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Mechanics of a molecular mousetrap—nucleation-limited innate immune signaling

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ABSTRACT Innate immune responses, such as cell death and inflammatory signaling, are typically switch-like in nature. They also involve "prion-like" self-templating polymerization of one or more signaling proteins into massive macromolecular assemblies known as signalosomes. Despite the wealth of atomic-resolution structural information on signalosomes, how the constituent polymers nucleate and whether the switch-like nature of that event at the molecular scale relates to the digital nature of innate immune signaling at the cellular scale remains unknown. In this perspective, we review current knowledge of innate immune signalosome assembly, with an emphasis on structural constraints that allow the proteins to accumulate in inactive soluble forms poised for abrupt polymerization. We propose that structurally encoded nucleation barriers to protein polymerization kinetically regulate the corresponding pathways, which allows for extremely sensitive, rapid, and decisive signaling upon pathogen detection. We discuss how nucleation barriers satisfy the rigorous on-demand functions of the innate immune system but also predispose the system to precocious activation that may contribute to progressive age-associated inflammation.

SIGNIFICANCE Innate immune responses are sensitive, rapid, and decisive. In this perspective, we review current knowledge of innate immune signaling pathways that involve self-templating polymerization of proteins into large complexes known as signalosomes. We propose that signalosome proteins' extraordinary sensitivity to noxious stimuli and switch-like activation result from their tenuous existence in physiologically unstable, supersaturated states in cells. We further suggest that precocious activation occurs as a consequence of this and contributes to progressive age-associated inflammation.

INTRODUCTION

Cells must sense, respond, and adapt to noxious agents, a responsibility borne by a network of interacting genes and proteins in the innate immune system that executes protective cellular programs such as inflammatory cytokine secretion, innate immune cell differentiation, and programmed cell death (1,2). Cellular commitments to these programs coincide with the formation of gigantic protein complexes mediated by orderly self-assembly of specific domain families (3,4). Referred to as supramolecular organizing centers (SMOCs) or more broadly as "signalosomes," these complexes couple the detection of pathogens or cellular damage with specific cellular responses mediated by caspases and other effector proteins (5,6).

The cellular consequences of signalosome formation are often rapid, switch-like, and decisive in nature. The most extreme among these is programmed cell death via the extrinsic apoptosis (death-inducing signaling complex), necroptosis (necroptosome), and pyroptosis (inflammasome) pathways. Similarly, the proinflammatory transcription factor NF-κB is activated in an all-or-none fashion by multiple signalosomes (7–9). The process of signalosome formation is closely associated with these switch-like innate immune signaling outcomes.

Some forms of protein self-assembly exhibit the functional properties of signalosomes. In particular, highly ordered polymers known as prions can assemble suddenly and irreversibly under normal cellular conditions. This property arises from a nucleation barrier that preserves the proteins in soluble form, even at high concentration, until the moment they encounter a preassembled seed of the polymer. Despite the polymer being thermodynamically favored, nucleation is highly unfavorable, and hence, polymerization occurs only rarely.

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In this perspective, we focus on the unusual biophysical and structural properties of innate immune signalosomes that contribute to the prion-like nature of their activation. We propose that the near crystalline order of major protein constituents implies that signalosome assembly is kinetically regulated through sequence-encoded nucleation barriers. Those nucleation barriers allow resting cells to express the proteins to deeply supersaturating levels, priming the signaling networks for abrupt activation upon detection of pathogens or cellular damage. We propose that this mechanism drives the rapid and frequently irreversible kinetics of activation that underlies signalosome functions in cell fate decisions, allows for executive redundancy in making those decisions, and suggests a thermodynamic basis for age-associated inflammation.

Signaling through crystallization not (just) liquid-liquid phase separation

Signalosomes assemble through homotypic protein-protein interactions of the death domain (DD), Toll/interleukin-1 receptor (TIR), and RIP homotypic interaction motif (RHIM) families (3,4). These modules are all ancient but appeared at different times in the evolution of innate immunity, with DD conserved into basal metazoans, TIR beginning to take cell death signaling roles in bacteria, and RHIM-related amyloid-forming motifs first appearing in multicellular bacteria (10,11). They occur in 97, 15, and 4 human proteins, respectively. Upon activation, DD, TIR, and RHIM domains polymerize into uniform filaments that can extend for hundreds of their subunits.

In their inactive, soluble state, RHIM modules populate a disordered ensemble of conformations. But in the polymer state—an amyloid fibril—they are locked into a very specific, tightly packed β-serpentine arrangement, with each molecule forming a precise conformational replica of the molecule preceding it in the fiber (12,13). The disorder-to-order transition propagates along the length of the RHIM polypeptide and across the interface of opposing β-sheets, rendering subunit addition highly cooperative (12) and imposing a quasi-multidimensional crystalline periodicity (14,15). The extraordinary loss of intra- and intermolecular entropy required for the nucleation, or de novo formation, of such a structure produces a kinetic barrier large enough to keep RHIM molecules from doing so spontaneously under normal cellular conditions and timescales. This nucleation barrier plays a significant role in regulating the kinetics of signalosome formation and will be elaborated below.

