


Control of the inflammasome by the ubiquitin system

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Inflammation is the body's response to danger. One of the first immune cell types to encounter danger is the macrophage. Macrophages sense danger signals such as extracellular ATP or bacterial toxins, derived from tissue damage or infection, and initiate the activation of an intracellular molecular complex called the inflammasome. The inflammasome consists of a cytosolic pattern recognition receptor, an adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD) and the protease caspase-1. Assembly of the complex leads to the cleavage and activation of caspase-1 that triggers processing and release of the cytokines interleukin (IL)-1 β and IL-18, and ultimately cell death via the process of pyroptosis. The ability to sense and respond to danger appropriately is critical for maintaining immune homeostasis. Dysregulation of inflammasomes contributes to the progression of chronic diseases prevalent in the ageing population, such as Alzheimer's disease, COPD and metabolic disease; hence, it is critical that activation of the inflammatory response and inflammasome activation are tightly regulated. Post-translational modifications (PTMs) such as ubiquitination have recently emerged as important regulators of inflammasome assembly. However, the mechanisms by which PTMs regulate the inflammasome are still not understood. This review aims to summarize our knowledge to date on how the ubiquitin system controls inflammasome activation and where this area of research is heading.

Introduction

It has been 15 years since Aaron Ciechanover, Avram Hershko and Irwin Rose received the Nobel Prize in Chemistry for the discovery of ubiquitin-mediated protein degradation. Since then, our understanding of how ubiquitin is fundamental to maintain cell homeostasis has exponentially increased. It has become clear

that the ubiquitin system is far more than just a system to control protein homeostasis through protein degradation and that it is fundamental for the control of signalling pathways such as DNA repair, receptor trafficking to the plasma membrane and immune signalling [1,2]. The discovery of a wide variety of

Abbreviations

AIM2, absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a CARD; DAMPs, damage-associated molecular patterns; DUBs, deubiquitinases; E1, E1 ubiquitin-activating enzyme; E2, E2 ubiquitin-conjugating enzyme; E3, E3 ubiquitin ligase; IL-1 β , interleukin-1 β ; Le-Tx, anthrax lethal toxin; LPS, lipopolysaccharide; LUBAC, linear ubiquitin assembly complex; MAMPs, microbe-associated molecular patterns; MINDY, MIU-containing novel DUB family; MJD, Machado–Joseph disease/Josephin domain; NLRC4, NLR family CARD domain containing 4; NLRP3, nucleotide binding and leucine-rich repeat-containing protein 3; OUT, ovarian tumour domain-containing proteases; PTM, post-translational modifications; SCF, Skp, Cullin, F-box containing complex; SHARPIN, SHANK-associated RH domain interactor; SUG-1, protease regulatory subunit 8 homolog; TRIM, tripartite motif-containing protein; Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolases; USP, ubiquitin-specific proteases; ZUFSP, zinc finger-containing ubiquitin peptidase 1.

polyubiquitin chains, including phosphorylated ubiquitin and how each of these controls different protein fates, has uncovered the great complexity of this system.

One of the key roles of the ubiquitin system is the control of the inflammatory response. Inflammation is the response of the body to tissue injury or infection. The initiation of inflammation leads to the activation of the innate immune response, as a first line of defence, followed by the adaptive immune response, with the ultimate goal of removing the threat, promoting tissue repair and restoring health. Although inflammation is a beneficial process in life, its deregulation can lead to chronic inflammation that results in the initiation and progression of disease [2]. It is therefore necessary that initiation and progression of the inflammatory response are tightly regulated at different levels, and this is achieved amongst other things through PTMs such as phosphorylation or ubiquitination [3].

One key process in the initiation of inflammation is the assembly and activation of molecular complexes called inflammasomes. Inflammasomes form when cells such as macrophages encounter danger signals, either derived from tissue damage, such as extracellular ATP, or infection, such as bacterial toxins. Assembly of these complexes leads to the activation of the protease caspase-1 which triggers the processing and release of the active forms of the pro-inflammatory cytokines interleukin (IL)-1 β and IL-18, and a process of cell death called pyroptosis [4]. The ability to sense and respond to danger appropriately is critical for maintaining immune homeostasis. Dysregulation of inflammasomes contributes to the progression of many chronic diseases such as Alzheimer's disease, chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis; hence, it is critical that inflammasome activation is tightly regulated [4].

In the recent years, it is becoming increasingly clear that the ubiquitin system plays an integral role in the control of the inflammasome activation. The aim of this review is to address the current knowledge on the ubiquitin system and inflammasomes and the challenges and future directions that this area of research faces.

