Initiation and perpetuation of NLRP3 inflammasome activation and assembly

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Summary

The NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome is a multiprotein complex that orchestrates innate immune responses to infection and cell stress through activation of Caspase-1 and maturation of inflammatory cytokines pro-interleukin-1β (pro-IL-1β) and pro-IL-18. Activation of the inflammasome during infection can be protective, but unregulated NLRP3 inflammasome activation in response to non-pathogenic endogenous or exogenous stimuli can lead to unintended pathology. NLRP3 associates with mitochondria and mitochondrial molecules, and activation of the NLRP3 inflammasome in response to diverse stimuli requires cation flux, mitochondrial Ca^{2+} uptake, and mitochondrial reactive oxygen species accumulation. It remains uncertain whether NLRP3 surveys mitochondrial integrity and senses mitochondrial damage, or whether mitochondria simply serve as a physical platform for inflammasome assembly. The structure of the active, caspase-1-processing NLRP3 inflammasome also requires further clarification, but recent studies describing the prion-like properties of ASC have advanced the understanding of how inflammasome assembly and caspase-1 activation occur while raising new questions regarding the propagation and resolution of NLRP3 inflammasome activation. Here we review the mechanisms and pathways regulating NLRP3 inflammasome activation, discuss emerging concepts in NLRP3 complex organization, and expose the knowledge gaps hindering a comprehensive understanding of NLRP3 activation.

Keywords

NLRP3; inflammasome; caspase-1; mitochondria

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Introduction

NLRP3 (NOD-like receptor family, pyrin domain containing 3) belongs to the nucleotide-binding domain and leucine rich repeat (LRR) containing (NLR) protein family (1). NLR family members that have a pyrin domain (PYD) are further subcategorized to the NLRP subfamily, whereas NLRC proteins possess a caspase activation and recruitment domain (CARD) (1). NLRP3 is one of four confirmed inflammasome-forming pattern recognition receptors (PRR) for activation of caspase-1 along with NLRP1, NLRC4, and AIM2 (which has a pyrin domain and a HIN2000 domain for sensing dsDNA). Other innate sensing molecules have also been proposed to form inflammasomes, including NLRC5, NLRP6, NLRP7, NLRP12, RIG-I, Pyrin, and IFI16, but additional biochemical evidence is needed for some of these (2–12).

Understanding the steps involved in activation of the NLRP3 inflammasome is critical given its role in numerous aspects of human health and disease. This was first recognized when gain-of-function mutations within NLRP3 were found to be responsible for Muckle-Wells syndrome, familial cold autoinflammatory syndrome, and neonatal onset multisystem inflammatory disease, collectively called the cryopyrin-associated periodic syndromes (CAPS) (13–15). Additionally, the NLRP3 inflammasome recognizes and responds to infection with numerous bacteria, viruses, fungi, and parasites (16). NLRP3 has also been implicated in the pathogenesis of many diseases including arthropathy, ischemic heart and renal disease, vascular disease, diabetes and obesity, Alzheimer's dementia, and others, which are well-reviewed elsewhere (17). Despite receiving much attention and investigation, the mechanism of NLRP3 inflammasome activation is still controversial, the direct ligands remain enigmatic, and the relationship between complex assembly and activation is unclear. The purpose of this review is to clarify the current models for NLRP3 inflammasome assembly and activation and to highlight persisting knowledge gaps that require further research. A more complete understanding of NLRP3 inflammasome complex activation and assembly will aid development of directed therapies for treatment of NLRP3-driven diseases.

Activating Signals for the NLRP3 Inflammasome

Licensing activation by priming

Activation of the NLRP3 inflammasome by macrophages is a two-step process, which requires 'priming' prior to or coincident with a secondary NLRP3-specific activating signal (18). Priming can be accomplished by activation of receptors that signal via MyD88 (myeloid differentiation factor 88)/TRIF (TIR-domain-containing adapter-inducing interferon-β) or other NFκB (nuclear factor-κB)-activating pathways, including Toll-like receptors (TLRs), IL-1R, tumor necrosis factor receptor (TNFR), and NOD2 (19, 20) (Fig. 1). The importance of priming for NLRP3 activation is well-reviewed elsewhere (21), but here we provide a brief background and update. The main role of priming was initially thought to be the NFκB-mediated upregulation of NLRP3 and inflammasome-dependent cytokine pro-IL-1β (19). However, it is now apparent that priming occurs rapidly and new protein translation is dispensable for caspase-1 activation (22, 23). Priming requires IRAK1 activation and proteasome-dependent ERK activation (with downstream MEK1/2

activation), and it involves post-translational modifications including: (i) BRCC3-mediated NLRP3 deubiquitination, (ii) LUBAC-mediated ASC ubiquitination, and (iii) SYK- and JNK-dependent ASC phosphorylation (18, 22, 24–30). However, additional protein modifications (e.g. acetylation, succinylation, myristoylation, and SUMOylation) and other feasible protein targets (e.g. caspase-1) have not been investigated (31, 32). NLR phosphorylation has been studied in the context of NLRC4 activation but is yet to be examined for NLRP3 (33). Additionally, recent work highlights the importance of metabolic changes during activation of macrophages and dendritic cells (DCs) (34, 35). Therefore, altered glycolysis and oxidative phosphorylation could influence reactive oxygen species (ROS) production, new fatty acid synthesis, and membrane dynamics might be involved, and changes in citrate and succinate utilization could influence post-translational modifications (acetylation and succinylation respectively), which could be relevant for NLRP3 priming (36). For instance, tubulin acetylation plays an important role in NLRP3 activation (37).

Potassium flux

Extracellular ATP and pore-forming toxins (e.g. nigericin and maitotoxin) activate the NLRP3 inflammasome (38, 39). Extracellular ATP induces IL-1β secretion by activating the purinergic $P2X_7$ receptor (formerly P2Z) and inducing K^+ efflux (40–42). Interestingly, priming alone can activate NLRP3 in certain types of cells (e.g. monocytes) due to autocrine and paracrine $P2X_7$ activation by secreted ATP (38, 43). Similarly, K^+ efflux is required for IL-1β secretion by pore-forming toxins (40, 44, 45). However, a point of confusion is the role of pannexin-1 pore-formation in ATP-induced NLRP3 inflammasome activation. Pannexin-1 associates with $P2X_7$, and NLRP3 activation by ATP, pore-forming toxins, and fungal components is attenuated by pannexin-1 knockdown or inhibition (46–48). Pannexin-1 was also implicated in lysosomal disruption induced by particulate activators of the NLRP3 inflammasome (49). However, studies using pannexin-1-deficient mice have not supported a role for pannexin-1 in NLRP3 inflammasome activation (50, 51). It is possible that the pharmacologic pannexin-1 inhibitors used may have off-target effects resulting in inhibition of NLRP3 inflammasome activation. What remains as the relevant mechanism for all known NLRP3 inflammasome agonists to date, including particulate agonists, is K^+ efflux independent of pannexin-1 (52). K^+ flux may have additional downstream consequences (e.g. Ca^{2+} flux, mitochondrial disruption, and cell volume regulation response), which are discussed below (53, 54).

Lysosomal disruption

Phagocytosis of certain exogenous and endogenous particulate matter, including monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD), amyloid-β, silica, asbestos, and alum, activates the NLRP3 inflammasome (49, 55–62) (Fig. 2). These agonists induce and require K^+ efflux, and $P2X_7$ is dispensable in this process (55–58). No precise mechanism linking particle phagocytosis to K^+ efflux has been established, but phagolysosomal disruption is critical to achieve NLRP3 inflammasome activation (49). Particulate phagocytosis causes lysosomal swelling, leakage of contents, and loss of phagosomal acidity (49). Furthermore, inducing lysosome rupture directly, either by hypotonic incubation or by treating with Leu-Leu-OMe, leads to NLRP3 inflammasome

activation (49). Inhibiting lysosomal acidification with the H$^+$ ATPase inhibitor bafilomycin A attenuates particle-induced NLRP3 inflammasome activation (49). The requirement for lysosomal acidification and disruption led to the hypothesis that introduction of active lysosomal enzymes to the cytosolic compartment activates NLRP3, and indeed the cathepsin B inhibitor CA-074-Me was found to partially inhibit NLRP3 activation (49). However, results using cathepsin B-deficient cells are mixed and have revealed discrepancies between pharmacologic inhibitor and genetic-based approaches (60, 63–68). It appears that CA-074-Me has off-target effects and inhibits other cathepsins, but cathepsin B ablation might be compensated by a redundant lysosomal cathepsin (69, 70). Additionally, it should be noted that lysosomes have high concentrations of calcium (Ca^{2+}), and destabilization could cause release of lysosomal Ca^{2+} which may trigger ER Ca^{2+} release and subsequent NLRP3 activation (71). In an effort to understand how lysosomal rupture leads to NLRP3 activation, a recent study investigated the roles of lysosomal Ca^{2+}, CaMKII, TAK1, and JNK activation using inhibitors and siRNA (72). A major obstacle is that JNK and CaMKII have broad roles in phagocyte function, including inflammasome priming (24, 73–75). Indeed, kinase inhibition does not only impair Leu-Leu-OMe- and MSU-induced activation, but also ATP- and poly(dA:dT)-mediated IL-1β secretion, suggesting a role for these kinases beyond lysosomal disruption-induced NLRP3 activation (72). It remains unresolved how lysosomal disruption (and cytoplasmic release of lysosomal proteases, lipases, Ca^{2+}, etc.) induces K$^+$ efflux and ultimately NLRP3 activation. A better understanding of this pathway is relevant to specifically protecting against adverse effects of exposure to environmental irritants (e.g. asbestos and silica) or endogenous molecules (e.g. urate crystals and amyloid) that activate the NLRP3 inflammasome.