In contrast to RHIM, DD and TIR domains are well folded even in the soluble state (16). The DD superfamily includes the eponymous DDs, caspase recruitment domains (CARDs), pyrin domain (PYD), and death effector domain (DED) subfamilies. Importantly, the monomers of DD and TIR domains contain charged patches on their surfaces that correspond to pairs of complementary binding sites. These binding sites give rise to asymmetric protein-protein interfaces that allow for open-ended self-assembly into tightly twisted two-dimensional crystalline lattices whose self-intersection in three-dimensional space results in long tubular filaments (Fig. 1).

The fact that DD and TIR assemble in two dimensions is informative from an evolutionary standpoint. This is because newly arising mutations on protein surfaces overwhelmingly favor symmetric self-interactions that do not lead to open-ended assembly (17). Additionally, even the relatively rare asymmetric interfaces more frequently allow for one-dimensional polymerization—such as that of SAM and DIX domains found in some 300 human proteins (18)—than two-dimensional polymerization because the former is less entropically restrictive (19). SAM and DIX domains do not assemble cooperatively and lack the switch-like kinetics of DD and TIR (20–22). Nevertheless, their polymerization frequently contributes alongside other interacting modules in the same proteins to liquid-liquid phase separation (LLPS) (see below) in many dynamic signaling responses (5,18). Given the restricted sequence space supporting two-dimensional polymerization, we can infer that a functional requirement for cooperative and/or switch-like activation, rather than higher-order assembly per se, is driving the evolution of innate immunity signalosome structure.

The existence of stabilizing interactions in more than one dimension means that RHIM, DD, and TIR monomers can assemble cooperatively. Indeed, innate immune signalosomes are prototypical examples of signaling by cooperative assembly formation (SCAF) (23). Cooperativity narrows the range of concentrations and stimulus parameters over which proteins respond and thereby enhances the sensitivity of signalosomes (23,24). This property is central to innate immune signaling and has shaped the evolution of signalosomes. Cooperativity is, however, an equilibrium property of molecular systems and is therefore inadequate to describe kinetic properties of signalosomes. In our view, the notion of SCAF does not sufficiently account for their peculiar structural features. This is underscored by recent descriptions of other signaling assemblies that achieve effectively infinite cooperativity and extraordinarily sharp response thresholds through the structurally much less restrictive process of LLPS or "biomolecular condensation" (25–28). LLPS involves the segregation of multivalent proteins into discrete regions of low and high density without concomitant ordering of the proteins and therefore tends to preserve protein dynamics and allows for rapid dissolution of the condensate when cellular conditions change. Although LLPS has been observed for the cGAS-STING and T cell receptor pathways (29,30), the fact that DD, TIR, and RHIM domains instead undergo crystalline deposition suggests that some requirement of signaling through most innate

