can allow multiparametric measurements at the single cell level (see Outstanding Questions). Techniques such as **Cy-TOF** and multi-color flow cytometry analysis are already being used to study individual cells in suspension [108,109]. Nevertheless, there is still a need to develop techniques that allow these measurements to be performed on adherent cells. We posit that considering valency as an important parameter might lead to a better understanding of biological processes, which in turn could potentially lead to the development of better therapeutic interventions for a variety of immune-related pathologies. Indeed, the idea of using valency to modulate drug efficacy is already being exploited through the development of multivalent drugs, highlighting the potential applications of this exciting concept [110–112].

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## Glossary

**AIM2**
cytosolic DNA sensor activated upon DNA binding to assemble the AIM2 inflammasome and initiate pro-inflammatory cell death through pyroptosis

**ASC**
adaptor protein required for inflammasome assembly that bridges the sensor and the catalytic actuator caspase-1

**Avidity**
measure of the accumulated strength of an interaction between biomolecules depending on the combination of all non-covalent interactions

**cGAS**
cytosolic DNA sensor that catalyzes the synthesis of cyclic AMP-GMP (cGAMP) to induce the innate immune response by activating STING-mediated signaling

**Cy-TOF**
a flow cytometry-based mass spectrometry technique to study single cells using cells labeled with lanthanide metal-tagged antibodies

**DAMP**

molecules generated from cell and tissue injury

**FUS**
nucleic acid binding protein that can undergo phase transitions that have pathophysiological consequences for amyotrophic lateral sclerosis disease

**IRAK4**
component of the myddosome complex

**IDR**
region that does not form a defined secondary structure in a protein; critical for the assembly of large protein complexes

**Inflammasome**
protein complex formed in the cytoplasm after sensing intracellular damage and pathogen-associated molecular patters that contains a sensor, executioner caspase-1 or caspase-4/5/11, and often the adaptor ASC

**Lattice-like structures**
biomolecules with a valency of 3 or more can form supramolecular assemblies that contain these structures

**Myddosome**
signaling platform formed upon stimulation of toll-like receptors containing MYD88 and other proteins

**NLRC4**
inflammasome sensor activated in response to intracellular bacteria by sensing components of the flagella and type III secretion system

**NLRP1B**
inflammasome sensor activated by proteolytic processing of its inhibitor domains by bacterial toxins

**NLRP3**
inflammasome sensor activated downstream of potassium efflux, dispersed Golgi network, mitochondrial DNA replication, and mitochondrial reactive oxygen species

**Optimal codons**
codons within the cell that allow for the fastest possible translation rate; these are generally the most abundant codon for a given amino acid

**PAMP**
molecular signatures of infection

**Phase separation**
property of liquids where two liquids with sufficiently different physiochemical properties become immiscible

**PTM**
molecular modifications, such as phosphorylation or ubiquitination, of proteins that occur after translation

**P-bodies**
membraneless cytoplasmic compartments involved in RNA processing; formed in response to stress and which contain RNase enzymes

**Pyroptosis**
pro-inflammatory cell death program activated downstream of inflammasome activation; characterized by cleavage of the pore-forming proteins gasdermin D/E and release of IL-1β and IL-18

**Repeat expansion disorders**
class of human diseases characterized by an increase in the number of times a repeated pattern of nucleic acids appears

**STING**
five transmembrane protein that plays a critical role in the innate immune response downstream of cyclic AMP-GMP synthase

**Stress granules**
membraneless cytoplasmic compartments formed in response to stressors; may act as storage compartments for translation initiation complexes allowing cells to survive stress

**TBK1**
serine/threonine kinase activated in response to innate immune sensing of bacterial and viral infections

**tRNA**
type of RNA cofactor involved in protein translation

**TLR**
type of pattern recognition receptor; helps cells sense damage- and pathogen-associated molecular patterns

**Valency**
number of distinct binding sites for a specific interaction on a biomolecule. In the case of cellular valency, it is the effective number of a cellular component (product of the number of binding sites on a component and the total number of that component present in/on the cell); e.g. if a receptor can bind two ligand molecules, and there are three copies of that receptor on the cell, the cellular valency for that receptor will be six

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## Highlights

- The valency of a biomolecule determines its biophysical interactions.
- Innate immune signaling can require multivalent interactions for the proper functioning of signaling platforms such as the myddosome.
- Inflammasome activation can exploit the multivalency of sensors, adaptors, and ligands.
- We posit that the concept of valency might be applied at the whole-cell level to study differences in individual cell responses.