Calcium signaling

There is much support for a role for intraceullular Ca^{2+}-signaling in NLRP3 inflammasome activation (Fig. 3): ATP-induced IL-1β release requires Ca^{2+} influx and ER-mediated Ca^{2+} release, ultraviolet-induced NLRP3 activation requires elevated intracellular Ca^{2+}, and Ca^{2+} influx is required for cholesterol-dependent cytolysins to activate NLRP3 (76–78). During the time that endoplasmic reticulum (ER) stress and mitochondrial disruption were emerging as important events for NLRP3 activation, it seemed appropriate to more closely study Ca^{2+} due to the well-defined role of ER-to-mitochondrial Ca^{2+} flux in cell death regulation (79). Osmotic stress and the cell volume response activate the NLRP3 inflammasome, and this appears to require transient receptor potential (TRP) channels, cytosolic Ca^{2+} signaling, and TAK1 kinase activation (54, 80). Two studies reported NLRP3 inflammasome activation in response to high extracellular Ca^{2+} mediated by two Gα$_q$ protein-coupled receptors (GPCR), the calcium-sensing receptor (CASR), and the GPCR family C group 6 member A (GPRC6A) (81, 82). NLRP3 was also activated by CASR agonists Gd^{3+}, Al^{3+}, and R-568 and phospholipase C (PLC) activator *m*-3M3FBS (81, 82). Interestingly, activation of other GPCRs by platelet-activating factor and thrombin also induces IL-1β maturation, which could be a promising area for future study (82). Importantly, Rossol *et al.* (82) used *in vitro* and *in vivo* strategies to demonstrate that extracellular Ca^{2+} released from necrotic cells may serve as a danger signal for NLRP3 activation. The finding that high extracellular Ca^{2+} alone is an NLRP3 activating stimulus has not been recapitulated, and it is possible that concentrated extracellular Ca^{2+} precipitates as calcium-phosphate crystals and activates

NLRP3 by lysosomal disruption (52). However, activation of NLRP3 by Ca^{2+} crystals would not be expected to require CASR and GPRC6A, hence the effects of CASR- and GPRC6A-deficiency on phagocyte function warrant closer examination.

NLRP3 inflammasome activation by soluble and particulate agonists is attenuated by inhibiting PLC, inositol trisphosphate receptor (IP_3R), and store-operated Ca^+ entry (SOCE), and by chelating cytosolic Ca^{2+} (65, 71, 81, 82). Thapsigargin (an inhibitor of SERCA-mediated ER Ca^{2+} reuptake) and Ca^{2+}-free media both partially impair NLRP3 activation, suggesting redundancy between ER and extracellular Ca^{2+} pools (71, 76). Murakami *et al.* (71) generated a more complete model for NLRP3 inflammasome activation by linking K^+ efflux, Ca^{2+} influx, mitochondrial dysfunction, and the C/EPB homologous protein (CHOP)-mediated ER stress response. The mitochondrial calcium uniporter (MCU) is important for mitochondrial Ca^{2+} uptake, which buffers elevations in cytosolic Ca^{2+}, but excessive uptake leads to mitochondrial dysfunction (79, 83, 84). Triantafilou *et al.* (85) showed that MCU is required for complement membrane attack complex-induced NLRP3 inflammasome activation. Therefore, a comprehensive view of the mechanism of NLRP3 activation appears to involve K^+ efflux, extracellular Ca^{2+} influx and ER Ca^{2+} release, mitochondrial Ca^{2+} uptake and finally mitochondrial dysfunction.

Lee *et al.* (81) also reported negative regulation of NLRP3 by adenylyl cyclase (ADCY) and its product cAMP, which was relieved by CASR-mediated $G_i\alpha$ activation. NLRP3 inflammasome activation was increased with ADCY inhibitors and reduced by activators of ADCY and inhibitors of cAMP degradation (81). Rossol *et al.* (82) failed to corroborate the finding that cAMP suppresses NLRP3 activation or that extracellular Ca^{2+} reduces cAMP to relieve inhibition of NLRP3. Critically, Ca^{2+} signaling and Ca^{2+} flux alone is not sufficient for NLRP3 activation because only certain $G\alpha_q$-coupled receptors and receptor tyrosine kinases (RTK) activate the inflammasome and the Ca^{2+} ionophore ionomycin does not activate NLRP3 (71, 80). It is ultimately unclear why Ca^{2+} flux activates NLRP3 in certain settings but not in others, but it might support the hypothesis that a concomitant decrease in cAMP is required. Alternatively, NLRP3 activation may depend entirely on Ca^{2+} concentration and sublocalization.

There are many additional regulators of Ca^{2+}-dependent signaling pathways that have not been fully investigated. Transient receptor potential (TRP) channels are a large group of plasma membrane proteins that form transmembrane channels permeable to cations and can therefore mediate both Ca^{2+} influx in response to various extracellular stimuli and mechanical stress, depending on the specific receptor (86). TRPV2 activation by tetrahydrocannabinol (THC), sphingosine, or cell regulatory volume decrease leads to NLRP3 inflammasome activation, and TRPM2 activation by mitochondrial ROS is required for NLRP3 inflammasome activation by liposomes and particulate agonists (54, 66, 80). However, because TRPM2 inhibition and ablation does not completely inhibit NLRP3 activation, other Ca^{2+} channels may also be involved (66). SOCE (or calcium release-activated channels) respond to transient increases in cytosolic Ca^{2+} by permitting additional extracellular Ca^{2+} influx, and include channel-forming proteins ORAI1, ORAI2, and ORAI3 and sensor proteins STIM1 and STIM2 (87, 88). While 2-APB blocks SOCE and inhibits NLRP3 inflammasome activation, these proteins have not been examined genetically in the

context of NLRP3 activation (71). Whether voltage-gated Ca^{2+} channels could theoretically activate NLRP3 has also not been investigated. Additionally, the contribution of individual PLC enzymes to NLRP3 activation has not been assessed. Furthermore, it is uncertain why diacylglycerol-mediated protein kinase C activation downstream of PLC activation was not implicated in NLRP3 inflammasome activation, especially given the putative role of PKC in positive feedback regulation of IP_3R-mediated Ca^{2+} release (89). Finally, it is unclear why not all Ca^{2+}-signaling events result in NLRP3 inflammasome activation; factors that distinguish GPCRs and RTKs that induce NLRP3 activation from those that do not should be studied by correlating Ca^{2+} flux with different downstream events (e.g. mitochondrial disruption).

TXNIP and ER stress

Thioredoxin-interacting protein (TXNIP) interacts with reduced thioredoxin (TRX), but NLRP3 agonist-induced ROS generation oxidizes TRX and causes TXNIP to dissociate from TRX and to then associate with NLRP3 (90). Silencing or ablating TXNIP attenuates glucose-induced NLRP3 activation in pancreatic β cells (90). TXNIP also translocates to mitochondria along with NLRP3 during inflammasome activation (91). During ER stress, the ER stress sensors IRE1α and PERK upregulate TXNIP transcription and inhibit TXNIP mRNA degradation by reducing miR-17, which in turn regulates NLRP3 inflammasome activation (92, 93). Importantly, these studies confirm earlier findings that ER stress leads to NLRP3-dependent caspase-1 activation and IL-1β secretion but contradict the unfolded protein response (UPR)-independence posited by Menu *et al*. (92–94). Critically, Menu and colleagues (92–94) directly examined the effect of IRE1α and PERK knockdown and Atf6α deficiency on inflammasome activation, whereas the other studies either employed a pharmacological inhibitor of IRE1α to inhibit NLRP3 activation or did not directly test UPR-inhibition in the context of inflammasome activation. Kim *et al.* (95) confirmed that TXNIP interacts with NLRP3, demonstrated a partial requirement for TXNIP in ER stress-induced NLRP3 activation, and found that the IRE1α-mediated ER stress activates NFκB to prime inflammasome activation. UPR-induced priming may be dispensable for inflammasome activation in PMA-primed THP-1 cells but required in the absence of another priming stimulus (94, 95). The UPR also regulates ER-mitochondria Ca^{2+} flux via IP_3R (96, 97). Indeed, Murakami *et al.* (71) reported that UPR-associated transcription factor CHOP regulates ER Ca^{2+} release and NLRP3 activation. In summary, it remains unclear if the UPR regulates priming or ER-derived Ca^{2+} flux required to achieve effective NLRP3 inflammasome activation. Importantly, not all studies support a requirement for TXNIP in NLRP3 activation; for example, Masters and colleagues (98) found TXNIP is dispensable for inflammasome activation by islet amyloid polypeptide, MSU, and ATP. TXNIP knockdown also reduces transcription of pro-IL-1β and IL-6 (93); hence, it seems TXNIP may play a limited role in NLRP3 priming downstream of ER stress, but not in direct NLRP3 inflammasome activation. The potential effects of TXNIP modulation (and subsequent liberation of TRX) on priming, ion flux, ROS generation, and mitochondrial function warrant further evaluation. Additionally, the requirement for TXNIP in NLRP3 inflammasome activation may be restricted to tissue-specific agonists in a cell type-specific manner.

Mitochondrial damage in NLRP3 inflammasome activation

Mitochondrial ROS and NLRP3 inflammasome activation

Nearly all NLRP3 agonists induce and require reactive oxygen species (ROS) for activation (56, 57, 99, 100). The lysosomal NADPH oxidase appeared to be a good candidate for the source of ROS and silencing NADPH oxidase reduced NLRP3 activation by asbestos and MSU (57). However, NLRP3 inflammasome activation is unaffected in murine and human cells deficient for NADPH oxidase subunits (49, 101). Furthermore, NADPH oxidase inhibitor VAS2870 fails to inhibit liposome-induced NLRP3 activation (66). Therefore, the phagocyte NADPH oxidase is dispensable for ROS-dependent activation of the NLRP3 inflammasome.