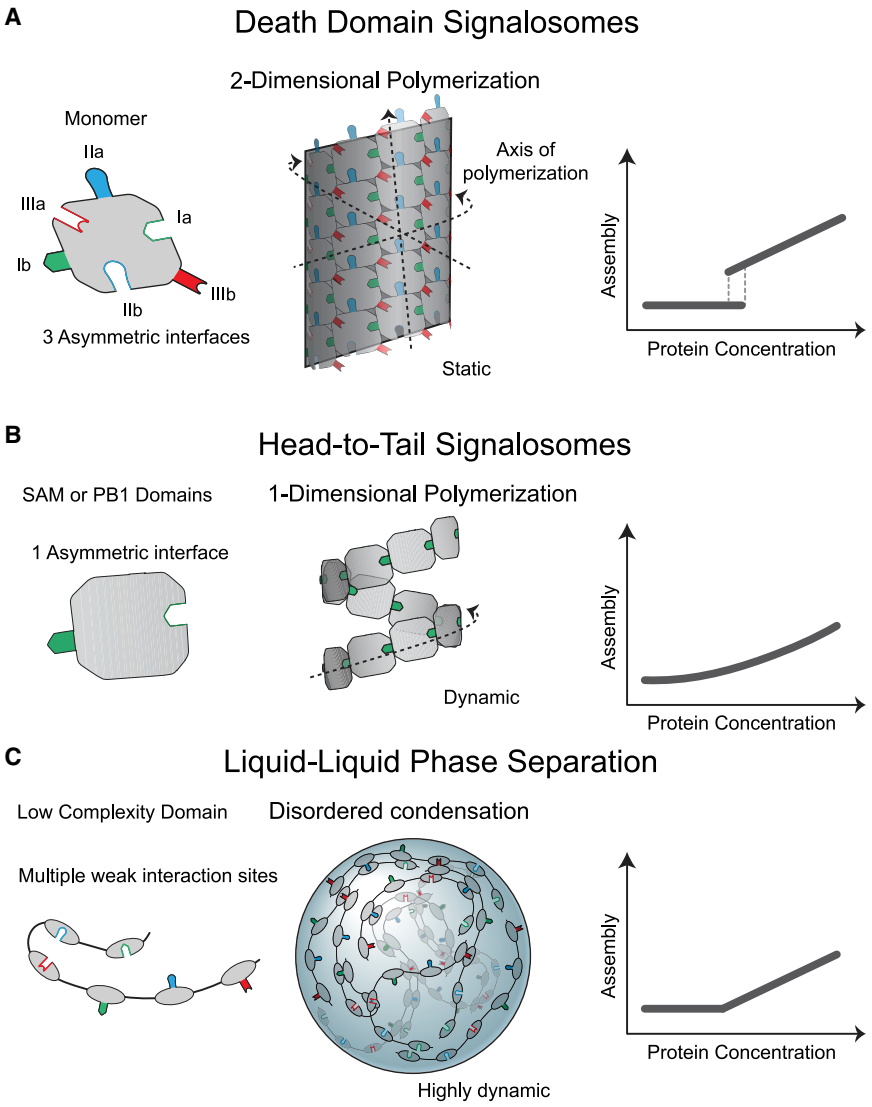

FIGURE 1 Switch-like kinetics emerge from the mechanism of innate immunity signalosome assembly. (*A*) DD monomers contain three asymmetric protein interfaces that allow for two-dimensional polymerization into tubular filaments. The improbability of forming these geometrically constrained interactions simultaneously de novo allows the protein to exist at supersaturing concentrations wherein signaling through this mechanism is probabilistic and switch like. (*B*) PB1 and SAM domains interact across one asymmetric interface to form dynamic polymers involved in transcriptional regulation and autophagy. Assembly through this mechanism increases continuously with concentration. (*C*) Multivalent disordered regions drive dynamic phase separation through multiple low-affinity interactions that are geometrically unconstrained. Assembly through this mechanism is sharply cooperative but not switch like.

immune signaling pathways cannot be met through LLPS. What might that requirement be?

Beyond cooperativity: function emerges from nucleation-limited kinetics rather than the material polymers

Phase separation involves a nucleation barrier that describes the improbable formation of the first bit of the condensed phase within a supersaturated solution. "Supersaturation" refers to the metastable or temporary nature of the soluble state of the molecules with respect to a more stable state (the assemblies) they can form under the same condition if given enough time. The saturating concentration, above which the solute is said to be supersaturated, is governed by the relative affinity of the solute for other molecules of itself versus molecules of solvent. Different proteins therefore have different saturating concentrations. A protein is physiologically supersaturated if its saturating concentration lies below that of its endogenous concentration, which is typically in the nanomolar to micromolar range. For LLPS, the nucleation barrier involves only a density fluctuation and is too insignificant to prevent sufficiently abundant proteins from spontaneously assembling in cells (31). In contrast, deposition onto a crystalline lattice, as in the case of signalosomes, involves a change not only in density but simultaneously in the orientation, translational position, and intramolecular conformations of the molecules. DD have been found to polymerize unidirectionally as a consequence of allosteric conformational changes that occur in the monomeric subunit upon joining the templating end of the fiber (Fig. 2; (36,37)). The combined entropic cost of losing so many degrees of freedom increases the nucleation barrier to such an extent that it allows DD, TIR, and RHIM to remain soluble in cells even at concentrations that are deeply supersaturated with respect to the assembled state.