## Outstanding Questions

- How do we determine the exact valency of a biomolecule? Homotypic and heterotypic interactions between well-studied domains are easy to predict. For example, interactions between PYRIN, CARD, RHIM, and DED domains play critical roles in regulating immune responses. However, post-translational modifications and interactions between under-studied proteins make it hard to accurately predict biophysical interactions. There is an important need to develop sets of tools to accurately predict such interactions.
- How do we determine the exact valency of a cell? The number of a specific cellular component can determine the valency of a cell with respect to that component. However, in many cases multiple ligands can signal through the same receptor to induce different cell fates. IL-1α/IL-1β and IFNα/IFNβ are examples of pairs of ligands that share receptors. Alternatively, the ligand can signal through multiple receptors, with TNF signaling through TNFR1 and TNFR2 as an example. We need tools that allow us to count the numbers of each cellular component at the single-cell level. We also need a computational framework enabling the interrogation of diverse datasets (for example, proteomic and transcriptomic data) to build accurate models.
- Can the valency of a cell determine its response to stimuli? We hypothesized that the valency of a cell and the extent to which the valency is satisfied might determine the responses of individual cells. Although it makes intuitive sense and agrees with the published literature, the hypothesis still needs to be directly tested.
- How do we account for the valency of intrinsically disordered regions (IDRs) in proteins? IDRs are capable of engaging in promiscuous interactions. They drive the assembly of many cellular supramolecular structures. However, IDRs are also prone to inducing irreversible phase transitions to the insoluble phase, with potential pathophysiological consequences. IDRs are present in a vast number of proteins, including proteins involved in immune signaling, such as DDX3X. We need to find a way to clearly understand how IDR-IDR interactions work so that we can better predict which of the IDRs are capable of interacting with each other. Alternatively, is there any specificity to IDR-IDR interactions? What are the different ways in which IDR-IDR interactions are regulated?

