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REVIEW

Recent insights into the regulatory networks of NLRP3 inflammasome activation

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ABSTRACT

The NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome is a fascinating cellular machinery endowed with the capacity for rapid proteolytic processing of the pro-inflammatory cytokine IL-1β and the cell death effector gasdermin D (GSDMD). Although its activity is essential to fight infection and support tissue homeostasis, the inflammasome complex, which consists of the danger sensor NLRP3, the adaptor apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD), caspase-1 and probably other regulatory proteins, also bears considerable potential for detrimental inflammation, as observed in human conditions such as gout, heart attack, stroke and Alzheimer's disease. Thus, multi-layered regulatory networks are required to ensure the fine balance between rapid responsiveness versus erroneous activation (sufficient and temporally restricted versus excessive and chronic activity) of the inflammasome. These involve multiple activation, secretion and cell death pathways, as well as modulation of the subcellular localization of NLRP3, and its structure and activity, owing to post-translational modification by other cellular proteins. Here, we discuss the exciting progress that has recently been made in deciphering the regulation of the NLRP3 inflammasome. Additionally, we highlight open questions and describe areas of research that warrant further exploration to obtain a more comprehensive molecular and cellular understanding of the NLRP3 inflammasome.

KEY WORDS: NLRP3, Inflammasome, Interleukin-1, Inflammation, Bruton's tyrosine kinase, BTK, Macrophage, Receptor signaling

Introduction

Inflammation is vital for resolving infections or sterile insults, but, when chronic or deregulated, it becomes a health problem of global importance. Among the major driving forces of inflammation are pro-inflammatory cytokines, released by and mediating crosstalk between immune and tissue cells (Broz et al., 2020). Besides tumor necrosis factor (TNF) and interleukin (IL)-6, IL-1β is the most critical pro-inflammatory cytokine (Dinarello, 2009). Its maturation from an inactive pro-protein to a biologically active soluble mediator is governed by so-called inflammasomes, most notably the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (Broderick et al., 2015). Inflammasomes are multi-protein molecular machineries that consist of a danger sensor, for example, NLRP3, the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC, also known as PYCARD) and the enzyme caspase-1 (Mangan et al., 2018). Assembly of the inflammasome is initiated by NLRP3 upon activation by external or endogenous danger signals and entails controlled homo-oligomerization and the stepwise recruitment of downstream components (ASC, and subsequently caspase-1) in a maturation process that leads to the formation of the fully assembled NLRP3 inflammasome multi-protein complex, observable by microscopy as a so-called ASC speck (Stutz et al., 2013). This assembly ultimately leads to the activation of caspase-1, which then cleaves gasdermin D (GSDMD) and leads to pyroptotic cell death (also known as pyroptosis), mediated by the formation of plasma membrane pores composed of the GSDMD N-terminal fragment (Broz et al., 2020). The inactive pro-forms of IL-1β and IL-18 are also processed by caspase-1 and released during pyroptosis, together with other so-called inflammatory alarmins (Liu et al., 2016). Broz et al. helpfully depict the inflammasome maturation process in an excellent recent review (Broz et al., 2020).

Collectively, inflammasomes are major regulators of cell death and the release of inflammatory signals that amplify the initial danger signals, which activate innate immune cells. As a major driver of inflammation in multiple experimental models of disease – from infections via gout, atherosclerosis, myocardial infarction or stroke to Alzheimer's and Parkinson disease – the NLRP3 inflammasome has emerged as one of the most attractive therapeutic targets in current translational immunology (Mangan et al., 2018). The interest in deciphering the dense network regulating the activity of this potent and potentially devastating inflammatory mechanism has also been fueled by the many studies that illustrate that IL-1β blockade can have therapeutic benefits, not only in patients with *NLRP3* gain-of-function mutations – so called cryopyrin-associated periodic syndrome (CAPS) (Broderick et al., 2015) – but also in those with cardiovascular disease (Ridker et al., 2017). In this context, IL-1 negatively influences the so-called post-ischemic 'remodeling' phase in experimental models (Abbate et al., 2015). Since anti-IL-1 biologicals are restricted to the control of IL-1α and/or IL-1β, but do not block other NLRP3-dependent alarmins, targeting the NLRP3 inflammasome itself would be highly desirable (Mangan et al., 2018). Unfortunately, there are still many gaps in our understanding of the cellular processes regulating NLRP3, and therefore recent research has strongly focused on the mechanisms of activation and regulation of this complex. Here, we review the recent work relating to afferent signals leading to NLRP3 activation, subcellular localization of the inflammasome components, NLRP3 structure, its regulation by other cellular proteins, mechanisms of IL-1 release and, finally, open questions in the field.

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Afferent signals leading to NLRP3 activation

The core NLRP3 inflammasome assembles when, upon activation, the tripartite protein NLRP3 self-oligomerizes upon incompletely understood cues and conformational changes (see below), and recruits the adaptor ASC. This in turn, drives the formation of a supramolecular complex – the ASC speck – that recruits and activates pro-caspase-1 to caspase-1 in a prion-like fashion (Franklin et al., 2014). Caspase-1 is responsible for processing pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 (Agostini et al., 2004). NLRP3 contains three domains – the N-terminal pyrin domain (PYD), the central nucleotide-binding and oligomerization NAIP, CIITA, HET-E and TEP1 (NACHT) domain, and the C-terminal leucine-rich repeat (LRR) domain (Agostini et al., 2004). The PYD is required for the interaction of NLRP3 with the PYD of ASC (Vajjhala et al., 2012), whereas ATP binding and hydrolysis by the NACHT domain is required for the self-oligomerization of NLRP3 after activation (Duncan et al., 2007). The LRR domain has previously been thought to induce auto-inhibition of nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), such as NLRP3, by impairing self-oligomerization (Hu et al., 2013); however, this view has been challenged by recent studies (Hafner-Bratkovic et al., 2018). Novel work indicates that the LRR is required for (constitutive) interaction with a co-regulator, the Nima-related kinase 7 (NEK7) (Sharif et al., 2019), but how this precisely relates to the activation process is unclear and has also been challenged in the human system in a recent preprint (Schmacke et al., 2019 preprint).

The activation of the canonical inflammasome pathway typically requires two signals, priming (Fig. 1A) and activation (Fig. 1B). Priming involves both transcriptional and post-translational regulation of NLRP3 that render NLRP3 'armed' and responsive for a second activation signal. This means priming stimuli (e.g. lipopolysaccharide; LPS) alone are not able to trigger NLRP3 inflammasome assembly or IL-1 maturation. However, activation stimuli (e.g. pore-forming toxins) are ineffective in driving IL-1 maturation in the absence of prior priming (Bauernfeind et al., 2009). Whilst the two steps – priming and activation – can be defined well in experimental *in vitro* settings, the spatial and molecular delineation *in vivo* is far less clear. *In vitro*, priming is typically elicited by diverse pattern recognition receptors (PRRs), such as the Toll-like receptor 4 (TLR4), which senses LPS through TNF, or generally stimuli that converge on nuclear factor κB (NF-κB) activation; this results in the upregulation of *NLRP3* and *IL1B* transcription (Bauernfeind et al., 2009). In addition, many PRR agonists induce post-translational priming of NLRP3 in the form of (de-) ubiquitylation, (de-)phosphorylation or other modifications that, while keeping NLRP3 in an inactive state, make it receptive to activation stimuli (Juliana et al., 2012; Lopez-Castejon et al., 2013; Stutz et al., 2017). The second NLRP3 activation signal can be elicited by multiple agonists, such as extracellular ATP, pore-forming toxins [such as nigericin from *Streptomyces hygroscopicus*, and LukAB and Panton–Valentine leucocidin (PVL) from *Staphylococcus aureus*], alum, silica and other particles, and intracellular RNA (Franchi et al., 2014; Munoz-Planillo et al., 2013; Perregaux and Gabel, 1994; Subramanian et al., 2013). Most of these agonists frequently converge on K^+ efflux, alone or in combination with lysosomal damage or mitochondrial dysfunction and reactive oxygen species (ROS) production, ultimately leading to disturbances in cellular homeostasis (Liston and Masters, 2017; Munoz-Planillo et al., 2013). We summarize the current knowledge on how NLRP3 activity is regulated by ion fluxes in the Box 1. Although ionic regulation seems central, the molecular signaling steps that lead to the activation of NLRP3, recruitment of ASC and caspase-1, and proteolytic activity of the complex remain unclear and await further elucidation.