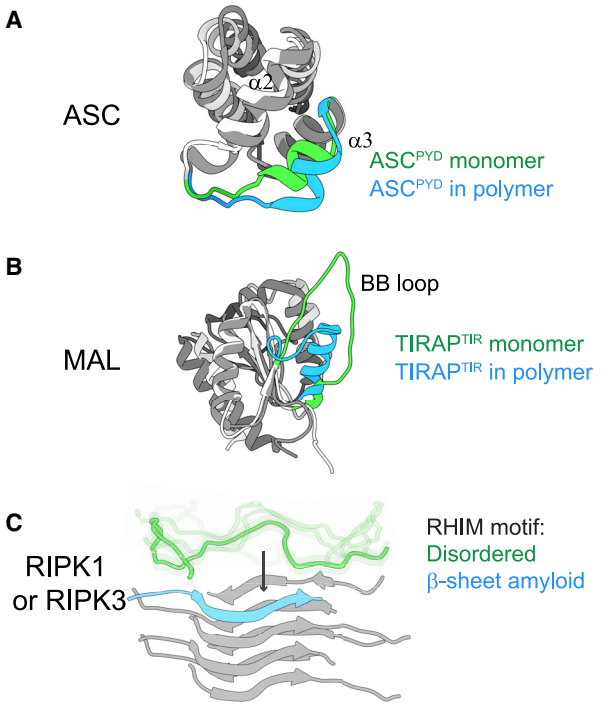

FIGURE 2 Conformational fluctuations in DD, TIR, and RHIM modules enhance nucleation barriers. (*A*) The pyrin subfamily DD of ASC undergoes a conformational change in the α2-α3 loop and short α3 helix upon polymerization, as evident by comparing its structure in the monomeric (*green* and *gray*; 1UCP (32)) and polymeric (*blue* and *white*; 3J63 (33)) forms. This region participates in all three asymmetric interactions within the DD polymer. (*B*) The TIR domain of MAL likewise undergoes a conformational change upon polymerization. The BB loop that is disordered in the monomer (*green* and *gray*; 2NDH (34)) becomes α-helical in the polymer (*blue* and *white*; 5UZB (35)). (*C*) The RHIM motifs of RIPK1 and RIPK3 are largely disordered in their monomer forms (*green*) but fold into highly ordered β-sheets upon polymerizing into functional amyloid fibers (5V7Z (12)).

This capability, we believe, explains why ordered polymerization features so prominently in innate immune signaling.

Although the structures of DD, TIR, and RHIM filaments grossly differ, those differences are less important for function than are the differences between each protein's soluble and filamentous form. If our view is correct, then the specifics of polymer structure should be irrelevant to the activities of signalosomes once they form. That prediction is borne out in the mechanisms by which effector proteins activate. The proximal molecular function of signalosomes is to activate a specific effector protein—often a caspase—that then executes downstream cellular changes. Caspases activate at signalosomes, not because of any particular configuration adopted there, but because the signalosome sequesters them to high enough local concentration that autocatalytic activation ensues simply because of their increased proximity (38). In fact, caspase activation is commonly controlled experimentally by replacing the caspase DD with an unrelated domain that can be induced to dimerize (39). These experiments demonstrate that neither the structure nor open-ended stoichiometry of DD assemblies is required for signalosome activity. Although oligomerization is the most straightforward way to bring caspases within proximity of one another, the particular form those oligomers take is more strongly shaped by the cell's imperative that they not form before stimulation because doing so, even a little bit, could be lethal. In other words, the DD, TIR, and RHIM assemblies are so highly ordered in the active state because that degree of order is so inaccessible from the soluble state that it will almost never happen spontaneously. Crystallization, but not LLPS or one-dimensional polymerization, provides the requisite kinetic barrier to meet that need.

The utility of nucleation barriers to signalosomes—a mousetrap analogy

Cells must detect nefarious agents such as viruses and destroy them before the agent can seize control of the cell to copy itself and thereby threaten the entire organism. This sometimes requires the cell to destroy itself, a difficult decision that is nevertheless made immediately and with limited information. Innate immune signaling, therefore, evolves toward maximal speed, sensitivity, and output. We discuss here how innate immunity signalosomes exploit nucleation barriers to meet these requirements.

Signalosomes generally contain three protein components: sensors, adaptors, and effectors. Of these, adaptors most prominently feature DD, TIR, and/or RHIM modules. We have summarized innate immune signaling network architecture around the known protein adaptors in Table 1. As for a spring-loaded mousetrap, each component has a hierarchical function in the overall mechanics. Adaptor proteins are the tensed spring; sensor proteins are the trigger; effector proteins are the hammer (Fig. 3).

Adaptor protein supersaturation sets the trap

The rate at which protein-protein interactions occur increases with the proteins' concentrations. The higher the concentration of the innate immune signaling machinery, the faster the cell can respond and more likely it will be to defeat the pathogen. How can cells maximize the concentration of signaling proteins without their activating spontaneously? Nucleation barriers provide one solution. Nucleation barriers are a physical property of proteins that emerges from their structures. As for protein structure, they are encoded by the polypeptide's sequence and its interaction with the cellular environment. They allow the protein to accumulate beyond the concentration required for assembly—that is, to become supersaturated—while still remaining soluble. The more supersaturated a particular innate immunity signaling protein, the faster will be its assembly and activation once a pathogen triggers its nucleation. We previously showed that self-assembly of the

TABLE 1 Exemplary innate immunity signalosomes, their ligands, and domains of interest