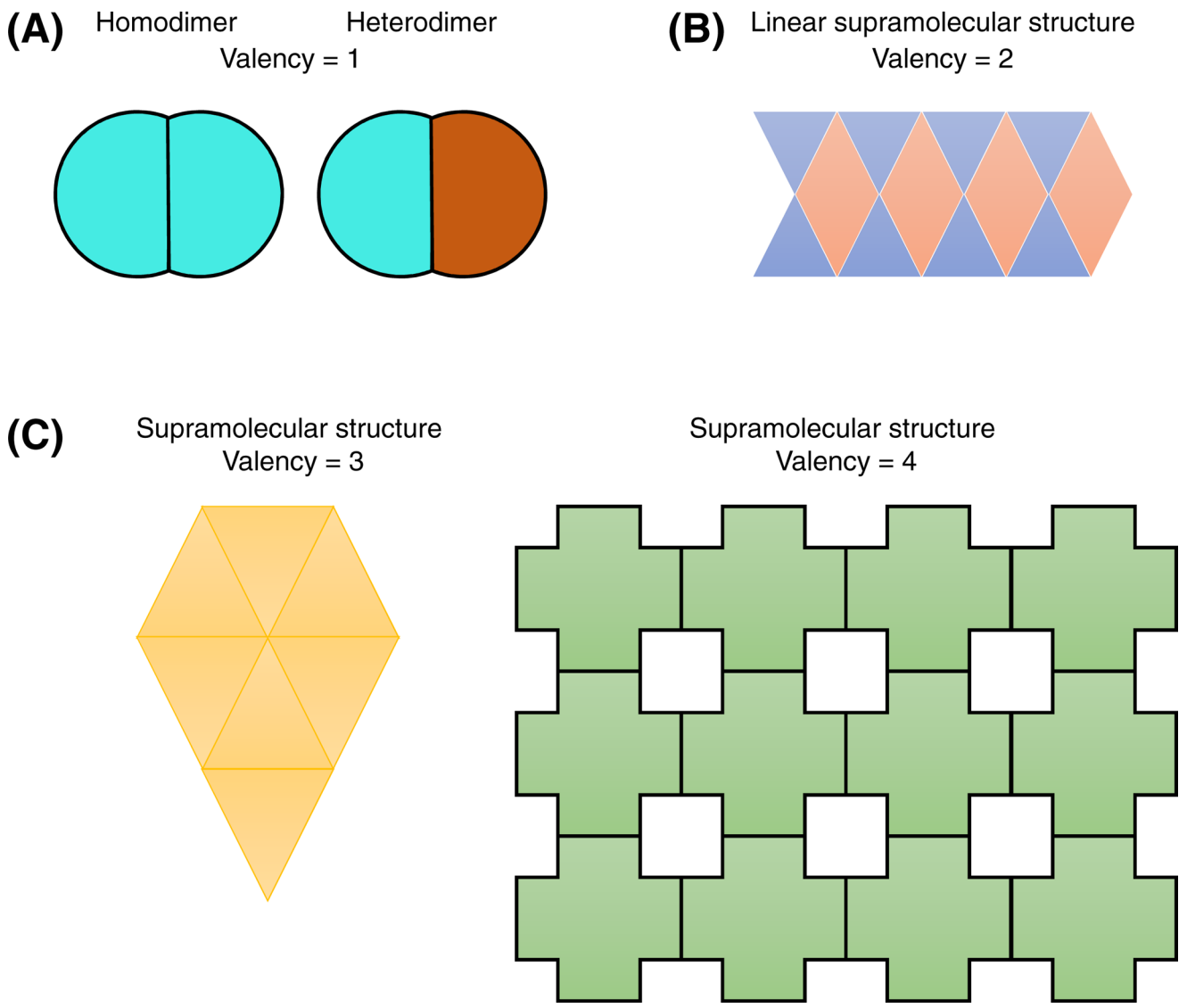



**Figure 1. Valency can determine the architecture and size of a biomolecular complex.**

(A) A valency of one limits the size of a complex to a dimer. (B) A valency of two can lead to formation of large, linear complexes. (C) A valency of three or more can allow assembly of complexes with varying architecture and sizes.