The ubiquitin system

The post-translational modification of proteins by ubiquitin constitutes a delicate equilibrium between the addition and the removal of this 8.5kDa molecule from its targets. Addition of ubiquitin to its substrate is mediated by the sequential activation of E1-E2-E3 enzymes. First, the E1 ubiquitin-activating enzyme and

ubiquitin form a thioester bond that is then transferred to an E2 ubiquitin-conjugating enzyme forming an ubiquitin-thioester intermediate that is finally transferred to the substrate protein by an E3 ubiquitin ligase [5] (Fig. 1A). While the E1 and E2 families have around 2 and 40 members, respectively ([6], the E3 ubiquitin ligase family is formed by around 600 members that can be grouped in three major classes: (a) RING (really interesting new gene) domain-containing and U-box-containing E3s, which function as scaffolds for E2 enzymes; (b) HECT (homologous to the E6-associated protein C terminus) domain-containing E3s, which form a thiol ester bond with ubiquitin before conjugation to the substrate; and (c) RBR (RING1-between-RING2) E3s, which function as hybrids of RING and HECT E3s [7].

The ubiquitin system presents a great level of complexity compared to other PTMs. Ubiquitin is conjugated to lysine (K) residues in its substrate by its C-terminal G75 and G76 residues. Ubiquitination can occur just in one residue (monoubiquitination) and occur repeatedly in the same protein at different sites (multi-monoubiquitination) or at the same site (polyubiquitination). In this case, the first ubiquitin serves as the acceptor of additional ubiquitins that conjugate to each other at any one of their lysine residues (K6, K11, K27, K29, K33, K48, K63 or methionine 1 (M1, linear chains)) [5] (Fig. 1Bi,ii). In addition to these eight different types of polyubiquitin chains (poly-Ub), ubiquitin (Ub) can also form hybrid polyubiquitin chains, where additional ubiquitins conjugate to each other through any of their different K residues (Fig. 1Biii). It is also worth noting that noncanonical ubiquitination of residues other than lysine, such as serine or threonine, can also occur, although this type of ubiquitination is not yet well understood [8]. An even higher level of complexity has been uncovered by recent discoveries showing that ubiquitin and members of the ubiquitin system can be modified by PTMs such as phosphorylation, acetylation or deamidation [9].

The type of polyubiquitin chains found in a substrate is key to determine the fate, function and regulation of the target protein, hence explaining the existence of this high level of complexity in the ubiquitin system. The best characterized chains are K48 poly-Ub chains being responsible for protein degradation by the proteasome and K63 poly-Ub chains, which are involved in signalling pathways, by acting as scaffold for the recruitment of other proteins. Linear M1 poly-Ub chains are also involved in signalling pathways. Other poly-Ub chains, such as K11 poly-Ub, also act as a strong degradation signal, while K33 poly-Ub chains are involved in anterograde protein