Apart from homeostasis disruption, certain cell surface PRRs, such as the carbohydrate-binding lectins Dectin-1 and Dectin-2 (also known as CLEC7A and CLEC6A, respectively), have been directly connected with NLRP3 inflammasome activation during fungal infection (Chang et al., 2017; Gross et al., 2009; Hise et al., 2009; Kankkunen et al., 2010; Ritter et al., 2010). *Candida albicans*, an opportunistic fungal pathogen that can cause severe infections in immunocompromised hosts, can engage Dectin-1 via binding to β-glucan, a component of the fungal cell wall (Brown et al., 2003), to induce activation of the downstream spleen tyrosine kinase (Syk) (Gross et al., 2009). Syk signaling in turn primes (signal 1) and activates (signal 2) the NLRP3 inflammasome to induce antifungal innate immunity (Gross et al., 2009; Hise et al., 2009; Joly and Sutterwala, 2010). Both, Syk-dependent K^+ efflux and ROS production were found to serve as signal 2 for NLRP3 activation in response to *C. albicans* (Gross et al., 2009). However, recent work contests the hypothesis of a Syk-dependent K^+ efflux, and suggests that the activation signal is provided by the hyphal peptide toxin candidalysin, which disrupts host membrane integrity and may thereby mediate K^+ efflux directly (Kasper et al., 2018; Rogiers et al., 2019). Thus, fungal hyphae may first elicit TLR- and/or Dectin-1-mediated priming and phagocytosis, and subsequently the secreted toxin candidalysin may function as a canonical K^+-dependent signal 2. A priming-independent and NLRP3 inflammasome-dependent form of rapid pyroptosis has instead been described in response to live intracellular bacteria (Lin et al., 2014). This acute form of pyroptosis and subsequent release of IL-18, but not IL-1β, appears to be triggered by stimuli that simultaneously activate TLRs and NLRP3, and is independent of NF-κB and transcriptional upregulation of *IL1B*. At least for bacterial infection, this requires instead the IL-1 receptor-associated kinase 1 (IRAK1), which interacts with and promotes the re-localization of ASC from the nucleus to the cytosol, thus enabling NLRP3 complex assembly (Lin et al., 2014). Generally, additional scenarios and the underlying molecular steps for conditions in which NLRP3 encounters dual signals simultaneously warrant further analysis.

In addition to the 'canonical' inflammasome signaling, two other signaling pathways result in the activation of NLRP3 and IL-1β maturation, the so-called non-canonical and the alternative inflammasome pathways. The non-canonical inflammasome is triggered by caspase-4 and caspase-5 in humans and caspase-11 in mice (Kayagaki et al., 2011; Vigano et al., 2015). In this non-canonical pathway, caspases directly bind to intracellular LPS, resulting in caspase activation and pyroptosis by GSDMD processing (Kayagaki et al., 2015; Shi et al., 2015; Yang et al., 2015b). Furthermore, ATP release activates P2X7 (also known as P2RX7), triggering K^+ efflux and the canonical NLRP3 pathway (Mariathasan et al., 2006) (Box 1). Several years ago, another pathway of NLRP3 activation had also been observed in human monocytes, which were able to respond to LPS stimulation alone (i.e. without a signal 2) by secreting IL-1β while remaining alive (Wang et al., 2013). This so-called alternative inflammasome pathway was later characterized by Gaidt et al., who discovered that the pathway involves the sequential activation of TLR4, the TIR-domain-containing adapter-inducing interferon-β (TRIF), the receptor interacting protein kinase (RIPK) and caspase-8 (Gaidt et al., 2016).

In summary, despite species and cell type differences, it is generally recognized that the NLRP3 inflammasome integrates multiple danger or damage signals. These perturb cell homeostasis and trigger a cascade of events that control not only the assembly of the complex at the transcriptional and post-transcriptional level, but

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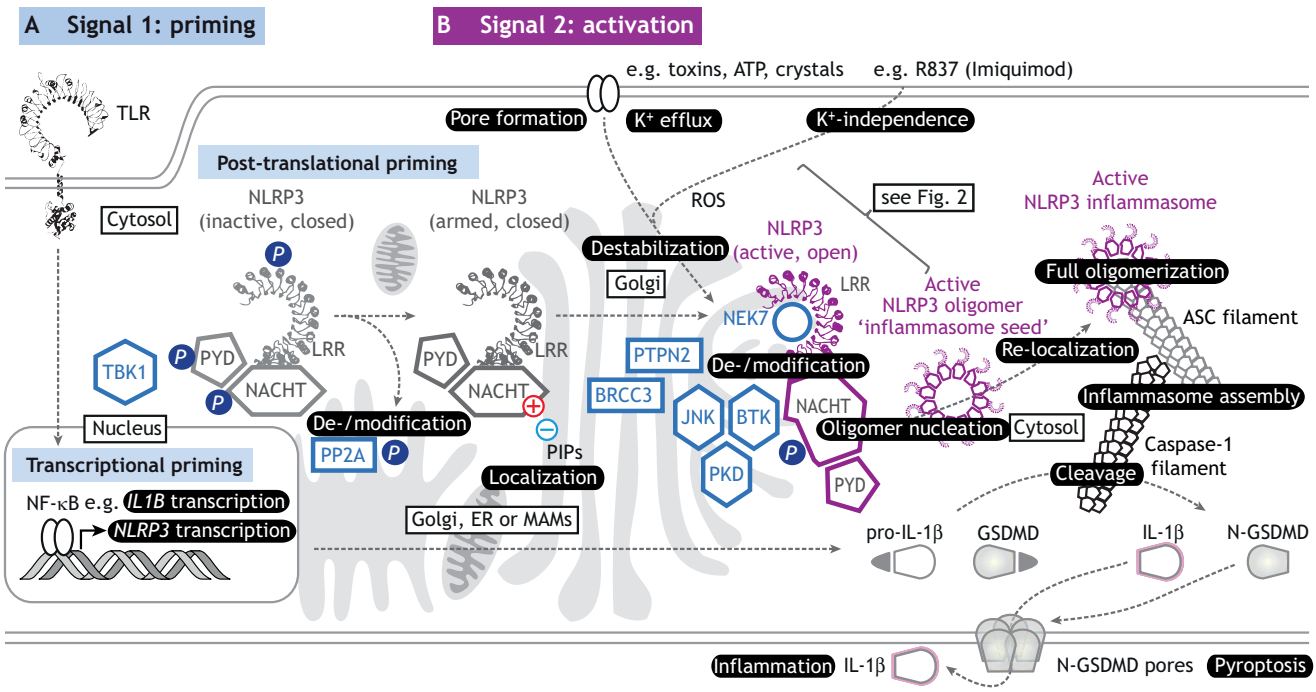