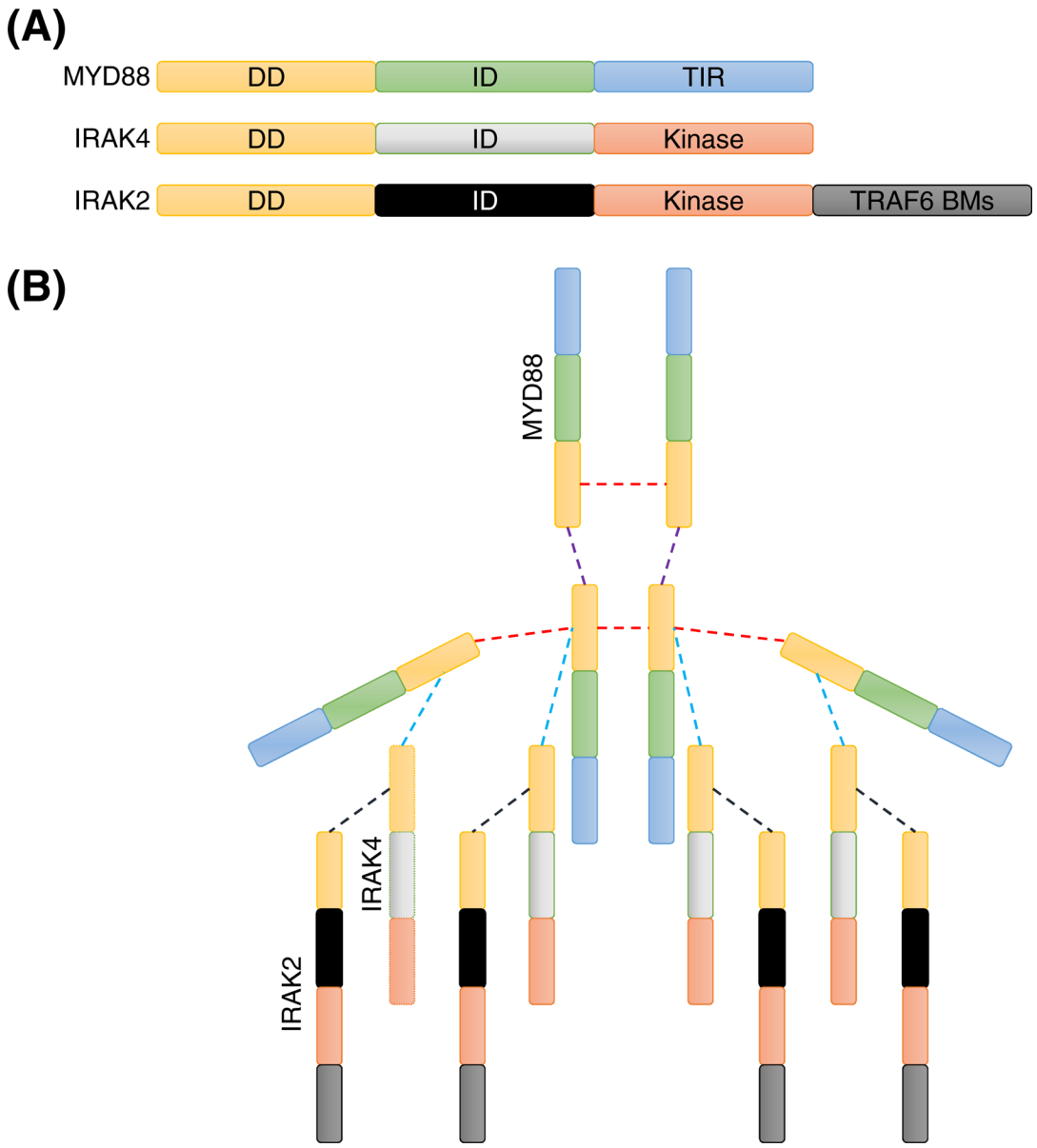



**Figure 2. Mammalian myddosome assembly is driven by the multivalency of its components.**

(A) Valencies of MYD88, interleukin-1 receptor-associated kinase (IRAK) 4, and IRAK2 help assemble the myddosome. DD is the death domain, ID is the intermediate domain, TIR is the Toll/interleukin-1 receptor (TIR) homology domain, and TRAF6 BMs is the TRAF6 binding motif. (B) Different types of interactions suggest distinct valencies in the DD domains of MYD88, IRAK4, and IRAK2 that allow assembly of the myddosome [22]. Domains are color-coded as in panel A. Dashed lines represent physical interactions between the DDs. Homotypic interactions between the different molecules representing distinct valencies in the DD are depicted with different colored dashed lines.