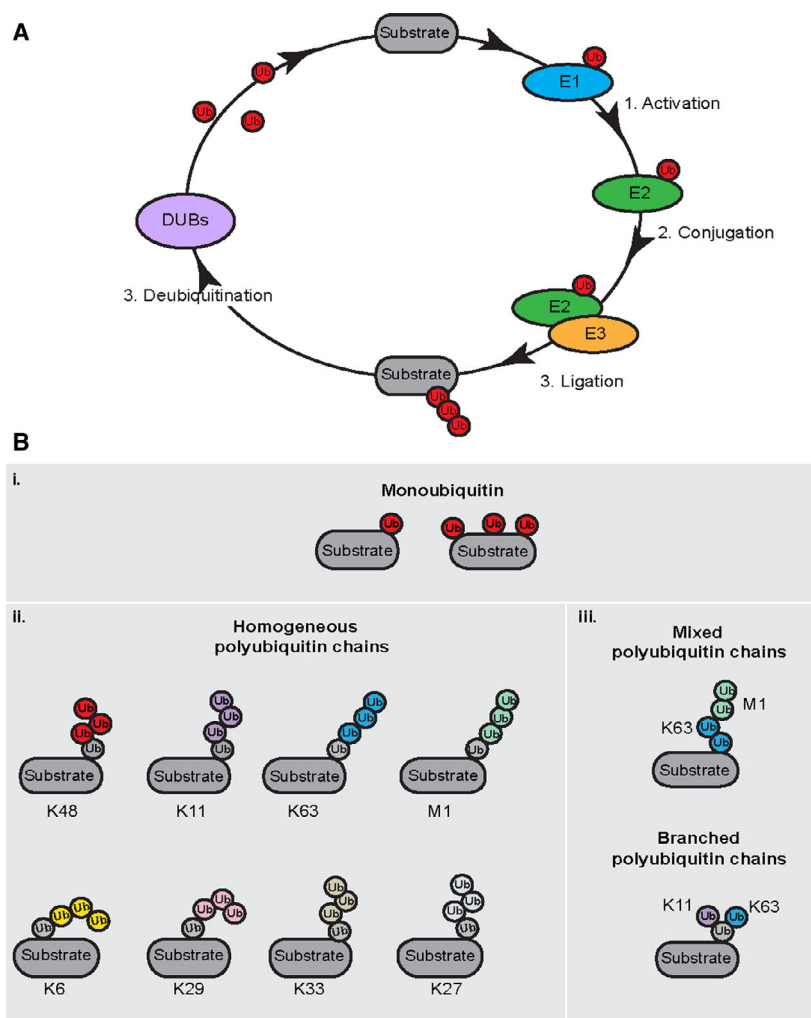


Fig. 1. (A) The ubiquitin system equilibrium. Addition of ubiquitin to a substrate protein is mediated by an E1 ubiquitin-activating enzyme which forms a thioester bond with ubiquitin. Ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme forming an ubiquitin-thioester intermediate that is finally transferred to the substrate protein by an E3 ubiquitin ligase. Deubiquitinase enzymes (DUBs) remove or edit ubiquitin chains from the substrate protein completing the circle of the ubiquitination system. (B) Different types of substrate modification by ubiquitin. Substrates proteins can be (i) by just one ubiquitin either just once or repeatedly in the same protein at different sites (monoubiquitination); (ii) at the same site repeatedly (polyubiquitination). Homogeneous polyubiquitination occurs when the first ubiquitin serves as the acceptor of additional ubiquitins that are conjugated to each other at the very same lysine residue each time (K6, K11, K27, K29, K33, K48, K63 or M1). (iii) Mixed polyubiquitination occurs when additional ubiquitins are conjugated to each other through any of their different lysines.

trafficking and K27 chains are involved in the DNA damage response [10].

Ubiquitination is reversed by a family of proteins called deubiquitinases (DUBs). Deubiquitinases are a family of ~100 members, divided into seven subfamilies. There are 6 thiol-protease families: the ubiquitin-specific proteases (USP); ubiquitin C-terminal hydrolases (UCH); ovarian tumour domain-containing proteases (OTU); Machado–Joseph disease (MJD)/Josephin domain DUBs; MIU-containing novel DUB family (MINDY); and zinc finger-containing ubiquitin peptidase 1 (ZUFSP) and one zinc-metalloprotease group, the JAB1/MPN/Mov34 metalloenzyme family [11–13]. DUBs can release free ubiquitin from Ub precursors (*de novo* Ub synthesis), remove whole polyubiquitin chains and edit ubiquitin chains from their substrates. DUBs are essential to keep the ubiquitin system equilibrium and have to be therefore tightly regulated. Which DUBs bind to which substrates will

depend on cellular localization, the nature of the substrate and the different ubiquitin chains that modify it [14].

Inflammasome activation

A wide range of innate immune cells, including monocytes, macrophages and neutrophils, mediates recognition of danger signals, DAMPs (damage-associated molecular patterns) and MAMPs (microbe-associated molecular patterns) that initiate inflammation. These cells have pattern recognition receptors (PRRs) that sense these signals and trigger the activation of signalling pathways that initiate inflammation. One of the pathways is the activation of the inflammasome which elicits a potent pro-inflammatory cascade key in this initial steps of the inflammatory response [15].

Inflammasomes are protein complexes responsible for the activation of two potent pro-inflammatory

cytokines, interleukin-1 β (IL-1 β) and interleukin-18 (IL-18). Inflammasomes are formed by a cytosolic PRR responsible for sensing the danger signal; caspase-1 responsible for the cleavage and activation of IL-1 β and IL-18 precursors; and in many cases the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD) that facilitates the interaction between the sensor and the effector molecules to assemble the inflammasome complex. Different PRRs assemble different inflammasomes. The type of PRR recruited to the complex will depend on the nature of MAMPs or DAMPs recognized. The best characterized up to date, and the main focus of this review, is the NLRP3 (nucleotide binding and leucine-rich repeat-containing protein 3) inflammasome although there are many others including NLRP1 (NLR family pyrin domain containing 1), NLRC4 (NLR family CARD domain containing 4) and AIM2 (absent in melanoma 2) [4].