Ligand	Sensor	Adaptor	Effector	Pathway	References
dsDNA	AIM2	ASC	CASP1	pyroptosis	(40,41)
	PYD				
PtdIns4P	NLRP3				(42)
	PYD				
NAIP|flagellin[a]	NLRC4				(43)
	CARD	*PYD|CARD*	*CARD*		
Z-DNA, Z-RNA	ZBP1	RIPK1	RIPK3	necroptosis	(44)
	RHIM	*RHIM|DD*	*RHIM*		
FAS-L trimer	FAS	FADD	CASP8	apoptosis	(45)
	DD	*DED|DD*	*DED*		
TNF trimer	TNFR1	TRADD	RIPK1	NF-κB activation	(46)
	DD	*DD*	*RHIM|DD*		
dsRNA	RIG-I	MAVS	TRAFs		(47)
	CARD	*CARD*			
Peptidoglycan	NOD1	RIPK2	IKKs		(48,49)
	CARD	*CARD*			
LPS micelle|LBP|CD14	MD-2|TLR4	MAL|MyD88	IRAK4,2		(50)
	TIR	*DD|TIR*	*DD*		
β-glucan	Dectin-1|CARD9	BCL10	MALT1		(51)
	CARD	*CARD*	*DD*		

[a]All of the ligands listed act in a multivalent fashion with the exception of flagellin; the monomeric subunits of which act in complex with the indicated host factor.

adaptor protein ASC, an event that signals pyroptosis, is indeed controlled by a nucleation barrier large enough for the protein to remain monomeric inside cells despite accumulating to deeply supersaturating concentrations (31,52). Upon nucleation, the transition from fully dispersed monomers to a single punctate inflammasome—corresponding to a roughly 1000-fold increase in caspase-1 proximity—takes less than a minute (5,31). In the course of subsequent pyroptotic cell death, the ASC polymers are released into the extracellular space and are then engulfed by macrophages, wherein ASC continues to polymerize and signal in a prion-like fashion (53). This latter property confirms that ASC is physiologically supersaturated within macrophages in vivo, at least under proinflammatory conditions, because polymerization could not have occurred if the concentration of ASC in macrophages was below the saturating concentration. Based on their comparable domain architecture, functions, and in vitro polymerization kinetics, it is likely that at least some of the other adaptors will share the ASC's property of nucleation-limited signaling (24). The elucidation of prion-like behavior among other adaptors will be very impactful in our understanding of these crucial signaling pathways. Despite the fundamental implications of protein supersaturation, the tools to detect and quantify it in vivo remain limited. Notable advances in this area include optogenetic nuclei (54,55) and distributed amphifluoric Förster resonance energy transfer (31,56), low- and high-throughput approaches, respectively, that can detect nucleation barriers to protein self-assembly in living cells.

As illustrated in Fig. 2, the nucleation barriers for ASC and other adaptors likely involve unfavorable conformational changes in the polymerizing module itself. But interdomain interactions can also limit nucleation. Recent structural and biochemical evidence suggests a tertiary conformational change may underlie the kinetic barrier for MyD88, an adaptor protein of Toll-like receptor (TLR) signaling (57–59). MyD88 consists of a DD and TIR joined by a flexible linker. Before TLR stimulation, MyD88 appears to exist as a preassembled nascent polymer wherein the DDs at one end are fully configured to recruit additional subunits but—as proposed by Moncrieffe et al.—are occluded by TIR domain(s) reaching back (via the flexible linker) from the other end of the short polymer. We note that in this model, inhibition will only occur when the ends of the nascent polymer (a hexamer) are closely apposed; hence, the nucleation barrier will be breached upon elongation of the polymer beyond that critical length. This energetically unfavorable event is presumed to be facilitated by displacement of the occluding TIR domains through homotypic interactions with the TIR domains of the activated receptor.

Once nucleated, the self-templating polymerization of adaptor proteins will continue until the available soluble subunits deplete to their saturating concentration. This process is fundamentally irreversible except in cases in which a biological mechanism exists to do so. This is because the energy that had been stored by supersaturation, and subsequently dissipated through polymerization, cannot be regained until the templating polymer is either dissolved or expelled to allow the protein to again accumulate in soluble form. The process of crystalline deposition thereby acts as a feed-forward mechanism that renders the network either on or off (25). As a consequence of the switch-like activation, the response output of innate immune signaling at