Fig. 1. Layers of cellular regulation of the canonical NLRP3 inflammasome pathway. (A) Priming phase (signal 1). During the priming phase, NF-κB-activating stimuli (TLR activation) drive *IL1B* and, to a lesser extent, *NLRP3* transcription (transcriptional priming). Additionally, changes in post-translational modifications, e.g. de-phosphorylation of serine 5 (PYD) by PP2A, license NLRP3 activation (post-translational priming). NEK7 and TANK-binding kinase 1 (TBK1) also modulate the NLRP3 activation state in an as yet unknown way. (B) Activation phase (signal 2). Signal 2 typically triggers K^+ efflux, changes in post-translational modification of NLRP3 [e.g. de-ubiquitylation by the BRCA1-BRCA2-containing complex subunit 3 (BRCC3), dephosphorylation by the protein tyrosine phosphatase non-receptor type 2 (PTPN2), or phosphorylation by JNK, PKD or BTK] and ASC and the destabilization of cellular organelles, such as the trans-Golgi network (TGN), but this can also result from K^+-independent stimuli. On Golgi membranes, phosphatidylinositol phosphates (PIPs) interact with NLRP3, prompting its congregation on Golgi structures, which leads to the formation of oligomeric NLRP3 (11- or possibly 12-mers). Through NLRP3 modification, for example by PKD or BTK, oligomeric NLRP3 is released from these membranes to enter the cytosol (NEK7 omitted for clarity). The NLRP3 PYD domains in these 'inflammasome seeds' interact with ASC to form ASC filaments that provide an assembly platform for caspase-1 filaments. Proximity-induced activation of caspase-1 leads to the cleavage of pro-IL-1β and GSDMD. The GSDMD N-terminal fragment can form pores in the cell membrane through which IL-1β is released, although GSDMD-independent secretion routes exist (for example via microvesicles, not shown). GSDMD activation may also lead to cell death through pyroptosis. Note that for clarity not all de/-modifications are shown, please refer to main text and text boxes.

also the localization of its core components, NLRP3 and ASC, as described in detail below.

Role of subcellular compartmentalization in the assembly of the NLRP3 complex

Another area of exciting developments, as well as controversies, has been the dynamic subcellular localization of NLRP3 over the course of its activation. Earlier studies suggested NLRP3 to be cytosolic (Wang et al., 2013), but others proposed NLRP3 to reside at the ER prior to activation and then move towards perinuclear, mitochondria-associated ER membranes (MAMs) (Zhou et al., 2011). This concept was corroborated further, implicating microtubules, the mitochondrial membrane component cardiolipin, the mitochondrial antiviral-signaling protein (MAVS), the microtubule affinity-regulating kinase 4 (MARK4) or the stimulator of interferon genes protein (STING, also known as STING1) in directing NLRP3 to the MAMs (Iyer et al., 2013; Misawa et al., 2013; Park et al., 2013; Subramanian et al., 2013; Wang et al., 2020b). Other studies have focused on NLRP3 ER–Golgi transitions (Chen and Chen, 2018; Hong et al., 2019). It was reported that perturbing vesicular trafficking between the ER and the Golgi attenuated NLRP3 inflammasome activation (Hong et al., 2019), but recent work has proposed that multiple K^+-dependent and -independent NLRP3 activators trigger the disassembly of the trans-Golgi network (TGN) to form a dispersed TGN (dTGN) (Chen and Chen, 2018). Moreover, the same study shows that NLRP3 binds to phosphatidylinositol phosphates (PIPs) in the membranes of the dTGN via a polybasic region in the PYD-NACHT linker (Fig. 2). At the dTGN, NLRP3 is then proposed to self-oligomerize at multiple sites, which serve as nucleating mini-complexes for the recruitment of ASC and maturation of the core complex into full active inflammasomes in the cytosol (Chen and Chen, 2018). Another recent study proposed that release from MAMs is vital for NLRP3 to interact with cytosolic ASC and for full inflammasome formation (Zhang et al., 2017). These data provide exciting new insights for establishing a unifying concept that would explain how multiple diverse stimuli converge upon NLRP3 activation (Fig. 2). Although further steps remain to be resolved, disassembly of the Golgi network may be the common cellular danger signal that is sensed by NLRP3 in response to different stimuli. However, as phosphatidylinositol-4-phosphate (PI4P) is also found in other cellular membranes, the specificity of dTGN recruitment is not clear yet. For example, other aforementioned studies implicate the ER and MAMs; recent evidence suggests that the cyclic GMP-AMP synthase (cGAS)–STING pathway is involved in the direct recruitment of NLRP3 to the ER, in response to viral DNA (Wang et al., 2020b). At the ER membrane, the interaction of STING with NLRP3 dampens NLRP3 polyubiquitylation, thus promoting the activation of the inflammasome (Wang et al., 2020b). Another layer of complexity is added to this scenario by the dynamic localization of ASC (Box 2). Hence, although it is possible that different trafficking pathways converge on inflammasome assembly, depending on the nature of the activating signal, as well as the cell

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Box 1. Regulation of NLRP3 inflammasome activity by ion flux

K^+ efflux is considered to be the unifying response that activates NLRP3 oligomerization and downstream signaling (Munoz-Planillo et al., 2013). However, K^+-independent inflammasome activation has been reported for the drug imiquimod (a TLR7 ligand) and its derivative compound CL097, which trigger ROS production for subsequent inflammasome formation (Gross et al., 2016). In addition to K^+ efflux, Ca^{2+} translocating from the extracellular space or from the ER lumen into the cytosol in response to ATP, nigericin or alum, also contribute to NLRP3 inflammasome activation (Lee et al., 2012; Murakami et al., 2012). On the molecular level, P2X purinoceptor 7 (P2X7) is a non-selective ion channel that promotes the influx of Na^+ and Ca^{2+} in response to ATP, for example, during injury (Di Virgilio et al., 2018). This influx in turn acts as a driving force for the two-pore domain weak inwardly rectifying K^+ channel 2 (TWIK2; also known as KCNK6) to extrude K^+ (Di et al., 2018). K^+ efflux cooperates with Cl^- in promoting NLRP3 activation and ASC speck formation. Cl^- efflux can induce NLRP3-dependent ASC speck formation; however, the complex requires K^+ efflux to activate caspase-1 (Green et al., 2018), warranting further investigation into the regulation of these Cl^--induced, non-functional ASC specks in the presence of NLRP3. Second, Cl^-intracellular channel proteins (CLICs) have been implicated downstream of K^+ efflux and consequent ROS generation (Domingo-Fernandez et al., 2017; Tang et al., 2017). While these results show that K^+ and Cl^- may act independently, but complementarily, at different stages in the inflammasome activation process, questions arise regarding which proteins ultimately sense and integrate or relay the changes in ion levels and how. Furthermore, how K^+ efflux or ROS leads to conformational changes in NLRP3 commensurate with its activation remains elusive and warrants further investigation.

type, compartmentalization of the core components is an essential regulatory step in the control of inflammasome activity. Regulation by 3D conformation, however, is another important layer of regulation as described in the next paragraph.