Activation of the canonical NLRP3 inflammasome in macrophages is a two-step process [16]. First, there is a priming step that involves activation of the NF- κ B pathway usually by TLR engagement (signal 1) and leads to the upregulation of some of the inflammasome components such as NLRP3 and proIL-1 β . This is followed by an activation step, where the cells sense DAMPs or MAMPs (signal 2) that results in the assembly and oligomerization of the inflammasome complex, the activation of caspase-1 and the consequent cleavage, activation and release of IL-1 β and IL-18. Step 2 is characterized by the induction of potassium efflux [17]. Inflammasome activation also leads to a process of cell death called pyroptosis mainly mediated by the pore-forming protein gasdermin-D upon its cleavage by caspase-1 [18,19] (Fig. 2). It was recently showed that signal 1 also has a non-transcriptional component and that if NLRP3 is already present in a cell, short-term priming with a TLR agonist is enough to render an active inflammasome upon signal 2 independent of transcription [20].

In addition to this canonical pathway, NLRP3 can be activated by other mechanisms. In the so-called noncanonical pathway, intracellular LPS activates caspase-11, which in turn cleaves gasdermin-D forming a membrane pore that leads to cell death and potassium efflux [18,21]. This ion efflux then acts as the signal 2 leading to inflammasome assembly as just described. The third described pathway is called the alternative pathway and has only been found in human monocytes so far. In this case, TLR4 activation with LPS is sufficient to induce NLRP3 activation independently of potassium efflux or ASC speck formation [22].

Post-translational modifications, including phosphorylation, play an important role in the regulation of NLRP3 inflammasome and have been reviewed elsewhere [23,24]. This review will focus on the role of the ubiquitin system in the control of the activation step of the canonical NLRP3 inflammasome, given that there is not much evidence on the role of the ubiquitin system in the regulation of noncanonical NLRP3 activation. How activation of other inflammasomes can be regulated by this PTM will also be discussed. The role of the ubiquitin system in NF- κ B activation (priming step) has been extensively reviewed elsewhere [25] and will not be covered here.

Role of E1-E2-E3 enzymes in canonical NLRP3 inflammasome activation

So far amongst all E1, E2 and E3 enzymes, only E3 ubiquitin ligases have been linked to the regulation of the NLRP3 inflammasome activation, by targeting either the NLRP3 itself or the other components of the inflammasome such as ASC and caspase-1 (Fig. 3).

E3 ligases can prevent inflammasome activation and hence act as negative regulators of NLRP3 inflammasome. One major way of achieving this is by controlling NLRP3 levels by the proteasome or autophagy degradation pathways. TRIM31 has been shown to directly bind to NLRP3, inducing K48 poly-Ub that leads to proteasomal degradation of the NLRP3 [26]. The E3 ligase SCF-FBXL2 also contributes to NLRP3 regulation [27]. SCF-FBXL2 ubiquitinates NLRP3 in the resting state and hence contributes to its degradation by the proteasome. However, upon LPS sensing (signal 1), the levels of the E3 ligase FBXO3 that ubiquitinates FBXL2 and targets it to proteasomal degradation increase. This consequently reduces FBXL2 levels leading to an increase in NLRP3 expression and therefore increased inflammasome activation. Although no direct link to a particular E3 has been yet established, it has been shown that cigarette smoke induces NLRP3 ubiquitination leading to its proteasomal degradation [28]. Similarly, Filardy *et al.* [29] showed that murine colonic macrophages express much lower levels of NLRP3 protein compared to bone marrow-derived macrophages (BMDM), despite levels of mRNA being much higher. This is due to basal NLRP3 levels being controlled by the proteasome since treatment with the proteasome inhibitor MG132 increased NLRP3 expression to levels equivalent to those seen in BMDMs. They also showed that blocking IL-10R in these macrophages restored NLRP3 levels, implying that IL-10 plays a role in such proteasomal control of NLRP3 inflammasome. This