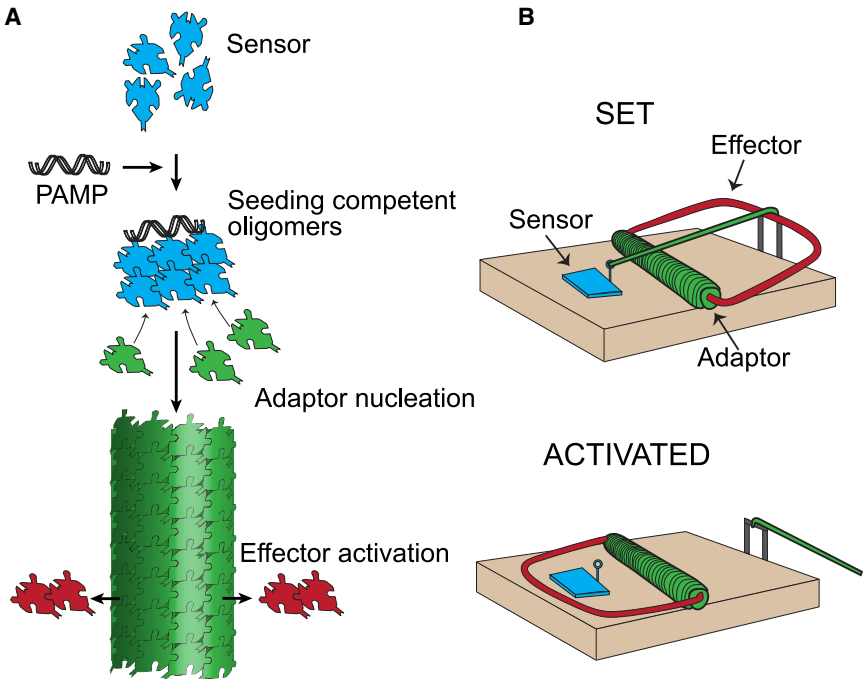

FIGURE 3 A mousetrap analogy for signalosome activation. (*A*) Signalosomes have three major protein components. Pathogen- or damage-associated molecular patterns allow for the oligomerization of sensor proteins into templates for adaptor protein polymerization. These in turn nucleate the rapid polymerization of adaptor proteins that had preaccumulated to deeply supersaturating concentrations. Finally, the adaptor protein polymers concentrate any effector proteins that bind them to high local concentrations, resulting in their autocatalytic *trans*-activation. (*B*) A mousetrap comprises three mechanical elements with analogous function. Upon sensing a mouse's touch, the trigger platform (the "sensor") releases the energy stored in the taught spring (the "adaptor"), which in turn propels the hammer bar (the "effector") onto the mouse. As for signalosome activation, the mousetrap operates with extraordinary sensitivity, speed, and decisiveness.

the cellular level does not depend on the degree of stimulation. Although this is obviously true when the outcome is cell death, it also appears to be true for nonlethal proinflammatory signaling. When cells engage either tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS), they respond by translocating NF-κB to the nucleus to promote the transcription of proinflammatory cytokines. Both pathways involve the formation of a signalosome (Table 1). In a population of cells, NF-κB activation is heterogeneous and digital at the single-cell level, and the probability of any single-cell responding increases with the intensity and duration of stimulation (7,60). We suspect that nucleation barriers encoded in the adaptor proteins underlie the digital activation of NF-κB by the TNF-α and LPS pathways. Thus, stimulating cells containing supersaturated adaptor proteins would produce a sensitive but semistochastic switch-like response in agreement with the observed kinetics and heterogeneity of innate immune signaling (Fig. 4).

Sensor protein oligomerization springs the trap

Innate immune signaling pathways have evolved to produce substantial cellular outputs—such as cell death or inflammation—in response to minimal stimuli. For example, fewer than 20 (and perhaps even one) molecules of the double-stranded RNA (dsRNA) genome of Sendai virus suffice to activate the RIG-I-MAVS signalosome, leading to cytokine secretion (61,62). This level of sensitivity requires tremendous and coordinated energy expenditure to amplify the signal. In the case of signalosomes, that exothermic event takes the form of supersaturated adaptor proteins depositing onto the crystalline lattice at signalosomes. The nucleation barrier to signalosome assembly allows the proteins to exist at supersaturating concentrations in anticipation of pathogen exposure. In effect, by synthesizing excess adaptor proteins, the cell has already paid the thermodynamic cost of a global cellular response. Sensor proteins are the trigger device for that response.

The critical principle of sensor function is the conditional oligomerization of their DD, TIR, or RHIM upon the protein's binding pathogen- or danger-associated molecular patterns (PAMPs or DAMPs). The newly formed oligomers serve as a nucleating template for the crystalline lattice of the corresponding adaptor protein. Sensor protein DD and TIR often have very low affinity for themselves such that oligomerization only appreciably occurs in complex with multivalent ligands. The multivalent nature of most innate immune stimuli (Table 1) reflects the importance of this principle.

Sensors are commonly maintained in an inactive form by autoinhibitory contacts between their DD or TIR and an adjacent module of the protein. For example, binding of cytosolic DNA or dsRNA by the helicase domain of AIM2, MDA5, or RIG-I induces a conformational change that exposes and triggers oligomerization of the respective protein's DD for subsequent interaction with the adaptor proteins ASC or MAVS.