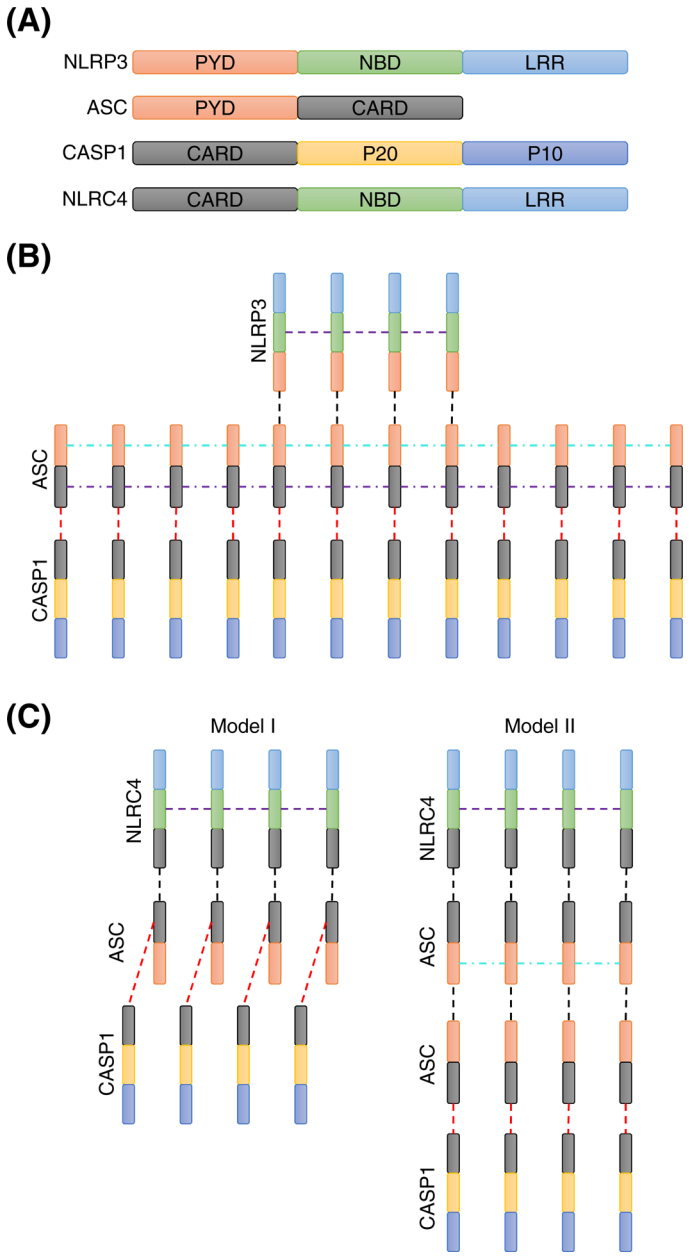
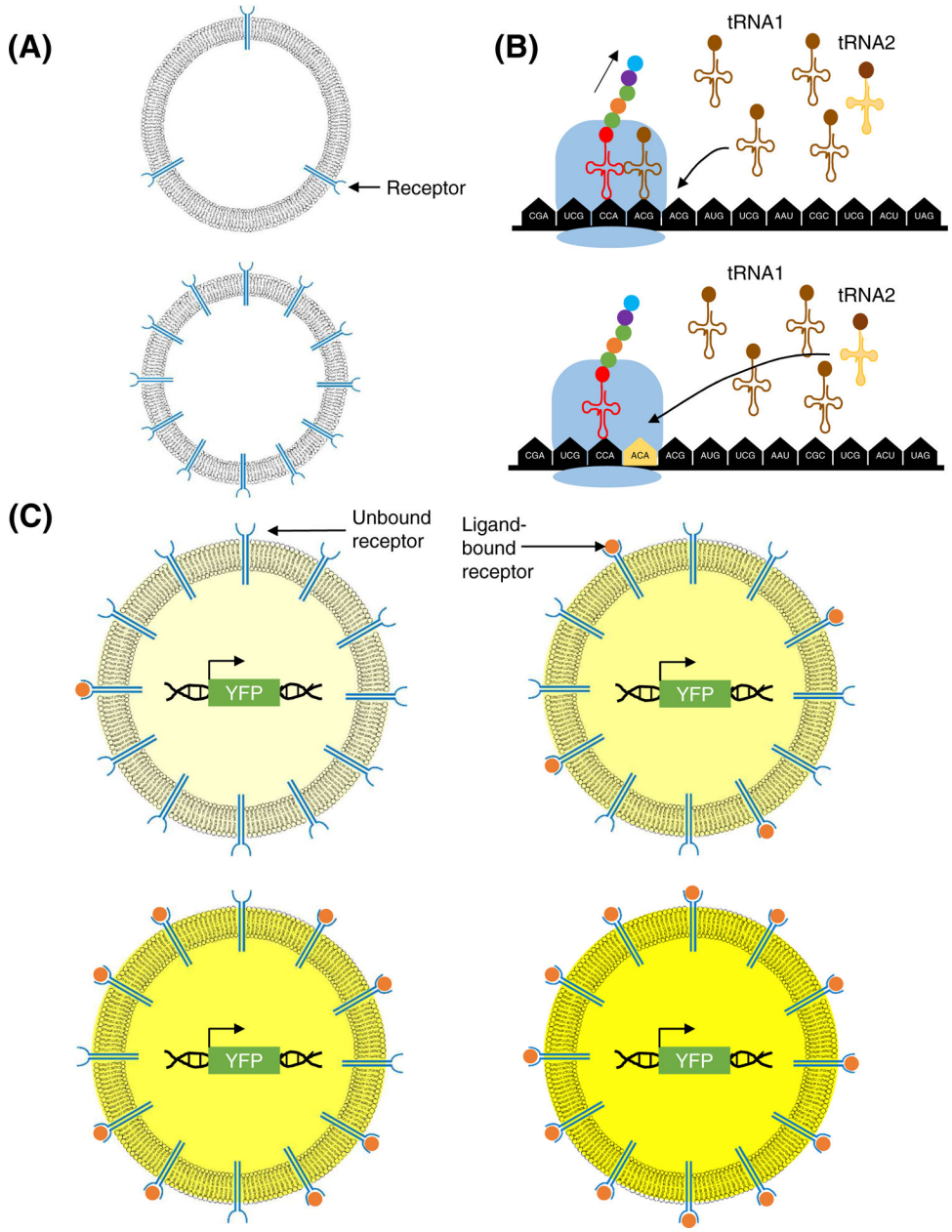