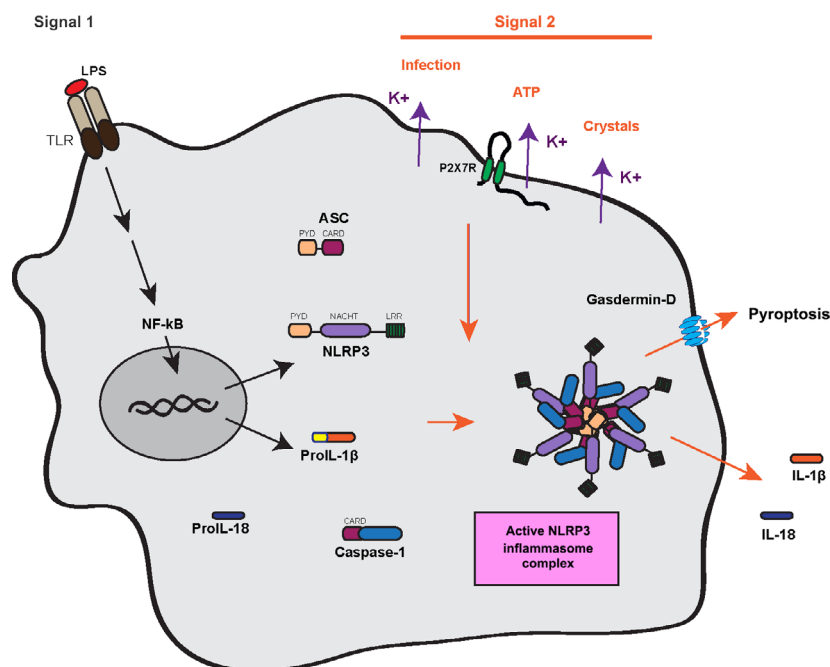


Fig. 2. Mechanism of canonical NLRP3 inflammasome activation. Inflammasome needs a priming signal (signal 1) such as lipopolysaccharide (LPS) that triggers the NF- κ B pathway leading to an increase in the production of proIL-1 β and NLRP3. A second signal, named activation signal (signal 2), is triggered after the recognition of MAMPs and DAMPs through specific receptors such as the purinergic receptor P2X7 that sense extracellular ATP, membrane disruption by bacterial toxins or lysosomal uptake of particulate matter such as monosodium urate crystals. This activating signal triggers a disruption of cell homeostasis that results in increased potassium efflux. This potassium efflux is sensed by the cytosolic receptor NLRP3 that in turn oligomerizes and forms the active complex of the inflammasome after recruitment of ASC and caspase-1. The active inflammasome will cleave procaspase-1 generating active caspase-1 which will lead to the cleavage and the release of the pro-inflammatory cytokines IL-1 β and IL-18. Caspase-1 will also cleave gasdermin-D and that will result in the creation of pores in the membrane. These pores are suggested to be one mechanism of release for IL-18 and IL-1 β . The gasdermin-D pore also contributes to pro-inflammatory cell death known as pyroptosis.

would provide a mechanism for other studies showing that IL-10 negatively regulates the NLRP3 inflammasome [30].

Contribution of the E3 ligase Parkin to NLRP3 regulation has been reported through two different pathways. The first one shows that in the context of polymicrobial sepsis, infection leads to the production of dopamine in wild-type (WT) mice, something impaired in PARKIN-deficient mice as well as in mice lacking the Parkin-upstream mediator PTEN-induced kinase 1 (PINK1). The authors proposed that dopamine controls HGMB1 release via HIF1 α -dependent anaerobic glycolysis and that this also controls NLRP3 levels, and its consequent activation, through proteasomal degradation [31]. The effect of dopamine on NLRP3-mediated degradation has been previously reported. Dopamine through the dopamine D1 receptor (D1DR) leads to the production of cAMP, which in turn binds NLRP3 promoting MARCH7-mediated K48 ubiquitination and its degradation by autophagy [32]. Although classically K63-polyubiquitin chains

mediate autophagy degradation, K48 chains can also lead to degradation by this pathway [33]. Whether this pathway was also involved in PARKIN-mediated NLRP3 regulation has not been investigated.

The second pathway links PARKIN to NLRP3 inflammasome through the DUB A20. PARKIN mediates the induction of A20 that negatively controls the priming as well as the activation steps of the NLRP3 inflammasome as will be discussed later. PARKIN deficiency leads to higher levels of caspase-1 activation and IL-1 β release compared to those in WT cells, an effect that is blocked by the NLRP3 inhibitor MCC950, suggesting this process is linked to NLRP3 nontranscriptional priming [34].

The other major mechanisms of E3s preventing NLRP3 inflammasome activation are by holding the NLRP3 in an inactive state, independently of degradation. The most recent E3 ligase linked to this mechanism is Cullin. This work showed that upon inflammasome priming (signal 1), Cullin1 binds to and ubiquitinates NLRP3, maintaining it in an inactive state. Upon