NLRP3 structure and conformational changes

Recent advances in NLRP3 inflammasome research also extend to structural aspects. The most informative has been a structural study reporting the cryo-electron microscopy (EM) structure of the NLRP3 NACHT-LRR domains (NLRP3ΔPYD) in complex with the NEK7 kinase domain (Sharif et al., 2019). In this structure (PBD 6NPY), NLRP3ΔPYD adopts an earring-shaped conformation that resembles the inactive conformations of the cytosolic PRRs NACHT, LRR and CARD domains-containing protein 4 (NLRC4) (Hu et al., 2013) and the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (Maekawa et al., 2016). Within the complex, the LRR domain is important for the binding of NEK7 (Sharif et al., 2019), as also suggested by another study where the lack of the LRR-encoding exon 5 in alternative transcripts of *NLRP3* was linked to loss of NLRP3 function (Hoss et al., 2019). Based on the active structures of NLRC4 and NOD2, NLRP3 was proposed to adopt an active conformation upon ATP hydrolysis in the NACHT domain (Vande Walle et al., 2019). However, the addition of ATP alone was insufficient to induce this conformational change and oligomerization experimentally (Sharif et al., 2019). Conformational change in NLRP3 is intrinsically linked to its activation; bioluminescence resonance energy transfer (BRET) experiments elegantly showed that NLRP3 undergoes a conformational change upon activation, leading to a more 'open' conformation, which resembles the conformation of an NLRP3 variant with CAPS-associated gain-of-function mutations (Tapia-Abellan et al., 2019). Binding of the most important currently available inhibitor of NLRP3, MCC950 (Coll et al., 2015), to the NACHT domain was proposed to lead to a stabilization of the closed conformation in both wild-type and mutant NLRP3 (Coll et al., 2019; Coll et al., 2015; Tapia-Abellan et al., 2019). However, where exactly MCC950 binds and whether MCC950 stabilizes a 'closed' NLRP3 conformation by blocking ATP hydrolysis remain a matter of debate (Coll et al., 2019; Tapia-Abellan et al., 2019; Vande Walle et al., 2019). In addition to MCC950, several other inhibitors that can

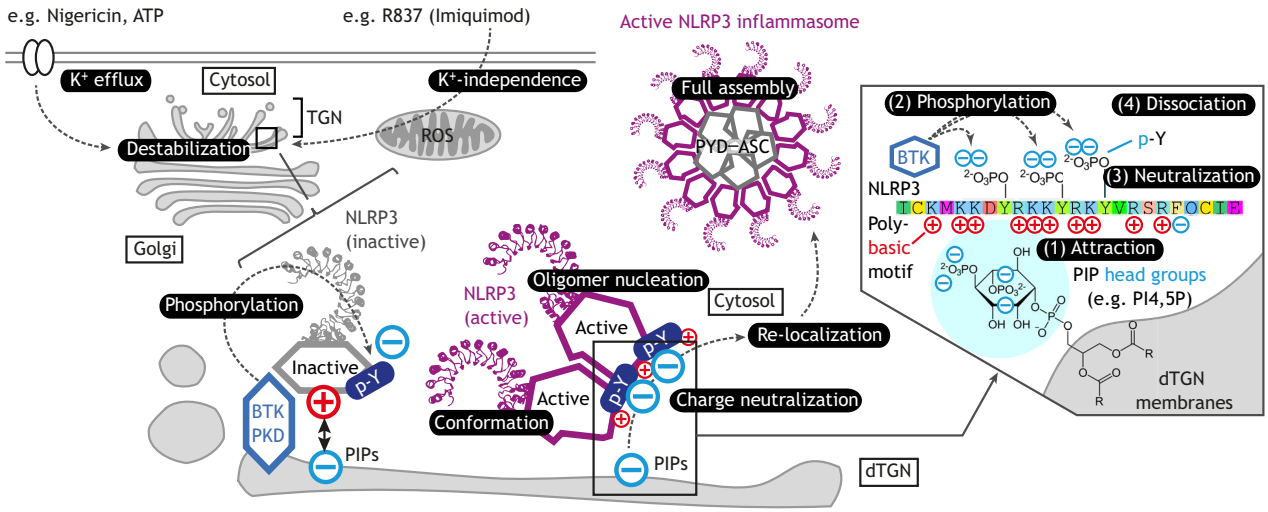

Fig. 2. Association and dissociation of NLRP3 with membranes. Through so far unknown molecular steps, both K^+ efflux-dependent (e.g. toxins, ATP and crystals) and -independent (e.g. R837/imiquimod, which acts via ROS) stimuli lead to an accumulation of NLRP3 on PIP-rich membranes via electrostatic interactions between the negatively-charged PIP head groups and the positively-charged polybasic motif that connects PYD and the core NACHT [inset step (1), attraction]. The local accumulation of armed NLRP3 monomers is thought to result in the formation of NLRP3 oligomers that can nucleate full inflammasomes. The required release of NLRP3 oligomers from the membranes of the dispersed trans-Golgi network (dTGN) could be mediated by neutralization of the electrostatic attraction through BTK phosphorylation of linker tyrosine residues [inset step (2), phosphorylation], leading to phosphorylated tyrosine residues (p-Y), which adds two negative charges per tyrosine, effectively neutralizing positively-charged lysine and arginine residues [insert, step (3) neutralization). Additionally, PKD may aid this process via serine 293 and serine 295 phosphorylation (not shown for clarity). With the positive charge of the linker neutralized, NLRP3 would be free to dissociate from the Golgi membranes (re-localization) [inset step (4), dissociation]. In the cytosol, NLRP3, which has by now adopted an active conformation, can recruit cytosolic ASC, resulting in full oligomerization and enzymatic activity.

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Box 2. Post-translational regulation and dynamic localization of the ASC protein

Similar to NLRP3, as a core component of the NLRP3 inflammasome, the adaptor ASC is also regulated by post-translational modifications, such as ubiquitylation and phosphorylation. ASC is ubiquitylated with different outcomes on complex activation; ubiquitylation of ASC by the linear ubiquitylation assembly complex (LUBAC) in response to signal 1 (priming) and 2 (activation) seems to be required for NLRP3 inflammasome assembly in a NFκB-independent manner (Rodgers et al., 2014). By contrast, in response to RNA virus infection, NLRP3 inflammasome assembly requires ASC ubiquitylation by the ubiquitin E3 ligase TNFR-associated factor 3 (TRAF3) in a mitochondrial antiviral signaling protein (MAVS)-dependent manner, which is responsible for TRAF3 recruitment and activation (Guan et al., 2015). More recently, it was reported that pharmacological and genetic ablation of the deubiquitylases USP7 and USP47 inhibited the activation of the canonical NLRP3 inflammasome pathway by blocking ASC polymerization and speck formation (Palazon-Riquelme et al., 2018). However, it remains unclear whether the two deubiquitylases target ASC or NLRP3, although the authors observed the formation of aberrant oligomers of NLRP3 in response to pharmacological inhibition of USP7 and USP47 (Palazon-Riquelme et al., 2018). Regarding phosphorylation, LPS- and nigericin-dependent phosphorylation of ASC by the Syk and the JNK kinases is required for ASC speck formation in BMDMs (Hara et al., 2013). Phosphorylation of ASC tyrosine 144 (Y146 in mouse ASC) by BTK also promotes its oligomerization (Ito et al., 2015). By contrast, phosphorylation of ASC by the IκB kinase α (IKKα) has been shown to sequester ASC in the nucleus, thus inhibiting NLRP3 inflammasome assembly (Martin et al., 2014), indicating that ASC phosphorylation can exert positive and negative effects. It seems likely that the interleukin-1 receptor-associated kinase 1 (IRAK1) may be involved in IKKα activation (Lin et al., 2014), but the identity of the phosphatase that releases ASC from the nuclear inhibitory complex with IKKα into the perinuclear area of the cytosol remains elusive. Collectively, these latter studies (Lin et al., 2014; Martin et al., 2014) and the observation that microtubules promote the apposition of ASC on mitochondria and transport ASC-loaded mitochondria towards the ER for ASC–NLRP3 interactions (Misawa et al., 2013) suggest that, similar to NLRP3, both ASC modification and localization are highly correlated and regulated.