Through normal respiratory functions mitochondria produce mitochondrial ROS (mtROS), but under stress mtROS production can increase enormously (102, 103). Most traditional NLRP3 agonists increase mtROS production and specifically scavenging mitochondrial superoxide attenuates NLRP3 activation (37, 66,71,91,104–112) (Fig. 4). Furthermore, mtROS production requires the PLC/IP$_3$R/Ca^{2+} flux pathway implicated in NLRP3 inflammasome activation (71). Curiously, the antibiotics linezolid and gramicidin both activate the NLRP3 inflammasome without inducing or requiring mtROS, but both could directly induce mitochondrial dysfunction either by interfering with electron transport or by creating mitochondrial membrane pores, respectively (52, 106). Similarly, Ichinohe *et al.* (112) found that mtROS were dispensable for NLRP3 inflammasome activation by influenza, encephalomyocarditis virus, and measles virus, but mtROS were still required for ATP, MSU, and nigericin. In sum, it appears that many but not all NLRP3 agonists require mtROS for activation, leading us to hypothesize that agonists that can directly induce mitochondrial dysfunction may still activate NLRP3 in the absence of mtROS. Besides leading to greater mitochondrial dysfunction, mtROS could possibly modify endogenous molecules to generate a distinct danger-associated molecular pattern (DAMP) for NLRP3. Alternatively, mtROS could directly oxidize inflammasome proteins to regulate activation. In fact, the NLRP3 PYD possesses a theoretical disulfide bond, which could be a target of redox activity (113). Interestingly, nitric oxide seems to stabilize mitochondria against dysfunction and suppress NLRP3 activation by S-nitrosylation (107, 108, 114). A final consideration is that mtROS may act on organelles other than mitochondria, as demonstrated in a recent study showing mtROS induces lysosomal damage, which could theoretically feedback to enhance NLRP3 inflammasome activation (115).

Mitochondrial Ca^{2+} uptake and NLRP3 inflammasome activation

Mitochondria-associated membranes (MAM) are contact sites where ER IP$_3$R and mitochondrial voltage-dependent anion channels (VDAC) are physically coupled by the chaperone glucose regulatory protein 75 (GRP75, mtHSP70) to regulate ER-mitochondrial Ca^{2+} flux (116–118). While mitochondria have the capacity to buffer ER Ca^{2+} release, excessive mitochondrial Ca^{2+} uptake leads to mitochondrial disruption (79, 119). Several lines of evidence implicate the MAM in NLRP3 activation, such as the importance of PLC/IP$_3$R activity, the requirement for mitofusins which tether ER and mitochondria, and the involvement of VDAC1 and VDAC2 that regulate mitochondrial Ca^{2+} uptake across the

outer membrane (71, 81,82,91,94,112, 120) (Fig. 4). VDAC is also involved in mitochondrial outer membrane permeabilization (MOMP), which regulates the release of proapoptotic inner-membrane space proteins [e.g. cytochrome c (cyt c), apoptosis-inducing factor (AIF), and SMAC/DIABLO] and VDAC may regulate release of a mitochondrial NLRP3 ligand (121). Anti-apoptotic protein Bcl-2 interacts with VDAC and IP_3R to suppress Ca^{2+} flux, and negatively regulates NLRP3 inflammasome activation (91, 105, 122–124). However, a recent study failed to replicate the findings that Bcl-2 affects NLRP3 activation, and also found no role for the proapoptotic MOMP regulators Bak and Bax (125–127). Overall, it seems likely that mitochondrial Ca^{2+} uptake is indeed important for causing the mitochondrial dysfunction associated with NLRP3 inflammasome activation, but perhaps MOMP is dispensable.

Mitochondrial Ca^{2+} uptake across the inner membrane into the matrix was long known to be inhibited by Ruthenium Red derivatives (such as RU360) and was thought to occur via a mitochondrial Ca^{2+} uniporter (MCU) (83, 128). The identity of the MCU was recently solved and MCU regulatory proteins are being identified (83, 84, 129, 130). Trianfilou *et al.* (85) found MCU was necessary for NLRP3 activation by the complement membrane attack complex, but other NLRP3 agonists have not been examined for MCU-dependence and there may be additional mitochondrial Ca^{2+} uptake pathways to be examined (Fig. 4).

Mitochondrial dysfunction model

Mitochondrial Ca^{2+} overload and accumulation of mtROS leads to loss of inner mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial permeability transition (MPT) (79) (Fig. 4). Zhou *et al.* (91) first identified a role for damaged mitochondria in NLRP3 activation, demonstrating that respiratory chain inhibitors such as rotenone activate NLRP3, and this involved loss of $\Delta\Psi m$ and mtROS accumulation. Subsequently, Nakahira *et al.* (104) confirmed that mitochondrial damage regulates NLRP3 inflammasome activation, but NLRP3 was also required upstream of certain aspects of mitochondrial disruption, namely mitochondrial DNA (mtDNA) release, which further amplified inflammasome activation. Using indicators of $\Delta\Psi m$ (e.g. TMRM, JC-1, and mitotracker red) multiple studies have confirmed that NLRP3 agonists decrease $\Delta\Psi m$ and induce MPT and this depends on K^+ efflux and mitochondrial Ca^{2+} uptake (37, 71,85,91,104, 105, 110). In contrast, Ichinohe *et al.* (112) reported increased $\Delta\Psi m$ during viral infection and reported that mtROS were dispensable for NLRP3 activation by certain viruses. Changes in mitochondrial oxygen consumption rate (OCR) have also been associated with NLRP3 activation (52, 105). Cyclosporine A (CsA) strongly inhibits NLRP3 inflammasome activation, but this inhibition may be independent of its effects on cyclophilin D (CypD)-mediated MPT (105, 106, 127). CsA has a number of targets in addition to CypD that could be responsible for inhibition of NLRP3 inflammasome activation.

Further support for the mitochondrial dysfunction model of NLRP3 inflammasome activation is the finding that cyt c is concurrently released (85, 104). It remains to be determined whether MOMP and intermembrane space factor-release are required for NLRP3 inflammasome activation, or whether these events represent a downstream consequence of caspase-1 activation or are entirely unrelated (104, 110, 131). Since Bak and

Bax are not required for NLRP3 activation, it is possible that MOMP is irrelevant (121, 127). With the recognition that NLRP3 and capase-1 contribute to mitochondrial damage, a major obstacle to studying NLRP3 activation is distinguishing which aspects of mitochondrial dysfunction are upstream or downstream of activation (104, 110). To summarize, mtROS generation and subsequent mitochondrial dysfunction appear to be required for NLRP3 activation; however, recent genetic studies suggest downstream MPT and MOMP are dispensable. Studies better defining the ligand recognized by NLRP3 will help inform our understanding of the requirement for mitochondrial disruption. For instance, if NLRP3 recognizes mitochondria DNA (mtDNA), then substantial disruption of mitochondrial membranes might be required for mtDNA extravasation. Part of the challenge in studying the requirement of mitochondrial dysfunction in NLRP3 activation is that the physical composition and regulation of MPT and MOMP are not fully understood. Learning more about the regulation of MPT and MOMP will permit investigators to test whether newly identified molecules and pathways are relevant for NLRP3 inflammasome activation.

Regulation of NLRP3 inflammasome activation by mitophagy

Clearance of damaged mitochondria by mitophagy is important to maintain cell viability (132). Chemical or genetic inhibition of autophagy/mitophagy leads to spontaneous NLRP3 inflammasome activation and enhances activation by known NLRP3 agonists in an ROS-dependent manner (91, 104). Microscopically, autophagy-deficient cells accumulate abnormal mitochondria with increased mtROS and reduced $\Delta\Psi m$ (104). RIPK2 ablation, palmitate treatment, and mevalonate kinase-deficiency also reduce autophagy/mitophagy, increase mtROS, and enhance NLRP3 activation (111, 133, 134). Active Caspase-1 degrades Parkin and inhibits mitophagy, suggesting positive feedback where NLRP3 activation reduces clearance of damaged mitochondria thereby increasing inflammasome activation (110). The finding that mitophagy inhibition generally enhances NLRP3 activation through accumulation of damaged mitochondria further supports a model where NLRP3 responds to mitochondrial dysfunction. However, autophagy is also involved in elimination of active inflammasomes, which might explain enhanced activation with autophagy inhibition (135, 136). Furthermore, some of the effects of autophagy inhibition might be to stimulate AIM2 inflammasome activation since AIM2 can sense mitochondrial DNA (104, 110, 137).

NLRP3 localization

During NLRP3 inflammasome activation or when overexpressed, NLRP3 has been shown to associate with mitochondria and MAM in the perinuclear region of cells (91). This association may depend on mitochondrial antiviral signaling protein (MAVS), cardiolipin, and/or c-FLIP (106, 138–140) (Fig. 4). NLRP3 also appears to associate directly with mitochondrial mitofusins during viral infection (112). To our knowledge, there is no known mitochondrial localization signal for NLRP3; however, PSORT bioinformatics analysis by Subramanian *et al.* (138) predicted that human NLRP3 should localize to mitochondria and experiments showed that N-terminal amino acids 2–7 were essential for the mitochondrial association of NLRP3. However, murine Nlrp3 differs in this minimal N-terminal sequence and PSORT predicts it will localize cytoplasmically, even though it too associates with mitochondria during activation (139).