**Figure 3. Valencies of the sensor, adaptor, and ligand are critical for mammalian inflammasome activation.**

(A) Valency of the inflammasome core components NLRP3, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), caspase-1 (CASP1), and NLRC4. The ASC caspase activation and recruitment domain (CARD) is multivalent with respect to homotypic CARD-CARD interactions that allow it to polymerize into ASC filaments [36]. PYD is the pyrin domain. NBD is the nucleotide binding domain. LRR is the leucine rich repeat domain. The P20 fragment contains the caspase active site. The P10 fragment is auto-proteolytically cleaved for CASP1 activation. (B) NLRP3 PYD and NBD domains provide multivalency for efficient assembly of ASC filaments. Domains are color-coded as in panel A. Dashed lines represent physical interactions. Homotypic interactions

between the different molecules representing distinct valencies are depicted with different colored dashed lines. (C) NLRC4 inflammasome assembly after ligand sensing can happen through two distinct molecular interaction pathways involving the CARD domains of ASC, CASP1, and NLRC4. In model I, the NLRC4 CARD binds to the ASC CARD, which can simultaneously recruit CASP1 through a CARD-CARD interaction. In model II, the NLRC4 CARD binds to the ASC CARD, leading to ASC polymerization through the ASC PYD. The ASC CARD domains in the ASC filament subsequently recruit CASP1 to facilitate its activation.




**Key Figure, Figure 4. Model: The valency of a cell might be determined by the number of molecules it possesses.**

(A) Cell surface receptor/sensor valency is the number of receptors/sensors, or a product of valency and number of receptors/sensors if the receptors/sensors can bind to more than one molecule of the ligand. (B) The number of tRNAs available may be another example of valency in the cell. The rate of mRNA translation can be modulated by the usage of redundant codons (tRNA1 and tRNA2 which code for the same amino acid here). For example, the use of a codon whose cognate tRNA abundance is low (tRNA2 here) will slow the rate of translation. (C) The fraction of receptors bound to their ligands can determine the response of individual cells. A higher fraction of bound receptors can lead to stronger cellular response. In the figure, we use an example of yellow fluorescent protein (YFP)

expression as a readout of cellular response. There is an increase in YFP fluorescence corresponding with the increase in the fraction of valency being satisfied by ligand binding.