directly interact with NLRP3 have been reported (Zahid et al., 2019). 3,4-methylenedioxy-β-nitrostyrene has been shown to bind the LRR and NACHT domains and suppress the ATPase activity of NLRP3 (He et al., 2014), whereas CY09 competes with ATP for binding to the Walker A motif of NLRP3 and abolishes direct NLRP3–NLRP3 interactions (Jiang et al., 2017). Oridonin interacts with cysteine 279 in the human NLRP3 NACHT domain through a covalent modification, thus abolishing the NLRP3–NEK7 interaction (He et al., 2018). Although the precise connections between structure and activity are still missing, these structural and inhibitor studies contribute to a growing structural and mechanistic framework for NLRP3 activation and for the development of additional chemical inhibitors (Chen et al., 2020; Mangan et al., 2018; Yang et al., 2020). Whether, molecularly, low intracellular K^+ or ROS directly or indirectly affect NLRP3 structure and conformation remains a pivotal open question. The same applies to the effects resulting from the binding of or modification by accessory proteins, which is discussed next.

Regulatory proteins and post-translational modifications – focus on recent additions to a growing list

Given the critical need to control a potentially harmful process and the number of danger signals converging on NLRP3, it is not surprising that numerous NLRP3 regulatory proteins have been discovered (Table 1). Whereas some of these proteins appear to function as chaperones, such as the small glutamine-rich tetratricopeptide repeat-containing protein 1 (SGT1; also known as SUGT1) and the heat-shock protein 90 (HSP90) family, the first identified NLRP3 regulatory proteins (Mayor et al., 2007), many seem to modify NLRP3 activity through post-translational modification. Of specific interest are phosphorylation and ubiquitylation of NLRP3 (reviewed in Haneklaus et al., 2013; Song and Li, 2018; Swanson et al., 2019). These modifications can either arm or disarm NLRP3, and activate or repress its activity (Swanson et al., 2019). From a therapeutic perspective, the discovery of NLRP3 modulators is exciting, as it expands the targeting possibilities beyond the two sole enzymatic activities of the core inflammasome, namely the ATPase activity of NLRP3 and the proteolytic activity of caspase-1 (Mangan et al., 2018; Randle et al., 2001; Swanson et al., 2019). Given the rapidly growing list of NLRP3-interacting proteins and regulators, an in-depth discussion of all of these proteins would vastly exceed the scope of this Review. We therefore hope to provide here a comprehensive list of modifiers (Table 1), but restrict our discussion to a selection of recently identified regulators.

NEK7, an extensively studied regulator of NLRP3, was discovered independently by three groups in 2016 (He et al., 2016; Schmid-Burgk et al., 2016; Shi et al., 2016). NEK7 had been known previously for its role in centrosome organization (Yissachar et al., 2006), but a function in the inflammasome process was unexpected. In murine and human cells, NEK7 binds to NLRP3, and the cryo-EM structure shows a 1:1 complex of NLRP3ΔPYD with NEK7 (Sharif et al., 2019). The precise role of NEK7 in the inflammasome process is still uncertain, as its kinase activity is apparently redundant (He et al., 2016), suggesting that targeting the inflammasome via NEK7 kinase inhibitors is probably not conceivable, as well as because of the anticipated side-effects on centrosome function. A recent preprint indicates that in primary human macrophages derived from induced pluripotent stem cells (iPSCs), NEK7 and transforming growth factor β-activated kinase 1 (TAK1; also known as MAP3K7) may have, at least partially, redundant functions in NLRP3 priming, with NEK7 acting at the transcriptional level and TAK1 at the post-translational level (Schmacke et al., 2019 preprint).

Bruton's tyrosine kinase (BTK) is another recently identified novel positive NLRP3 regulator (Ito et al., 2015; Liu et al., 2017), chosen for further discussion due to its status as a well-known drug target. Indeed, BTK is a pivotal component of the B cell receptor pathway (Khan, 2012; Weber et al., 2017), and BTK inhibitors are clinically approved against B cell malignancies (Wilson et al., 2015). Our laboratory was able to show that cells from patients suffering from X-linked agammaglobulinemia (XLA), due to loss-of-function mutations in *BTK,* or from patients treated *in vivo* with BTK inhibitors, exhibited a selective NLRP3 deficiency, as evidenced by reduced IL-1β release (Liu et al., 2017), suggesting that XLA may be the first known inherited NLRP3 inflammasomopathy. Although a function for BTK at the level of ASC has only been proposed and not fully explored (Ito et al., 2015) (Box 2), recent work from our laboratory (Bittner et al., 2020 preprint) suggests that BTK interacts constitutively with NLRP3 upon priming and directly modifies the aforementioned polybasic motif required for dTGN association. Our data show that blocking BTK kinase activity or mutating the targeted tyrosine residues in NLRP3 results in reduced IL-1β production (Bittner et al., 2020 preprint). Therefore, we speculate that BTK may utilize the post-translational modification of NLRP3 to prompt its relocation from the dTGN to the cytosol for ASC engagement (Fig. 2). We cannot exclude an additional effect of phosphorylation on the 3D structure and/or the ATPase activity of NLRP3, based on

the localization of the BTK-modified tyrosine residues in the structure of the inactive NLRP3ΔPYD–NEK7 complex (Sharif et al., 2019). Although BTK promotes NLRP3 activation at physiological concentrations of LPS (and thus TLR-priming stimuli), a recent study showed that high LPS levels appear to turn BTK into a negative regulator; this occurs by preventing de-phosphorylation of the NLRP3 PYD domain by the protein phosphatase 2A (PP2A) (Mao et al., 2020), which in turn seems to be required to license NLRP3 for activation (Stutz et al., 2017). This is further discussed in an e-Letter (https://www.jci.org/eletters/view/128322#sec1) accompanying Mao et al. (2020) and a recent preprint from our laboratory (Bittner et al., 2020 preprint). How these reported functions of BTK, activating and inhibiting NLRP3, can be mechanistically reconciled on the molecular and cellular level remains to be explored. Nevertheless, these studies have the merit to put forward the notion of BTK as a rheostat, that is, it promotes NLRP3 activity under normal settings of infection or sterile inflammation, but shuts off NLRP3 in the presence of high levels of TLR stimuli to avert excessive inflammation (Bittner et al., 2020 preprint; Mao et al., 2020). This potential role obviously needs to be studied further and may influence any therapeutic strategies based on the modulation of BTK activity. Interestingly, a BTK function in macrophages – probably via NLRP3 – was recently implicated in COVID-19-related lung inflammation. In an off-label trial of ibrutinib, the BTK inhibitor attenuated inflammatory parameters and improved lung function in hospitalized SARS-CoV2-infected patients (Roschewski et al., 2020). These data strengthen the earlier suggestion that BTK may be a way to target NLRP3 in the absence of clinically approved NLRP3 inhibitors (Banoth and Cassel, 2017; Henrickson, 2017; Liu et al., 2017).