Some autoinhibiting modules themselves oligomerize, which can bypass the need for multivalency in the ligand. Some two dozen human sensor proteins contain nucleotide-binding domains that couple ligand binding with an ATP-hydrolysis-powered conformational change leading

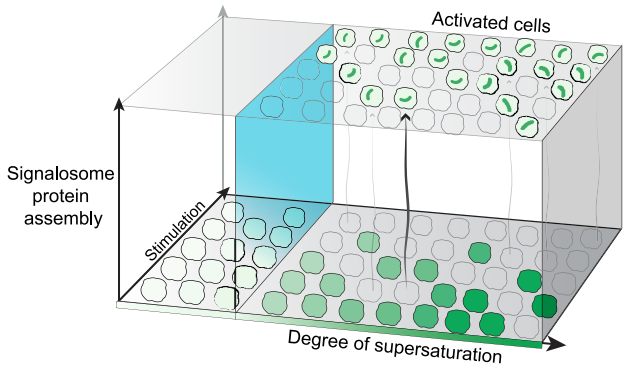

FIGURE 4 Adaptor protein supersaturation allows for switch-like probabilistic immune responses to ligand stimulation. Cells are arrayed with different concentrations of adaptor protein (*x* axis). Green intensity indicates concentration. Cells with assembled protein are depicted by a green punctum and reduced green background intensity. A blue divider at the saturating concentration for signalosome protein assembly partitions cells into those that cannot signal (to the *left*) and those that can (to the *right*). Signaling occurs upon protein assembly and is represented by cells rising off of the bottom surface. It is switch-like and unidirectional because of the nucleation barrier to assembly. Stimulation, as by pathogen-mediated oligomerization of sensor proteins, greatly increases the probability of nucleation.

to homo-oligomerization (63). For example, the sensor for intrinsic apoptosis, Apaf-1, oligomerizes upon binding monomeric cytochrome c released from mitochondria, which then triggers the autocatalytic activation of procaspase-9.

Multiple features of sensor proteins facilitate their activation in the presence of ligand. For example, many sensors are localized to membranes, which constrains their diffusion to two dimensions and hence reduces the entropic penalty for oligomerization, a concept expounded by recent studies of cell-adhesion-receptor- and T-cell-receptor-mediated signaling (64). Other sensors are constitutively oligomerized in an autoinhibited form. This preoligomerization effectively primes their DD or TIR for immediate assembly upon release of autoinhibition by ligand binding or associated post-translational modifications. Note that within such autoinhibited oligomers, direct interactions between DD or TIR of different subunits are typically prevented until activation. We recently reported that CARD9, an indirect sensor of fungal pathogens that triggers proinflammatory NF-κB activation, is blocked from templating its adaptor, BCL10, by interactions between its DD and an adjacent coiled coil module (65).

A supersaturated adaptor protein and uninhibited sensor protein is in effect a trap waiting to be sprung. This can pose a grave risk for the cell. To minimize that risk, cells avoid arming certain signalosomes until danger seems imminent. For example, TLR agonists "prime" the NLRP3 inflammasome for subsequent activation by inducing the transcriptional and post-transcriptional upregulation of NLRP3. Simultaneously, post-translational modifications including phosphorylation and ubiquitination release the autoinhibiting interactions of NLRP3 (66–68).

Effector proteins transduce cellular consequences

The hierarchical assembly of the signalosome concludes with the activation of specific effector proteins that transduce and execute the signal for each signalosome to other components in the cell. The DD and RHIM of certain effector proteins have also been found to polymerize in vitro (24,69), suggesting that as for adaptors, they may be able to amplify their own activation once nucleated on the surface of their cognate adaptor polymer. In the case of procaspase-1 (CASP1), the activation mechanism involves a direct nucleating interaction between its CARD and the exposed CARDs from ASC polymers. For both case of procaspase-8 (CASP8) and CASP1, activation leads to cell death, consistent with the switch-like output of signalosome activation (9,70). Additionally, some effectors have been implicated in enzymatic feed-forward loops after their initial activation at signalosomes (71,72).

This means that as for adaptors, these effectors must never appreciably oligomerize in the absence of stimulation because doing so would spring the trap prematurely. The solution is to have their affinities for self-interaction be so weak that they only appreciably occur when brought together through a shared affinity for the adaptor polymer, whose existence is in turn contingent on pathway activation (36,73).

How are self-templating signalosomes turned off for those that do not end with cell death? Complete elimination of the self-templating polymers is required for the cell to "reset" by again accumulating the proteins to supersaturating levels. Wholesale destruction of the polymers by autophagy appears to be a general way to accomplish this, as has been shown for the Bcl10, MAVS, MyD88, and ASC signalosomes (74,75). Any transient change in cytosolic pH or other control knobs of signalosome protein affinities could also allow for total dissolution of the polymers. In contrast, piecemeal proteolysis or enzymatic modifications to individual proteins is anticipated to be much less effective in terminating this form of signaling.