Misawa *et al.* (37) made the slightly different observation that during NLRP3 activation microtubules and dynein drive the perinuclear migration of mitochondria, which results in co-localization of ER-associated NLRP3 with mitochondrially associated ASC. Microtubule-dependent organelle transport was not driven by K^+ efflux or mtROS, but instead involved increased α-tubulin acetylation by MEC-17 and decreased SIRT2-mediated deacetylation due to mitochondrial dysfunction and NAD^+ depletion (37). These findings conflict with previous studies suggesting that prior to activation ASC is predominantly found in the nucleus and that during NLRP3 inflammasome activation ASC associates with mitochondria in a NLRP3-dependent manner (91, 141). Further biochemical validation will be required to reconcile these studies.

In contrast to these studies supporting mitochondrial or MAM localization of NLRP3 inflammasomes, microscopy experiments by Wang *et al*. (142) using a variety of organelle markers could not determine a specific localization of ASC specks, and because NLRP3 co-localizes with the specks the authors concluded that NLRP3 is cytosolic. However, association of NLRP3 with specific organelles was not independently examined, and since it is unclear whether the ASC speck represents an active NLRP3 inflammasome (discussed below), it is difficult to know whether or how these findings relate to NLRP3 localization. In summary, organelle rearrangement and a number of molecules regulate mitochondrial localization of NLRP3 to promote inflammasome activation. How ASC and casapse-1 localize is less clear, but there are two obvious models that need to be tested: (i) that ASC associates with mitochondria through interactions with NLRP3, or (ii) that ASC associates with mitochondria directly in unstimulated cells. Whether MAVS, mitofusins, c-FLIP and cardiolipin are the only mediators of the mitochondrial localization of NLRP3 remains to be determined. Further, it is possible that these different NLRP3-interacting molecules have temporally distinct, sequential roles in NLRP3 recruitment that need to be elucidated. If NLRP3 associates with ER, rather than mitochondria, is this through ER-associated mitofusin or another ER/MAM protein? If ASC associates with mitochondria upstream of activation, what governs its localization? Finally, why are organelle associations important for NLRP3 but appear to be dispensable for NLRC4 and AIM2?

Mitochondrial antiviral signaling protein

The role of the MAVS in NLRP3 inflammasome activation has been a topic of several studies and remains controversial. MAVS is an adaptor for the cytosolic dsRNA sensing proteins retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), which lead to IRF3 and NFκB activation (143). Several studies have reported that NLRP3 and MAVS physically interact and that MAVS regulates mitochondrial association of NLRP3 (138, 139, 144). Unfortunately, the role of MAVS in NLRP3 activation is unclear. Subramanian *et al.* (138) reported that MAVS was necessary for NLRP3 activation by soluble stimuli (e.g. ATP and nigericin) and cytosolic poly(I:C), but not particulate stimuli (e.g. alum, CPPD and MSU). While subsequent studies on MAVS have continued to suggest there may be a role for MAVS in NLRP3 inflammasome activation induced by RNA virus infection, they have failed to support a role for MAVS in non-viral NLRP3 stimuli such as ATP and nigericin (127, 131, 139, 144). MAVS is required for the NLRP3-dependent response to infection by the ssRNA Sendai virus, but this is due to

effects on priming since Sendai virus alone does not activate NLRP3 unless a secondary signal (e.g. nigericin) is provided (139). In contrast, Rift Valley fever virus alone induces minimal pro-IL-1β synthesis, but priming cells with lipopolysaccharide prior to virus infection induces robust MAVS-dependent IL-1β secretion indicating that MAVS mediates activation and not priming (144). Altogether, it appears that MAVS is uniquely required for NLRP3 activation by viruses, but whether MAVS mediates inflammasome priming or has direct effects on NLRP3 activation is uncertain. Another virus-specific mitochondrial pathway for NLRP3 activation involves activation of receptor-interacting serine/threonine protein kinases 1 and 3 (RIP1 and RIP3) and downstream mitochondrial fission mediated by DRP1 (145).

NLRP3 recognition of a mitochondrial DAMP

Mitochondrial DNA (mtDNA) resembles prokaryotic DNA and extracellular mtDNA is recognized as a danger signal by TLR9 (146). Treatment of macrophages with ethidium bromide leads to mitochondrial DNA (mtDNA)-deficient mitochondria (ρ^0), and ρ^0 macrophages have reduced inflammasome activation (104, 105, 112). Additionally, NLRP3 agonists cause mtROS-dependent, MPT-dependent release of mtDNA into the cytosol, which is diminished in ρ^0 cells and augmented in autophagy-deficient cells (104, 105). Furthermore, Shimada *et al.* (105) found that mtDNA interacts with NLRP3 and AIM2 and that oxidized mtDNA (ox-mtDNA) interacts specifically with NLPR3 in cells treated with ATP and nigericin (Fig. 4). Importantly, the interaction of NLRP3 with ox-mtDNA occurs in caspase-1-deficient cells, suggesting that mtDNA release is upstream of inflammasome activation (105). These findings have led to the hypothesis that mtDNA may be a ligand for NLRP3. However, ρ^0 mitochondria have reduced expression of normal proteins (e.g. cytochrome *c* oxidase) and abnormal respiratory function (104). Furthermore, mtDNA could be sensed as a priming agent or by a different inflammasome. Indeed, IL-1β secretion in response to mtDNA is almost entirely abrogated in $Aim2^{-/-}$ macrophages (104, 105). Whether the remaining activation is due to NLRP3, another inflammasome, or increased TLR9-mediated priming is yet to be determined. Generation of $Nlrp3^{-/-}Aim2^{-/-}$ double-deficient macrophages and inflammasome reconstitution experiments will be critical for establishing a role for mtDNA in NLRP3 activation. Interestingly, IL-1β secretion is greater in response to ox-mtDNA than mtDNA and AIM2 does not appear to mediate ox-mtDNA-induced inflammasome activation. However, NLRP3 deficiency also does not abrogate IL-1β release induced by ox-mtDNA (105). It is possible that ox-mtDNA binds to and is necessary for NLRP3 activation but alone is insufficient to drive NLRP3 activation. Overall, a model for mtDNA or ox-mtDNA as the sole activating signal for NLRP3 remains unclear.

The structurally unique diphospholipid cardiolipin is found in prokaryotes and in eukaryotic inner mitochondrial membranes and at contact sites between mitochondrial membranes (147–149). Cardiolipin is important for normal mitochondrial function, but it also regulates extrinsic apoptosis by recruiting Bid and caspase-8 to mitochondria, which is thought to require externalization of cardiolipin to the outer mitochondrial membrane (150–157). Additionally, cardiolipin anchors cyt *c* to the inner mitochondrial membrane and during intrinsic apoptosis cyt *c* oxidizes cardiolipin and is released into the cytosol (158, 159). Thus, cardiolipin functions as a foreign prokaryotic molecule, uniquely confined to

mitochondria, and is modified in structure and location during cell stress. This makes it an interesting candidate as a danger signal. Cardiolipin interacts with the N-terminal portion of the NLRP3 LRR *in vitro* (106) (Fig. 4). In addition, cardiolipin synthase (CLS) knockdown and cardiolipin depletion inhibit NLRP3 inflammasome activation (106). If cardiolipin is a ligand for NLRP3 activation, it is possible that its recognition requires accessory proteins as is the case for TLR4/MD2/Lipid A (160); therefore, determining other molecules that associate with the NLRP3 inflammasome complex is critical. Whether cardiolipin is redistributed in the mitochondria during NLRP3 inflammasome activation or whether cardiolipin is structurally modified (e.g. oxidation or lipolysis) has not yet been assessed. The development of a reconstitution systems or cell-free system for the NLRP3 inflammasome and structural studies are required to definitively assess ligand binding relationships between potential DAMPs and NLRP3.

Alternative hypotheses

While a role for mitochondria in NLRP3 activation is generally well supported there is also some evidence to the contrary. The production of mtROS seems to be essential for activation and the evidence that NLRP3 localizes to mitochondria and/or MAM during activation has been replicated in a number of independent studies. However, the greater challenge is elucidating the extent of mitochondrial disruption required for NLRP3 activation and determining whether it results in exposure or release of a mitochondrial ligand. Certainly, it appears that aspects of mitochondrial damage, especially loss of $\Delta\Psi m$ and release of mitochondrial contents, are actually downstream of NLRP3 activation and caspase-1 maturation (104, 110). Others suggest that mitochondrial disruption is entirely unrelated to NLRP3 inflammasome activation (52). Na^+/K^+-ATPase inhibition with ouabain prevents increased OCR without affecting IL-1β maturation, leading Munoz-Planillo *et al*. (52) to conclude that OCR is increased due to ATP expenditure as cells reestablish K^+ gradients. Activity of Na^+/K^+-ATPase might partially explain the reduction in ATP that other studies have observed during NLRP3 inflammasome activation, although Munoz-Planillo and colleagues did not directly examine the effects of ouabain on intracellular ATP (37, 52, 106). If cytosolic cation changes such as K^+ efflux or Ca^{2+} influx are sufficient for NLRP3 inflammasome activation, and mitochondria are dispensable as has been proposed, it will be critical to determine how cation concentration alone can induce structural changes and regulate protein-protein interactions to promote NLRP3 inflammasome activation. Certainly K^+ concentration could affect NLRP3 conformation, but evidence suggests K^+ efflux is not sufficient for activation since inhibiting Cl^- channels inhibits IL-1β processing without affecting K^+ efflux (54). Future studies should also consider whether mtROS could directly modify NLRP3 by cysteine or methionine oxidation to cause inflammasome activation.