Several other newly discovered regulators warrant further discussion. For example, the dual-specificity lipid and protein phosphatase PTEN, a key regulator of PIPs and a well-known tumor suppressor (Masson and Williams, 2020), was identified as a positive regulator of NLRP3 inflammasome activation in myeloid cells (Huang et al., 2020). Those authors were able to show that PTEN interacts with and dephosphorylates NLRP3 at tyrosine 32 and that this dephosphorylation is necessary for NLRP3 interaction with ASC and chemotherapy-induced NLRP3 inflammasome activation. Furthermore, protein kinase D (PKD; also known as PRKD1) was found to phosphorylate NLRP3 on serine 293 (human NLRP3 S295), thereby regulating NLRP3 localization. PKD is thought to act on membrane-associated NLRP3, thus promoting its relocation to the cytosol and complex assembly (Zhang et al., 2017). Intriguingly, serine 295 on human NLRP3 is also targeted by protein kinase A, which suppresses inflammasome activation in response to bile acids and prostaglandin E_2 (Guo et al., 2016; Mortimer et al., 2016). These studies for serine 295 phosphorylation are but two examples of a growing number of reports that describe opposing effects – either promoting or limiting NLRP3 activity – for the same post-translational modification. It has recently been reported that the transmembrane tyrosine kinase receptor EphA2 functions as a negative regulator of inflammasome activation during reovirus infection (Zhang et al., 2020) by targeting Y136, a residue also modified by BTK to promote NLRP3 activity (Bittner et al., 2020 preprint).

Further progress has also been made regarding other, non-phosphorylation post-translational modifications, such as ubiquitylation, small ubiquitin-like modifier (SUMO)-ylation or acetylation; as previously mentioned, deubiquitylation is required for NLRP3 post-transcriptional priming. Under basal conditions, ubiquitylation of NLRP3 by the F box protein L2 (FBXL2) or cullin-1 reduces NLRP3 stability (Han et al., 2015) and/or blocks ASC recruitment and thus inflammasome assembly (Wan et al., 2019), respectively. This ubiquitylation event can be counteracted by the activity of the F-box O3 (FBXO3), another member of the Skp, cullin, F-box-containing (SCF) complex, which, upon LPS stimulation, promoted FBXL2 degradation (Han et al., 2015). Intriguingly, in non-stimulated bone marrow-derived macrophages (BMDMs), NLRP3 was found to be conjugated to SUMO-2 and SUMO-3 by the SUMO E3 ligase mitochondrial-anchored protein ligase (MAPL), on six different lysine residues (Barry et al., 2018), but LPS plus nigericin or ATP triggered NLRP3 de-SUMOylation by the sentrin- and SUMO-specific protease (SENP) 6 and SENP7, enabling NLRP3 inflammasome assembly and activation (Barry et al., 2018). However, in the same inducing conditions, full inflammasome activation was also shown to require conjugation of NLRP3 to SUMO-1 on lysine 204 by UBC9 (also known as UBE2I), as recently reported (Shao et al., 2020). Another study reported that in LPS- and ATP-stimulated macrophages, acetylation of NLRP3 promotes its interaction with ASC and complex assembly (He et al., 2020); this modification was reversed by the NAD^+-dependent deacetylase and metabolic sensor sirtuin 2 (SIRT2), which dampens inflammasome activation (He et al., 2020). Intriguingly, the expression of SIRT2 was decreased in macrophages derived from old mice (2 years old). Consequently, 'old' macrophages responded to stimuli with increased inflammasome activation, suggesting that an increased inflammatory environment in aging and obese individuals might be caused by reduced SIRT2 activity. Interestingly, SIRT2 overexpression in these macrophages reduced caspase-1-dependent cleavage and release of IL-1β in response to LPS or ATP, and restored insulin signaling in the presence of adipose tissue (He et al., 2020), suggesting that aging-associated sterile inflammation and insulin resistance can be reversed.

Taken together, the core inflammasome components, and NLRP3 in particular, are embedded in a dense regulatory framework (Fig. 3), which has yet to be fully elucidated and may be highly dependent on cell type (Huang et al., 2020). How modifications at the same residue result in different or even opposite effects on NLRP3 function and inflammasome assembly remains unclear, and warrants further work into the structural and conformational consequences of each modification state. Nevertheless, we hope to provide here a comprehensive and up to date list of known NLRP3 regulators (Table 1), and to have illustrated the intricate effects of post-translational modifications on inflammasome assembly and activity.

Recent insights into IL-1β release and cell death

The release process of IL-1β and its relationship with cell death (Broz et al., 2020; He et al., 2015) are still not entirely elucidated. GSDMD cleavage and pore formation depend on caspase processing but may differ in terms of the recognition of their molecular client (pro-IL-1β or full-length GSDMD), according to a very recent study (Wang et al., 2020a). Although many studies have directly linked GSDMD pore formation and IL-1β release, it has become increasingly clear that, in response to specific triggers, innate immune cells can enter a state of hyperactivation and release IL-1β in a GSDMD-independent or -dependent manner, while retaining viability and not entering pyroptosis (Evavold et al., 2018; Pelegrin et al., 2008; Verhoef et al., 2004; Zanoni et al., 2016, 2017; Monteleone et al., 2018; Semino et al., 2018). It has long been known that mature IL-1β, which lacks a signal peptide, is not secreted through the conventional secretory pathway, that is, from the ER to the Golgi and, finally, to the plasma membrane via