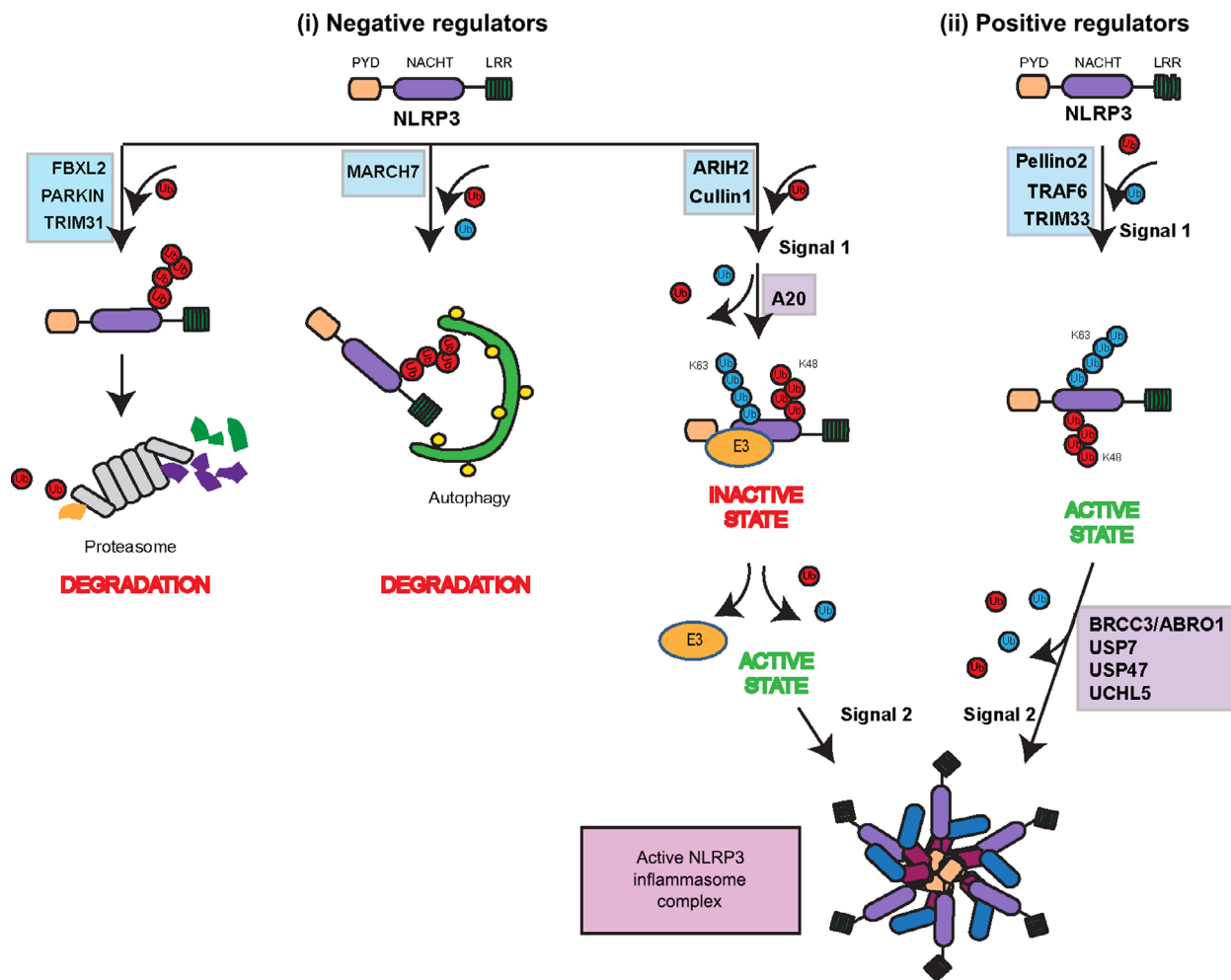


Fig. 3. Regulation of NLRP3 inflammasome by the ubiquitin system. The ubiquitin system contributes to both negative (i) and positive (ii) regulation of NLRP3. (i) E3 ligases such as FBXL2, PARKIN, TRIM31 or MARCH7 have been identified as negative regulators of the inflammasome by controlling its protein levels by either proteasome or autophagy pathways. Upon sensing inflammasome-priming signals (signal 1), ARIH2 and Cullin1 bind and ubiquitinate NLRP3 to maintain it in an inactive state, which prevents its assembly and inflammasome activation. Upon sensing activating signals (signal 2), they dissociate from NLRP3 reducing its ubiquitination and allowing for the formation of an active inflammasome. The deubiquitinase A20 also contributes to a negative regulation of the NLRP3 inflammasome. (ii). On the contrary, ubiquitination of NLRP3 by Pellino2, TRAF6 and TRIM33 upon signal 1 poises the NLRP3 into an active estate that facilitates its activation by signal 2. Upon sensing of these activating signals, deubiquitinases such as BRCC3/ABRO1, USP7, USP47 or UCHL5 contribute to inflammasome activation, although the exact mechanisms by which they do this are not completely understood.

sensing of the activating signal (signal 2; i.e. ATP or the bacterial toxin nigericin), Cullin1 dissociates from NLRP3 allowing it to form an active inflammasome [35]. ARIH2 (Ariadne homolog 2), an E3 ubiquitin ligase of the RBR family, interacts with NLRP3 through its NACHT domain inducing K48 and K63 poly-Ub chains [36]. Knocking out ARIH2 leads to overactivation of the inflammasome. The mechanism of how this occurs is unclear; however, levels of NLRP3 are not affected in ARIH2-deficient macrophages implying this effect is independent of protein degradation.