Inflammasome complex assembly

Activation of pro-caspase-9 by the apoptosome has been modeled as occurring by: (i) induced proximity, (ii) proximity-driven dimerization, or (iii) induced conformational change, and this has largely informed models of pro-Caspase-1 activation by the inflammasome (161). The relationship between inflammasome complex assembly and activation is complex since certain associations between inflammasome components may

occur prior to activation. Recent progress has been made in understanding the biochemical properties and functions of active ASC and this has led to new potential models for NLRP3 inflammasome assembly. Finally, technical advances in fluorescent and electron microscopy have the capability to more clearly elucidate the organization of inflammasome complexes.

Relationship between inflammasome activation and assembly

Competing models to describe the relationship between inflammasome activation and assembly can be summarized as follows: (i) activation of monomeric NLRP3 leads to complex assembly; and (ii) preassembled NLRP3 complexes undergo activation-induced conformational change leading to recruitment of additional proteins. The first conventional model is supported by the finding that NLRP3 and ASC co-precipitate following NLRP3 activation and that NLRP3 is evenly distributed throughout the cytosol prior to activation and colocalizes with ASC in specks during activation (54, 142, 162, 163). The alternative view is that portions of the complex are assembled at rest and activation results in conformational change that recruits additional members of the complex and activates caspase-1. This model is supported by studies showing protein associations and high molecular weight (HMW) complex assembly at rest. For instance, size-exclusion chromatography and co-precipitation experiments have shown wildtype NLRP3 elutes at 1,000 kDa and self-associates, while NLRP3 with a mutated NBD does not readily self-oligomerize (164). Similarly, bioluminescence resonance energy transfer (BRET) experiments by Compan *et al.* (54) showed that NLRP3 molecules are in proximity prior to the activation stimulus. NLRP3 and ASC even seem to interact at baseline because ASC co-expression increases NLRP3 BRET without activating the complex, but this interaction is probably weak because ASC did not co-precipitate with NLRP3 in the absence of an activating stimulus (54). In another study, NLRP3 inflammasome reconstitution in unstimulated HEK 293 cells resulted in spontaneous NLRP3-ASC-caspase-1 associations, although IL-1β processing was also induced suggesting that such complexes were active (15). Additionally, the ASC PYD has substantial ability to self-associate and could theoretically participate in pre-activation self-assembly (165). Whether pre-formed NLRP3 complexes exclude ASC or whether NLRP3 and ASC weakly interact prior to activation, there is sufficient evidence to conclude that oligomerization is not synonymous with activation. However, a weakness of some studies on NLRP3 complex formation is the reliance on overexpression systems, which could cause artifactual, activation-independent interactions, and the lack of examination of endogenous agonist-induced NLRP3-ASC interactions.

Inflammasome complex size

The initial description of the inflammasome by Martinon *et al.* (166) showed that complexes of NLRP1, ASC, and Caspase-1 elute in HMW fractions (about 700 kDa) from a Superdex S-200 column. Interestingly, NLRP1 eluted at this same MW even under resting conditions, suggesting baseline self-association of NLRP1 may occur in the absence of activation. In the study that first characterized the NLRP3 inflammasome, Agostini *et al.* (15) demonstrated an association of NLRP3 with the adapter ASC and caspase-1 by co-immunoprecipitation. Subsequently, Martinon *et al.* (167) reported that NLRP3 activation with MDP caused IL-1β to elute in in HMW fractions greater than 670 kDa. This study has been cited as evidence

that NLRP3, like NLRP1, forms a large ~700 kDa complex. However, whether NLRP3, ASC, and caspase-1 eluted in the HMW fraction with IL-1β was not directly assessed. Since these early studies, co-immunoprecipitation has confirmed NLRP3, ASC, and caspase-1 oligomerization, but the molecular weight of these NLRP3 complexes remains to be directly assessed. Oligomerization of NLRP3 and ASC in HMW complexes has also been supported by immunofluorescent confocal microscopy studies showing co-localization of NLRP3 and ASC in specks (142, 162, 163). However, biochemical determination of the predominant molecular weight of endogenous NLRP3 inflammasome complexes has yet to be determined and will be relevant to assess what constitutes an active inflammasome. Such studies would help determine which parts of the complex associate at baseline, with priming, and with activation, and would establish a clear model for step-wise activation and assembly.

Spoked wheel model

Early studies examining NLRC4 and NLRP1 noted structural and functional similarities to the APAF-1 apoptosome (166, 168). Faustin *et al.* (169) provided the first direct evidence of inflammasome structure by examining the *in vitro* assembly of NLRP1 and caspase-1 into five- or seven-member ring-like structures using electron microscopy. The similarity in structure and function of NLRs to APAF-1 led to the prevailing hypothesis that inflammasomes resemble a bicycle wheel with 7 spokes, with the sensory LRR at the rim and caspase-1 at the hub (169–171) (Fig. 5A). These electron microscopy experiments have yet to be performed with NLRP3, but other evidence has emerged supporting a spoke-like model for NLRP3.

BRET analysis of N- or C-terminus YFP-tagged and C-terminus luciferase-tagged NLRP3 molecules demonstrated that NLRP3 self-associates at baseline (54). More specifically, because BRET signal was greater in cells transfected with N-terminus YFP-NLRP3 compared to C-terminus NLRP3-YFP, the authors concluded that the N-terminus and C-terminus of neighboring molecules are in close proximity (54). This finding supports a heptameric spoke model in which the NLRP3 LRR is bent back towards the central hub prior to ligand binding. NLRP3 activation by hypotonic incubation appears to induce a conformational change distancing the C-terminus from the N-terminus and resulting in cessation of BRET (54). The NLRP3 LRR was hypothesized to switch from a bent, or 'closed', to an 'open' configuration upon activation. Overall, this work further supported a spoke-like organization for the NLRP3 inflammasome.

Branching tree model

ASC aggregates have the prion-like ability to convert soluble ASC PYD to polymeric ASC PYD, which can be propagated between cells *in vitro* by introducing PYD aggregates into the cytosol of a recipient cell (172). This prion-like property of ASC was reinforced in another study showing cytosolic mCherry-tagged ASC is recruited to phagocytosed mCerulean-tagged ASC specks (162). ASC mutations abrogating prion-like activity prevent activation of reconstituted inflammasomes, which suggests that prion-like activity is important for activation (172). However, it is possible that mutations disrupting ASC aggregation also disrupt other ASC functions that regulate its participation in inflammasomes. The finding that ASC PYD can be replaced with fungal prions while

retaining the ability to activate pro-caspase-1 further supports the model that prion-forming activity is relevant for inflammasome activation and also supports the induced proximity model of caspase-1 activation (161, 172). Prion formation by ASC PYD appears to be unique since the PYD of NLRP3, IFI16, and IFI204 are unable to induce yeast prion conversion (172). Keeping in mind the unique prion-forming properties of ASC, three-dimensional electron microscopy studies of ASC oligomers by Lu *et al.* (165) found ASC PYD forms fiber-like structures. The authors determined inflammasome-activating PRRs, either NLRP3 or AIM2, localize entirely to one pole of the ASC PYD filament (165). Fluorescence polarization assays demonstrated rapid polymerization of ASC PYD, which could be enhanced by addition of NLRP3 or AIM2 (165). Cryoelectron microscopy in conjunction with the previously published NMR structure of ASC PYD was used to model the ASC filament structure (165, 173). The results ultimately support an extension of the spoke-like model where heptamers of NLRP3 or AIM2 PYD form the root on which a trunk of ASC PYD polymerizes with caspase-1 branching laterally from the ASC CARD (165) (Fig. 5B). Importantly, full-length ASC polymerizes less extensively than ASC PYD, and inclusion of caspase-1 CARD also diminishes fiber length, demonstrating that overexpression of truncated ASC may result in non-physiologic behaviors (165). Moreover, while endogenous filamentous structures were found in MSU-activated THP-1 cells, data regarding fiber number and size in untreated cells are required to assess the relevance of ASC polymerization and whether it is activation dependent (165).

If a single PRR heptamer can induce fibrous extension of ASC that propagates indefinitely, a number of questions are raised regarding inflammasome complex assembly. Why is only a single ASC speck observed per cell when a single active NLRP3 heptamer nucleates extensive ASC polymerization, and might this suggest that few active NLRP3 complexes are required to achieve maximal caspase-1 activation? Additionally, NLRP3 has been thought to oligomerize via the NBD/NACHT domain, but the prion-like properties of PYD suggests a need to reassess the properties of the NLRP3 PYD in promoting self-association (164). Interestingly, when Bae and Park (113) published the crystal structure of the NLRP3 PYD, they predicted it has the capacity to dimerize. If PYD-PYD interactions cause irreversible prion-like polymerization, and if NLRP3 can oligomerize via the PYD, what prevents NLRP3-NLRP3 interactions from occurring via the PYD and excluding ASC from the complex? Furthermore, how are detergent-resistant, protease-resistant ASC prions eventually eliminated to prevent uncontrolled inflammatory responses?