Domino effects

Cells are replete with macromolecular surfaces that lower the entropic cost of nucleation relative to the homogeneous theoretical extreme. By extension, for any supersaturated protein, a hierarchy of other factors that accelerate its nucleation, each to a different extent, must exist in the cell. The efficiency of any such factor will be determined by the combination of its affinity for the protein, its valency, and whether it stabilizes favorable conformations for assembly. The latter is especially important for amyloid nucleation, as it involves a massive loss of conformational entropy (31).

Consequently, amyloids of other proteins can dramatically accelerate nucleation by providing a template for that conformational change. This phenomenon is called "cross-seeding." Disease-associated amyloids notoriously cross-seed each other, resulting in cascades of protein self-assembly that are believed to accelerate and enhance the pathogenesis of Alzheimer's and other degenerative diseases (76,77). Does cross-seeding also occur between different signalosomes or even from signalosomes to proteopathic assemblies?

Microglial neuroinflammation is a major etiological factor in Alzheimer's disease, and recent evidence suggests that ASC inflammasomes released by microglia directly cross-seed amyloid-β. Likewise, necroptotic signaling by RHIM-mediated RIPK1 and RIPK3 amyloids is an important mediator of axon degeneration (78), raising the possibility that cross-seeding between this signalosome and pathogenic amyloids may play an as-yet-unexplored role in neurodegenerative diseases.

"Cross talk" also occurs between different signalosomes (79). Many of these instances involve enzymatic transactivation between effector caspases (80), but protein-protein interactions consistent with cross-seeding have also been implicated. For example, the apoptosis effector CASP8 can directly nucleate ASC assembly (81,82), and possibly vice versa (83,84), resulting in a redirection of signaling to pyroptosis and PANoptosis, respectively. MAVS, an adaptor that normally elicits NF-κB activation, can also engage the sensor NLRP3 to nucleate ASC (85) or an as-yet-unknown factor to trigger apoptosis (86). The RIPK1 adaptor, which contains both a DD and an RHIM module, appears to be a particularly promiscuous link between signalosomes. Its activation culminates in a context-dependent manner with either NF-κB activation or the apoptosis, pyroptosis, and/or necroptosis modes of programmed cell death (87–90). To what extent the many modes of cross talk between signalosomes result from cross-seeding interactions remains to be investigated. In any case, the paradigm emerging from these reports is that cells are predisposed to die regardless of the particular pathway engaged. We submit that supersaturation of programmed cell death signalosome proteins provides a thermodynamic basis for that predisposition.

Even beyond cross-seeding, a system of supersaturated adaptor proteins poised for activation is fraught with danger. Mutational disruption of the autoinhibitory regions of sensor proteins can cause them to activate even without stimulation, leading to myriad autoinflammatory diseases (91,92). These cases strongly support our thesis statement that the corresponding DD, TIR, and/or RHIM-containing adaptors are indeed supersaturated in the resting state.

Because signalosome formation is governed by a kinetic barrier, which is probabilistic in nature, signalosomes almost certainly activate with some frequency spontaneously, and the cumulative risk of such aberrant signaling must increase with time. Inflammation is one of the driving hallmarks of aging, and senescent cells exhibit a proinflammatory secretory state that has been directly linked to age-associated pathologies (93). It would be extremely valuable to our understanding of aging to measure how signalosome supersaturation and nucleation barriers change in the course of cellular aging. For example, observing an accumulation of adaptor protein assemblies without a concomitant change in their stability would imply a kinetic basis for their accumulation and not just age-associated declines in protein quality control, and therefore, that supersaturation had contributed to aging at the cellular level. Such experiments would illuminate whether the inherent susceptibility of signalosome activation due to physiological supersaturation comprises an ultimate molecular driver of aging.

CONCLUSION

The initial discoveries of prion-like properties of innate immune signaling proteins opened a new view into spatiotemporal control of intracellular signaling wherein extremely sensitive, rapid, and decisive signaling emerges from the sudden appearance of near crystalline polymers. To date, at least one such polymer has been solved to atomic resolution for each of the signaling modules described here. Yet, we still lack an appreciation for how their extreme kinetics of polymerization emerge from their sequence-encoded monomer structures.

We argued in this perspective that monomer structure kinetically prevents the proteins from self-associating. Polymerization is a mere consequence of the functional imperative that the proteins never self-associate until the moment in time that doing so becomes essential. Any precocious sampling of oligomeric states by the soluble proteins would lead to the death of healthy cells, whereas any delay in assembly upon the appearance of a vanishingly small number of templates could compromise the viability of the whole organism. These dueling demands cannot be satisfied by soluble oligomerization, which lacks sufficient cooperativity, nor by phase separation, which lacks sufficient sensitivity. Instead, the innate immune system has met the challenge through nucleation-limited crystallization within deeply supersaturated resting states. In choosing this solution, however, the innate immune system may have predetermined the fates of cells, rendering programmed cell death and chronic inflammation inescapable with the passage of time.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and revising of the manuscript.

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