E3 ligases can also act as positive regulators and promote the activation of the inflammasome. As mentioned above, Juliana *et al.* [20] showed that nontranscriptional priming of the NLRP3 was sufficient to render an active inflammasome. Following this work, several E3 ligases, such as Pellino2, TRAF6 and TRIM33, have been implicated in the nontranscriptional priming step. The most recent is Pellino2. This E3 ligase plays a dual role in NLRP3 regulation. First, it interacts with NLRP3 during the LPS-priming phase facilitating NLRP3 K63 polyubiquitination, a step

required for consequent inflammasome activation upon signal 2. In addition, the authors found that Pellino2 also ubiquitinates IRAK1 and limits its interaction with NLRP3 and consequently the ability of IRAK1 to inhibit NLRP3. In Pellino2-deficient macrophages, this effect is lost, and IRAK1 mediates its blocking effect on NLRP3 activation [37].

TRAF6 has also been implicated in NLRP3 non-transcriptional regulation, in addition to its role in the NF- κ B pathway and hence the transcriptional priming step [38]. Here, a short priming step was used to show that TRAF6-deficient cells had impaired NLRP3 inflammasome activation and that this was caused by the E3 ubiquitin ligase function of TRAF6, although whether TRAF6 acted on NLRP3 or other components of the inflammasome was not described.

Positive regulation of NLRP3 inflammasome by E3 ligases can also be independent of the NLRP3

nontranscriptional priming. This is the case of NLRP3 inflammasome activation by dsRNA present in the cytosol mediated by the interaction between the RNA helicase DHX33, a cytosolic RNA sensor, and NLRP3 [39]. Weng *et al.* [40] showed that TRIM33-mediated K63 polyubiquitination of DHX33 is needed for this interaction and that TRIM33-deficient macrophages abolished the dsRNA-induced NLRP3 inflammasome activation.

Control of the NLRP3 inflammasome complex is also achieved by ubiquitination of the inflammasome adaptor protein ASC (Fig. 4). Similarly to NLRP3, ASC can be negatively regulated by ubiquitination. Shi *et al.* [41] initially reported that K63 polyubiquitination of ASC leads to its degradation by autophagy in a mechanism that keeps both AIM2 and NLRP3 inflammasomes in check. Similarly, it has been shown that far-infrared, used as treatment to ameliorate burn