Layered ASC speck model

In a portion of inflammasome-activated cells, focal aggregates of ASC are visible by fluorescent microscopy, but it remains uncertain whether these focal aggregates of ASC represent true inflammasome complexes. ASC oligomerizes spontaneously in low K^+ conditions in the absence of NLRP3, and Pyrin (a negative regulator of inflammasome activation) can induce ASC speck formation (174–176). These findings suggest that ASC speck formation is not always due to or correlated with inflammasome activity. This also raises the possibility that focal ASC sequestration in specks could represent a regulatory mechanism. However, ample evidence, including the prion-like biochemistry of ASC discussed above, points to ASC specks representing active inflammasomes. ASC specks

form at the site of initiation of inflammasome formation as NLRP3-dependent ASC aggregates localize to mitochondria (138). Recent studies suggest that NLRP3 and Caspase-1 colocalize with ASC specks (162, 163, 177). Bayesian localization superresolution immunofluorescent microscopy of endogenous ASC speck formation revealed that Caspase-1 is confined to the center of a ring of ASC (163). Meanwhile, NLRP3 (and NLRC4) were within the speck, but surrounded the region containing Caspase-1 (163) (Figure 5C). Importantly, the finding that an 'inflammasome' can be a heterooligomeric complex of multiple NLRs is an interesting advance (163). The model that the ASC speck represents an active NLRP3 inflammasome is also supported by evidence that they possess pro-Caspase-1 and IL-1β activating activity (162, 177). Introducing purified *in vitro*-formed ASC aggregates into ASC-deficient cytosols results in pro-caspase-1 and pro-IL-1β activation and processing (162). Although compelling, the relationship between inflammasome complex activation and ASC specks raises a number of questions. Does inflammasome activation always result in speck formation? Does ASC speck formation always correlate with caspase-1 activation and are caspase-1 and IL-1β activated within the speck? Finally, how is complex size correlated with activity and what is the minimal size of an active NLRP3 inflammasome?

Sandwich model

Misawa *et al.* (37) observed that in resting cells, ASC localizes with the mitochondria and NLRP3 with the ER. NLRP3 inflammasome activation was driven by microtubule-mediated approximation of the ER and mitochondria near the nucleus (37) (Fig. 5D). Proximity-ligation assays confirmed a microtubule-dependent association between ER NLRP3 and mitochondrial ASC in the perinuclear region (37). This model uniquely emphasizes the importance of distinct organelles and cytoskeletal elements as platforms for complex assembly. Whether pro-caspase-1 participates in this membrane-associated assembly or whether the associated NLRP3 and ASC are released into the cytosol to interact with pro-caspase-1 was not assessed. Given the fiber-like structure of ASC aggregates, it is also important to consider whether ASC might polymerize on cytoskeletal scaffolding (162, 165, 178). Further studies will be required to examine the relationship between mitochondria, other organelles, and cytoskeletal elements with the active NLRP3 inflammasome complex.

Post-inflammasome activation

Assembly of the NLRP3 inflammasome is well known to result in activation of caspase-1, and recent evidence suggests that caspase-8 can be activated by the complex as well (179). Caspase-1 activation leads to cytokine processing and secretion and in some circumstances results in pyroptotic cell death, which remains poorly understood (180, 181). Until recently, the events following activation were largely uninvestigated aside from studies showing that inflammasome complexes are cleared by autophagy (135, 136, 182). Active caspase-1 is known to be secreted with IL-1β and IL-18, but recent findings suggest that NLRP3 and ASC are also extruded from cells following activation (162, 177). The dynamics of release differ among components with monomeric ASC and caspase-1 resembling IL-1β release, and NLRP3 and ASC speck release correlating with LDH release and requiring caspase-1-dependent cell death (162, 177). Phagocytosis of extracellular ASC specks induces

lysosomal disruption and results in an inflammatory response, which is partially NLRP3-dependent due to lysosomal rupture but also NLRP3-independent due to polymeric ASC prions seeding the soluble cytosolic ASC (162, 177). The *in vivo* relevance of this inflammasome-induced inflammasome activation mechanism remains unclear. ASC specks appear to persist *in vivo* and resist extracellular proteases when directly injected, during *Pseudomonas aeruginosa* infection, in chronic lung inflammation, and in serum of patients with active CAPS (162, 177). However, the duration that they persist has not been fully determined. It is also possible that pro-caspase-1, pro-IL-1β, and pro-IL-18 released from necrotic cells can be activated extracellularly by these particles (162). A central challenge is to understand how inflammation resolves if extracellular ASC specks persist and self-perpetuate as detergent- and protease-resistant prions.

Conclusion

The NLRP3 inflammasome has garnered much attention given its involvement in the pathogenesis of numerous disease processes. Recent studies have implicated cation flux, ER stress, and mitochondrial dysfunction in the activation of the NLRP3 inflammasome. In addition, the assembly and structure of the inflammasome complex has also been examined. Despite these advances, numerous questions regarding the specific steps required for NLRP3 inflammasome activation remain. For example, the precise role for the mitochondria in NLRP3 inflammasome is unclear. A number of studies suggest that the mitochondrion acts as a platform for the recruitment and assembly of NLRP3 inflammasome components. In addition, it is postulated that NLRP3 senses mitochondrial damage and can potentially be activated through interactions with a mitochondrial DAMP, such as cardiolipin of mtDNA. Defining the ligand that is required for NLRP3 activation will be critical to determining if the mitochondrion serves as a simple platform for NLRP3 inflammasome complex assembly or if NLRP3's role is the surveillance of mitochondrial integrity. The physical structure and molecular composition of the functional NLRP3 inflammasome also remains unclear. It will also be important to determine whether the recently described prion-like properties of ASC and ASC specks are directly relevant to the formation and function of the NLRP3 inflammasome, or if they are a consequence of inflammasome activation or a separate independent process. Addressing these fundamental questions will be instrumental for understanding how NLRP3 coordinates caspase-1 activation and inflammation in response to cellular stress or damage, and may reveal novel pathways and targets for regulating NLRP3 activity in disease.

Acknowledgments

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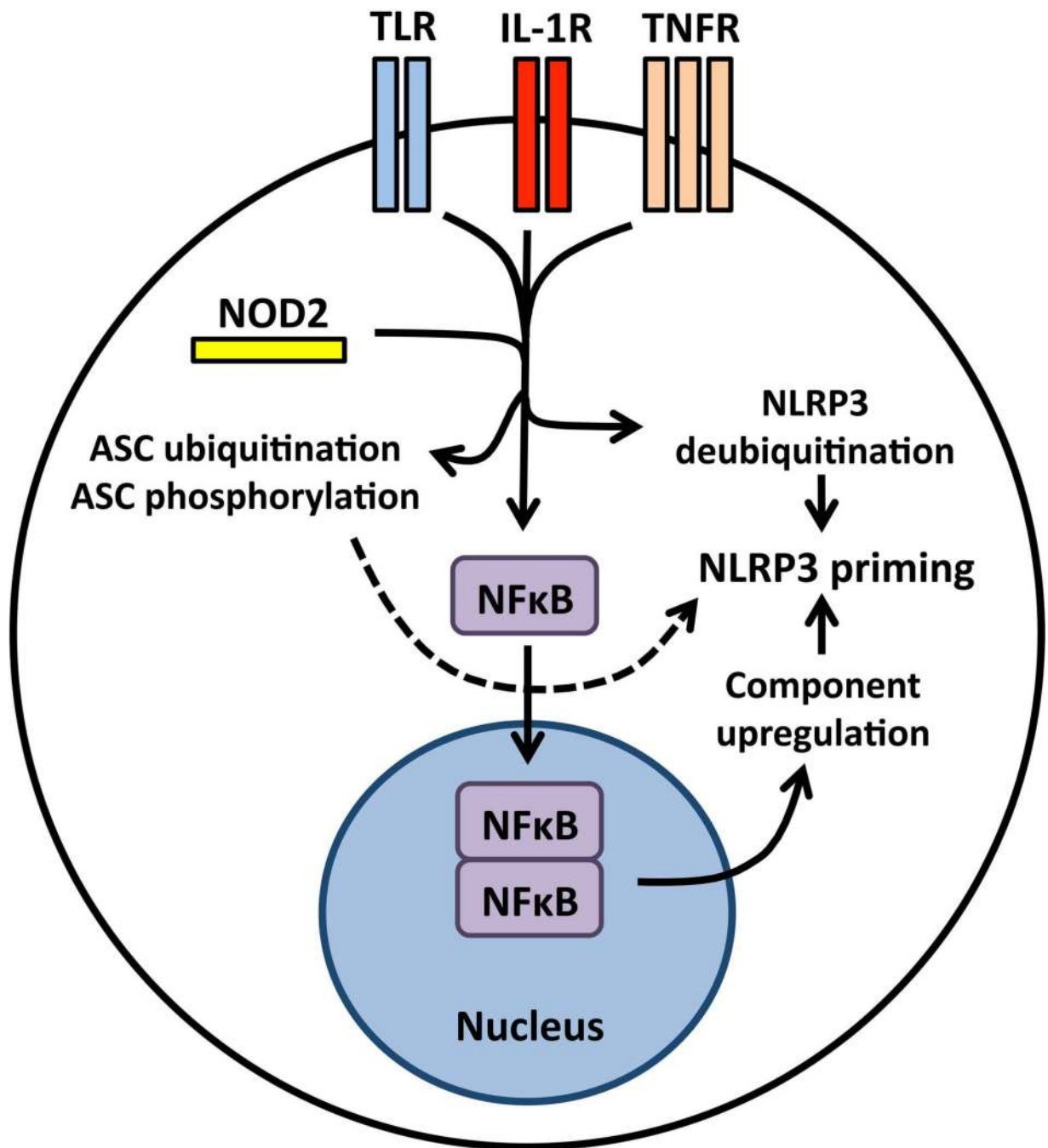
Fig. 1. Priming the NLRP3 inflammasome for activation
NLRP3 inflammasome priming is accomplished by NFκB-activating receptors including
Toll-like receptors, interleukin-1 receptor, tumor necrosis factor receptor, and the cytosolic
PRR NOD2. Nuclear translocation of NFκB leads to increased synthesis of NLRP3 and
inflammasome-dependent cytokine pro-IL-1β. Priming also induces post-translational
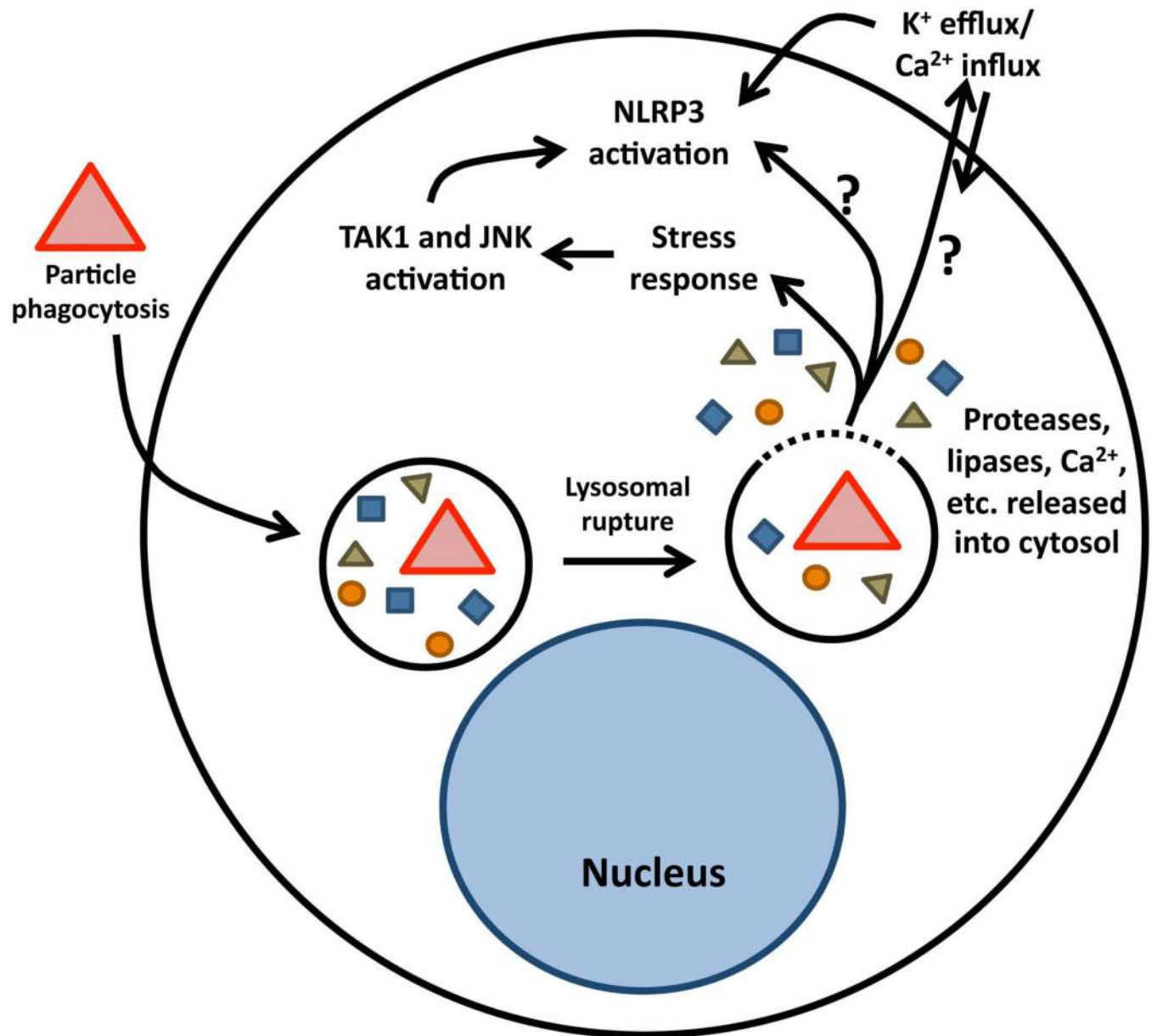modifications to inflammasome components including NLRP3 deubiqutination and ASC
ubiquitination and phosphorylation.