Table 1. Reported NLRP3 regulators or interactors

Protein or Gene	Mode of action	Effect	Signal	Cell type (species)	Reference
ARIH2	NLRP3 NACHT domain ubiquitylation	negative	2	THP-1 cells (h)	Kawashima et al., 2017
BRCC3	NLRP3 LRR deubiquitylation	positive	2	Immortalized (BMDMs) (m)	Py et al., 2013
BTK	NLRP3 polybasic region phosphorylation (Y136, Y140, Y143, Y168)	positive	2	BMDMs (m), PBMCs (h), primary macrophages (h), THP-1 cells (h)	Bittner et al., 2020 preprint; Ito et al., 2015; Liu et al., 2017
BTK	PP2A phosphorylation and consequent NLRP3 inhibition	negative	2	Primary monocytes (h), BMDMs, BMDCs (m)	Mao et al., 2020
CUL1	NLRP3 K689 ubiquitylation	negative	1 or 2?	THP-1 cells (h), BMDMs (m)	Wan et al., 2019
EPHA2	Phosphorylation of NLRP3 Y132	negative	1 or 2?	HEK293T (h)	Zhang et al., 2020
FBXL2	NLRP3 K689 K48-linked ubiquitylation leading to proteasomal degradation	negative	1	Primary monocytes, U937 cells (h)	Han et al., 2015
GBP5	Binds NLRP3 PYD and facilitates ASC speck formation	positive	1	THP-1 cells (h), J774A.1 (m) cells (h), BMDMs (m)	Shenoy et al., 2012
GNB1	NLRP3 PYD binding	negative	1	BMDMs (m)	Murakami et al., 2019
HSP70	NLRP3 binding	negative	1	BMDMs (m)	Martine et al., 2019
HSP90	Stabilizing NLRP3 inactive conformation	negative	1?	BMDMs (m)	Mayor et al., 2007
JNK1	Phosphorylation of NLRP3 S194	positive	1	Immortalized BMDMs (m)	Song et al., 2017
lnc RNA	Probably various, e.g. ANRIL (upregulation of BRCC3 expression via sponging miR-122)	various direct/ indirect	n/a	HK-2 epithelial cells (h)	Hu et al., 2019
MAPL	NLRP3 SUMOylation of K689	negative	2	BMDMs (m)	Barry et al., 2018
MARCH7	NLRP3 LRR ubiquitylation	negative	2	BMDMs (m)	Yan et al., 2015
MARK4	NLRP3 mitochondria targeting	positive	2	BMDMs (m)	Li et al., 2017
miR	Various, e.g. miR-21 (via A20-mediated IL-1 transcription and caspase-1 activation) or miR-122 (via BRCC3, see above), miR-233	various direct/ indirect	n/a	Various	Hu et al., 2019; Xue et al., 2019
NEK7	Binds NLRP3 NACHT and LRR domains	positive	1 or 2?	BMDMs (m), immortalized BMDMs (m), peritoneal macrophages (m), RAW264.7 cells (m), J774A.1 (m)	He et al., 2016; Schmacke et al., 2019 preprint; Schmid-Burgk et al., 2016; Shi et al., 2016
PELI2	NLRP3 ubiquitylation	positive	1	BMDMs (m)	Humphries et al., 2018
PKA	Phosphorylation of NLRP3 S295	negative	2	BMDMs (m)	Guo et al., 2016
PKD	Phosphorylation of NLRP3 S295	positive	2	BMDMs (m)	Zhang et al., 2017
PP2A	NLRP3 S5 dephosphorylation	positive	2	Immortalized BMDMs (m)	Stutz et al., 2013
PTEN	NLRP3 Y32 dephosphorylation	positive	2	BMDMs, *in vivo* (m), THP-1 cells (h)	Huang et al., 2020
PTPN22	NLRP3 Y891 dephosphorylation	positive	2	BMDMs (m)	Spalinger et al., 2016
SENP3	Counteracts UBC9 SUMOylation of NLRP3	negative	2	THP-1 cells (h), BMDMs (m)	Shao et al., 2020
SENP6/ SENP7	Counteracts MAPL SUMOylation of NLRP3	positive	2	BMDMs (m)	Barry et al., 2018
SFK-Cbl	Reduction of mitochondrial ROS	negative	2	THP-1 cells (h)	Chung et al., 2018
SGT1	Stabilizing NLRP3 inactive conformation	negative	1?	BMDMs (m)	Mayor et al., 2007
SHP	Competes with NLRP3 for ASC binding	negative	2	BMDMs (m)	Yang et al., 2015a,b
SIRT2	NLRP3 K21/K22 deacetylation	negative	n/a	BMDMs, immortalized BMDMs (m)	He et al., 2020
SREBP, SCAP	NLRP3 transport to mitochondria	positive	2	BMDMs (m)	Guo et al., 2018
TRIM31	NLRP3 PYD K48 linked-ubiquitylation leading to proteasomal degradation	negative	1	BMDMs (m)	Song et al., 2016
TXNIP	NLRP3 binding upon ROS	positive	2	Microglia BV2 cells (m)	Ye et al., 2017
UBC9	SUMOylation of NLRP3 K204	positive	2	THP-1 cells (h), BMDMs (m)	Shao et al., 2020
USP7/ USP47	Deubiquitylation of NLRP3 and ASC	positive	2	Primary macrophages, THP-1 cells (h), BMDM (m)	Palazón-Riquelme et al., 2018
n/a	*PYCARD*, *CASP1*, *IL1B* gene methylation (and stimulus-dependent demethylation)	negative (indirect)	1	Primary monocytes (h)	Vento-Tormo et al., 2017
n/a	*NLRP3* promotor demethylation during *M. tuberculosis* infection	positive	1?	THP-1 cells (h)	Wei et al., 2016

ANRIL, antisense non-coding RNA in the INK4 locus; ARIH2, ariadne RBR E3 ubiquitin protein ligase 2; BRCC3, BRCA1/BRCA2-containing complex subunit 3; BTK, Bruton's tyrosine kinase; CBL, Casitas B-lineage lymphoma proto-oncogene; CUL1, cullin 1; EPHA2, ephrin type-A receptor 2; FBXL2, F-box/LRR-repeat protein 2; GBP5, guanylate-binding protein 5; GNB1, G-protein subunit β1; HSP, heat-shock protein; JNK1, c-Jun N-terminal kinase 1; lnc RNA, long non-coding RNA; MAPL, mitochondrial-anchored protein ligase; MARCH7, membrane-associated RING-CH-type finger 7; MARK4, microtubule affinity regulating kinase 4; miR, microRNA; NEK7, NIMA-related kinase 7; PBMCs, peripheral blood monocytic cells; PELI2, pellino 2; PKA, protein kinase A; PKD, protein kinase D; PP2A, protein phosphatase 2A; PTEN, phosphatase and tensin homolog; PTPN22, protein tyrosine phosphatase, non-receptor type 22; SCAP, cleavage activation protein; SENP3/6/7, SUMO-specific protease 3/6/7; SFK, Src family kinase; SGT1, suppressor of G2 allele of SKP1; SHP, small heterodimer partner; SIRT2, sirtuin 2; SREBP, sterol regulatory element-binding protein; SUMO, small ubiquitin-like modifier; TRIM31, tripartite motif-containing 31; TXNIP, thioredoxin-interacting protein; UBC9, Ubiquitin-like conjugating enzyme 9; USP4/USP7, ubiquitin-specific-processing protease 4/7. Species: h, human; m, mouse.

secretory vesicles (Rubartelli et al., 1990; Semino et al., 2018). According to the most accepted model, processing and secretion of mature IL-1β in the canonical or non-canonical NLRP3 inflammasome activation pathway employs caspase-1- or caspase-11-dependent cleavage of GSDMD, which leads to pore formation and IL-1β release (Evavold et al., 2018; Heilig et al., 2018).

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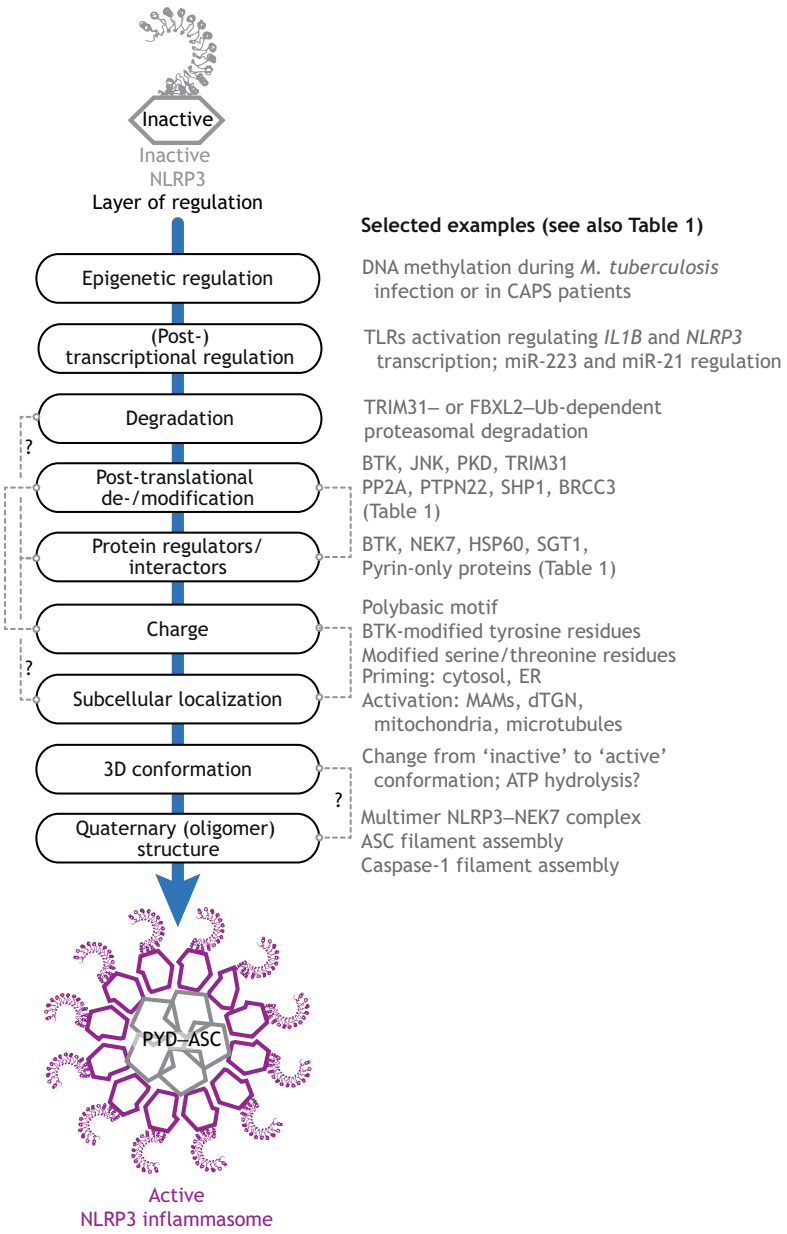