Fig. 2. Activation of the NLRP3 inflammasome by particulate agonists
Extracellular particles and crystals are initially phagocytosed. Destabilization of the
lysosome leads to release of lysosomal enzymes and calcium. It is unclear how release of
these lysosomal molecules leads to NLRP3 activation, but it appears that lysosomal
disruption induces cation flux through an unknown mechanism. It is also possible that
phagolysosomal destabilization leads to activation of cell stress responsive kinases, which
could affect upstream NLRP3 priming or inflammasome activation directly, but more work
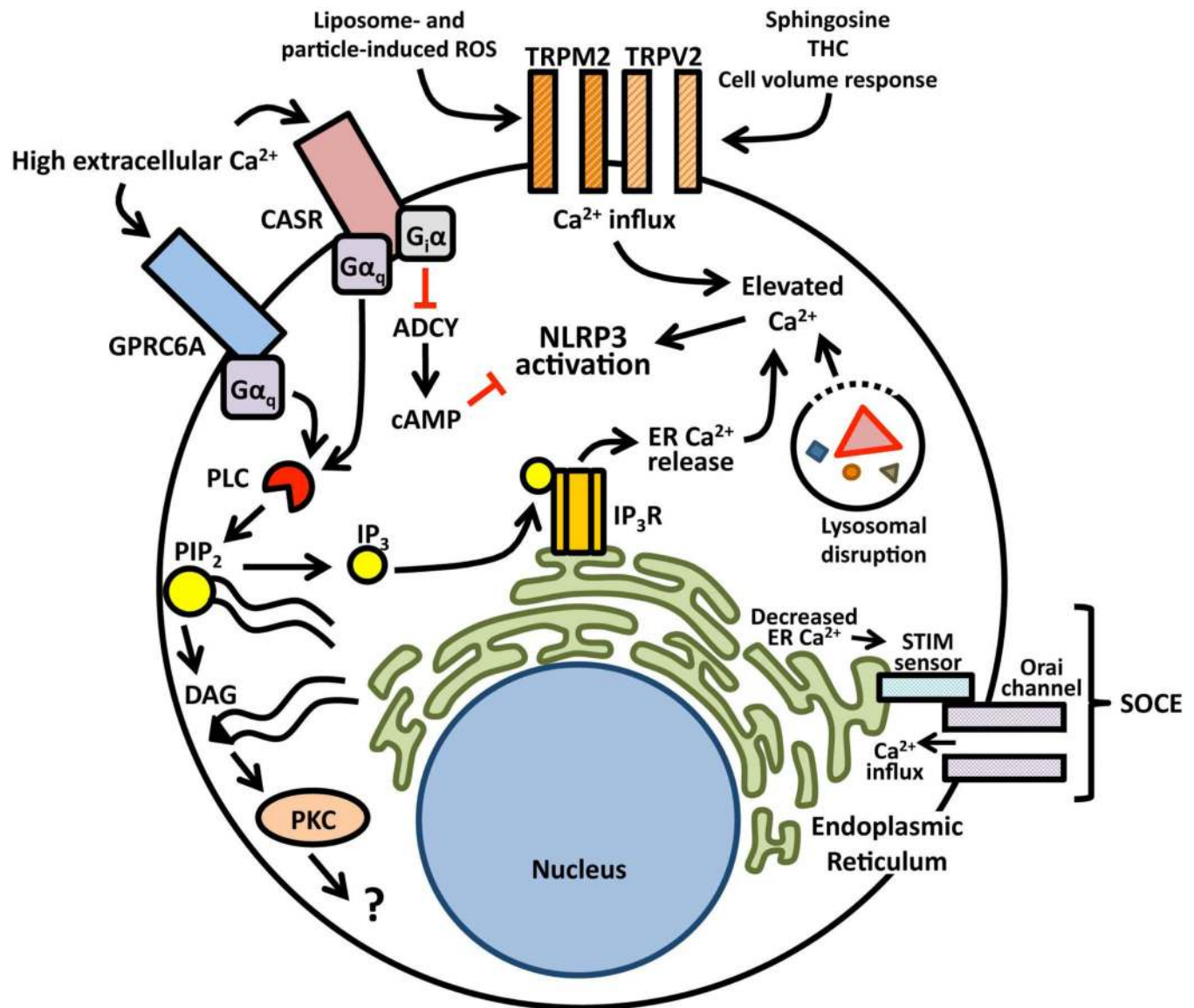is necessary to clarify these mechanisms.

Fig. 3. Regulation of NLRP3 activation by Ca^{2+} signaling
High extracellular Ca^{2+} from necrotic cells activates CASR and GPRC6A. Activation of these and other GPCRs leads to activation of PLC, which cleaves PIP_2 into DAG and IP_3. IP_3 activates IP_3R on ER membranes to trigger Ca^{2+} release. DAG activates PKC, but the role of this pathway in inflammasome activation is unclear. CASR also inhibits adenylate cyclase (ADCY), which may relieve the inhibition of NLRP3 by cAMP. Activation of NLRP3 requires SOCE, wherein STIM proteins sense ER Ca^{2+} depletion and trigger Ca^{2+} influx *via* Orai channels. NLRP3 inflammasome activation is also driven by Ca^{2+} uptake through TRPV2 and TRPM2 channels. Destabilized lysosomes could provide an additional source of Ca^{2+} for NLRP3 activation.
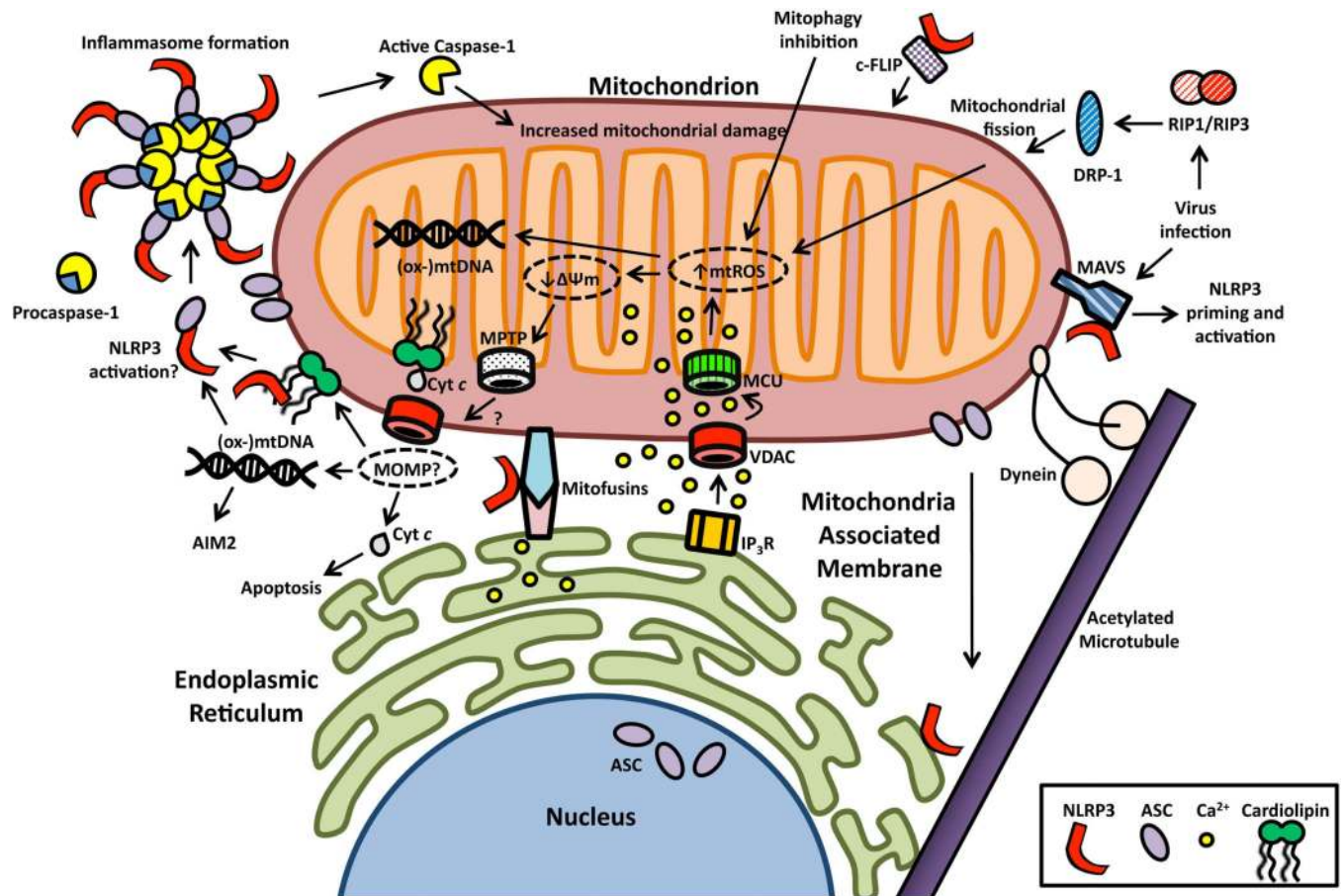
Fig. 4. NLRP3 inflammasome activation is regulated by the mitochondrion
NLRP3 associates with mitochondria by interacting with MAVS, mitofusins, c-FLIP and
cardiolipin. ER and mitochondrial membranes are tethered at the MAM via interactions
between VDAC and IP$_3$R as well as mitofusins. The IP$_3$R releases ER Ca^{2+} stores while
VDAC and MCU mediate mitochondrial Ca^{2+} uptake. Mitochondrial Ca^{2+} overload results
in increased mtROS, which is required for NLRP3 activation. The role of mtROS in NLRP3
activation could be to increase mitochondrial damage or to create a DAMP by oxidizing
mitochondrial molecules such as mtDNA. High mitochondrial Ca^{2+} and mtROS leads to
loss of $\Delta\Psi$m and MPTP, but it is unclear whether altered $\Delta\Psi$m and MPTP are required for
NLRP3 activation. MOMP permits release of cytochrome c from the inner-membrane space
into the cytosol to interact with Apaf-1 for initiation of apoptosis, but the role of MOMP in
NLRP3 activation is also uncertain. It is possible that MPTP or MOMP could regulate the
release of a mitochondrial DAMP for NLRP3 such as (oxidized) mtDNA or cardiolipin.
While the activating ligand of NLRP3 remains unknown, NLRP3 activation ultimately leads
to formation of an inflammasome containing ASC and procaspase-1 and results in caspase-1
activation. Activation of caspase-1 in turn leads to increased mitochondrial damage.
Inhibition of mitophagy prevents clearance of damaged mitochondria and increases mtROS
and NLRP3 activation. Additionally, the NLRP3 response to viruses may involve priming
and activation mediated by MAVS and RIP1/3-mediated activation of DRP-1 with

subsequent mitochondrial fission and mtROS production. Finally, dynein-mediated mitochondrial transport along microtubules may regulate NLRP3 and ASC interactions.
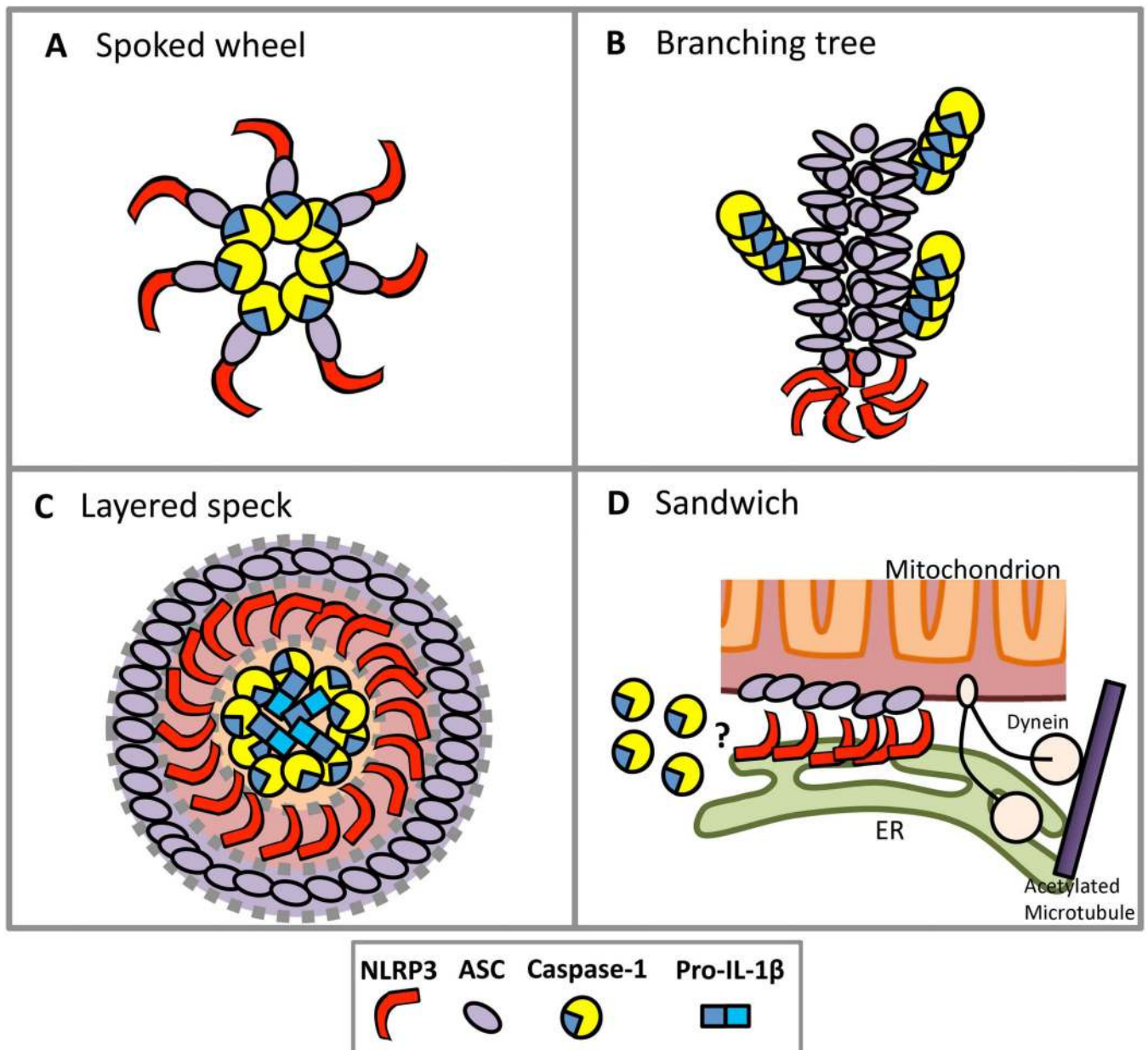
Fig. 5. Models of NLRP3 inflammasome complex assembly

(A) Traditionally, the inflammasome has been modeled after the apoptosome and thought to consist of peripheral NLRP3 spokes interacting indirectly with caspase-1 at the central hub through the adapter ASC. (B) The NLRP3 inflammasome resembles a tree where a heptameric NLRP3 root nucleates a fibrous ASC trunk, and caspase-1 recruited to the complex polymerizes laterally as branches. (C) The ASC speck is an inflammasome in which molecules of NLRP3 are contained within an ASC aggregate and caspase-1 and IL-1β are confined to the core of the complex. (D) The NLRP3 inflammasome is formed by the sandwich-like joining of ER membrane-associated NLRP3 with ASC on the outer

mitochondrial membrane, which is regulated by dynein-mediated transport along microtubules.