Fig. 3. Multiple layers of cellular regulation govern the activation of the NLRP3 inflammasome. For simplicity sake, the different layers are shown to act sequentially and independently from one another, although multiple inter-dependencies between these regulatory steps have been reported (some indicated by dotted lines). For example, a modification may affect charge or charge affects subcellular localization and/or the protein 3D structure. Unfortunately, the exact boundaries of these interactions and the regulators operating on them are not well defined. Please also note that not all layers of operation may apply concomitantly in a given context. Several selected examples are further discussed in the text and a comprehensive list is provided in Table 1. Ub, ubiquitin.

However, two studies have reported slow TLR- and vesicle-dependent, but GSDMD-independent, IL-1β secretion preceding cell death in human monocytes (Monteleone et al., 2018; Semino et al., 2018), similar to what occurs during the release of IL-1β in the absence of cell death described in the alternative inflammasome pathway (Gaidt et al., 2016). This slow IL-1β secretion is dependent on cleavage of IL-1β and exposure of a polybasic motif, which targeted IL-1β to phosphatidylinositol 4,5-bisphosphate (PIP2)-enriched membrane ruffles (Monteleone et al., 2018). Stimulation of multiple TLRs and subsequent rapid ROS- and GSDMD-dependent release of IL-1β contributes to increase secretion of this cytokine (Monteleone et al., 2018; Semino et al., 2018). Interestingly, in CAPS patients carrying *NLRP3* hyper-activating mutations, TLR4 stimulation alone (i.e. without subsequent 'activation' stimulus) is sufficient to trigger massive IL-1β release, probably by enhancing the already high basal levels of ROS (Semino et al., 2018). Another study has argued that signal 2 stimuli simply enable IL-1β release in a non-selective manner via necrotic cell death (Cullen et al., 2015). These differences are probably attributable to distinct cell types and host species and warrant further dissection.

Perspectives and conclusions

Much has been achieved in the past few years that expands our knowledge on the multiple regulatory layers of NLRP3 activation (Fig. 3). To harness the considerable translational potential of the NLRP3 inflammasome, several important open questions remain. First, priming with LPS and stimulation with nigericin or ATP to measure IL-1β is the most commonly used *in vitro* approach to study NLRP3 activity. Nevertheless, studying a wider panel of agonists, including K^+-dependent and -independent agonists, and analytes, such as IL-18, the high mobility group box 1 protein (HMGB-1), other alarmins or cell death, would be highly desirable. Furthermore, *in vivo* models and clinical trials will be informative (see below), bearing in mind that the delineation of priming (signal 1) versus activation (signal 2) is probably less paradigmatic in this context, and overall outcomes are more important. Second, whereas the macrophage NLRP3 inflammasome is relatively well-studied

in vitro (Table 1), inflammasome studies in dendritic cells, neutrophils, Kupffer cells, platelets and non-immune cells, such as cardiomyocytes, are lagging behind. Since use of NLRP3 inhibitors is likely going to be systemic, a wider exploration appears to be mandatory. Use of lineage-specific conditional NLRP3 knockouts in experimental *in vivo* models could complement the emerging *in vitro* picture and studies using inhibitors. Surprisingly, a floxed (conditional ready) mouse *Nlrp3* KO allele has not been published so far, but it is probably in preparation. Third, only a few studies have so far addressed the role of NLRP3 in cancer models, especially in cancers that originate from naturally inflammasome-competent cells, such as myeloid cells. We have previously mentioned the role of PTEN in NLRP3 inflammasome activation induced by chemotherapy (Huang et al., 2020). Results from a clinical study have also suggested that anti-IL-1 therapy may reduce the risk of developing lung cancer as well as lung cancer mortality. This was unexpected because this disease had not been previously been considered as an IL-1 or inflammation-driven malignancy (Ridker et al., 2017). Nevertheless, further work is needed to confirm and clarify the protective effects of anti-inflammatory therapy in lung cancer and to explore its effects on other forms of neoplasia. Furthermore, much of what we know is derived from the analysis of murine cells and *in vivo* models (Table 1). However, recent studies have flagged up important differences in terms of signaling cascades or importance of NLRP3 regulators between mice and humans (Gaidt et al., 2016; Schmacke et al., 2019 preprint). Thus, analyses in human cells will be essential to better understand the therapeutic potential of NLRP3, for example by performing studies in human cell lines or through iPSC-based approaches, although these are not yet broadly accessible. Additionally, more specific second- or third-generation inhibitors, for example those that are directed at some of the pathway modifiers discussed above, could be advantageous for *in vitro* studies. Certainly, analyses embedded within clinical trials for the approval of these molecules could also prove immensely insightful. Finally, a major bottleneck for *ex vivo* studies and clinical trials in humans is the virtually complete lack of NLRP3-proximal biomarkers (i.e. those monitoring the activity of NLRP3 itself, rather than downstream events, such as alarmin release). For example, in the CAPS field, serum amyloid A (SAA) and the C-reactive protein (CRP) are still the only currently recognized readouts to monitor disease activity, but they are relatively distal to NLRP3, that is, they cannot be used to estimate the extent of NLRP3 activity or inhibition. Therefore, readouts that would help to directly monitor NLRP3 conformation, modification or inhibitor target engagement *in vivo* or *ex vivo* would be highly desirable. Expanded efforts in this area could be fueled by the considerable potential for therapeutic applications still to be realized in this area, as new generations of NLRP3 inhibitors will require proximal markers and assays that inform on target-engagement and/or the degree of NLRP3 activity during clinical trials. We propose that NLRP3 post-translational modifications should be explored systematically as potential NLRP3-proximal biomarkers.

Despite the intense research efforts, defining the mechanism and the pathophysiological relevance of the NLRP3 inflammasome continues to pose considerable but exciting challenges in immunology and a growing number of related fields. Neuroinflammation, cardiovascular medicine, metabolic regulation and trained immunity are but a few of several areas in which a significant involvement of NLRP3 is anticipated. More research will be essential to safely and comprehensively harness the potential of NLRP3 inhibitors or agonists. We hope to have highlighted here at least some of the areas that have evidenced exciting new developments in this direction and some that may be fruitful to explore in the future.

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