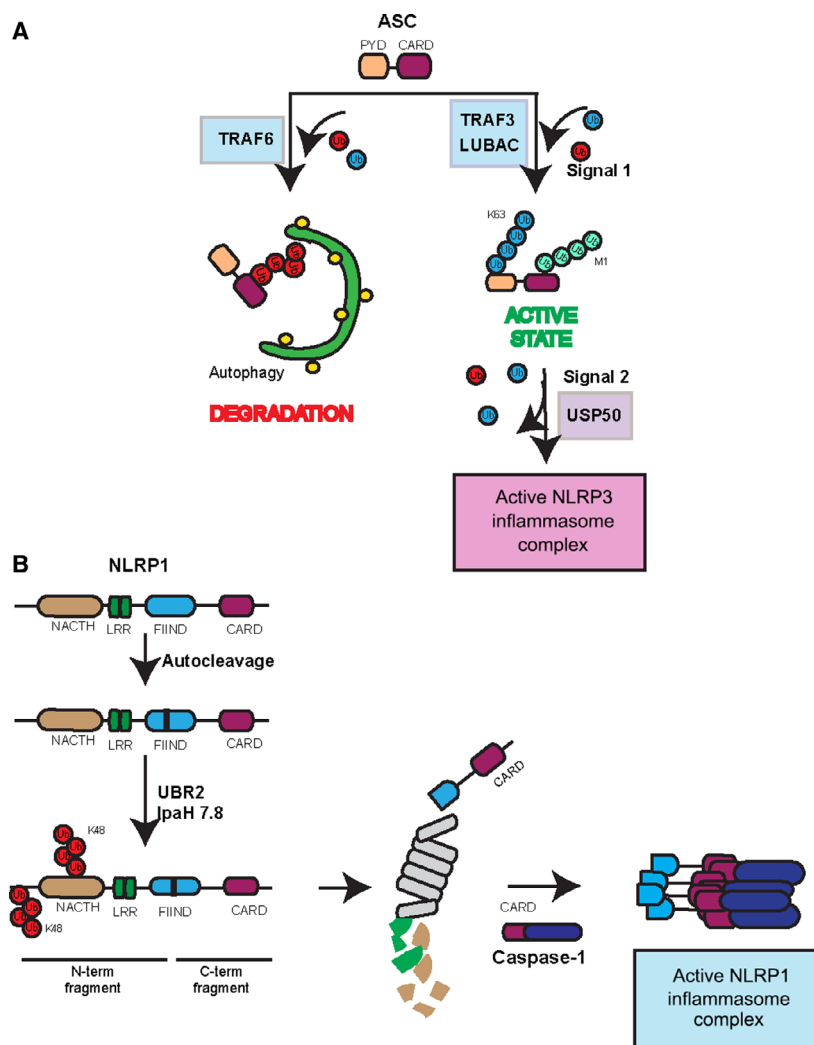


Fig. 4. (A) Regulation of the adaptor protein ASC by the ubiquitin system. Like NLRP3, ASC can be ubiquitinated by TRAF6 and potentially other E3 ligases leading to its degradation by the phagosome and hence negative regulation. TRAF3 and LUBAC on the other hand modify ASC with K63 and M1 polyubiquitin chains, and these modifications are required for successful inflammasome activation. The deubiquitinase USP50 also contributes to NLRP3 inflammasome activation through ASC deubiquitination. (B) Control of NLRP1 activation by the ubiquitin system. Initially, NLRP1 undergoes autocleavage of its FIIND domain. After that, the ubiquitin ligase UBR2 in the presence of the anthrax lethal toxin (Le-Tx) and/or *Shigella* E3 ligase IpaH7.8 modify the NLRP1 N terminus polypeptide which is consequently degraded in the proteasome. This frees the NLRP1 C terminus fragment that contains the CARD domain to oligomerize, recruiting and activating caspase-1.

wound progression, leads to TRAF6-mediated ASC polyubiquitination and its degradation by autophagy in macrophages [42], implying that TRAF6 plays different roles on inflammasome activation depending on the activating stimuli and cell type. Another report has shown that treatment with myricetin, a plant-derived flavonoid, inhibits NLRP3 inflammasome. This compound reduces ASC ubiquitination and increases NLRP3 ubiquitination; however, whether this leads to degradation of one of them or prevents nontranslational priming needs to be further investigated [43]. ASC ubiquitination also acts as a post-translational priming step important for inflammasome activation and regulates the response to some pathogens. For instance, severe acute respiratory syndrome coronavirus ORF3a protein activates the NLRP3 inflammasome by promoting TRAF3-dependent ubiquitination of ASC [44]. Similarly, K63 ubiquitination of ASC also occurs upon MAVS-mediated recruitment of TRAF3 upon vesicular stomatitis virus infection [45].

Linear polyubiquitination of ASC by the linear ubiquitin assembly complex (LUBAC) has been described. LUBAC is formed by HOIP, HOIL and SHARPIN (SHANK-associated RH domain interactor) and regulates the addition of a linear chain to the methionine 1 (M1) of ASC [46]. Macrophages deficient in HOIL-1 show reduced IL-1 β secretion after inflammasome activation induced by NLRP3 activators [47]. In line with this work, it has been shown that SHARPIN is required for activation of the canonical and noncanonical NLRP3 inflammasome, as SHARPIN-deficient macrophages have an impaired NLRP3 inflammasome response. SHARPIN-deficient cells also have an impaired NF- κ B activation and, however, do not show alterations in NLRP3 levels, suggesting that SHARPIN might be directly regulating ASC and not NLRP3 itself although this still needs to be shown [48].

While there is evidence of the contribution of E3 ligases to caspase-1 activation, how this process is controlled is poorly understood. Contrasting functions of the E3 ligase IAPs (inhibitor of apoptosis proteins) in the regulation of procaspase-1 activation have been reported, with positive and negative regulatory functions being described. Labbe *et al.* [49] showed that IAPs are required for inflammasome activation by inducing caspase-1 K63 polyubiquitination that is required for its consequent activation in the inflammasome. They also showed that cIAP1-, cIAP2- or XIAP-deficient mice showed attenuated caspase-1 activation. In contrast to these findings, however, Vince *et al.* [50] showed the simultaneous genetic depletion of cIAP1, cIAP2 and XIAP activated caspase 1 and

proIL-1 β cleavage. This study showed that the absence of these E3 ligases produces receptor-interacting protein kinase 3 (RIPK3)-dependent reactive oxygen species (ROS) generation and is sufficient to activate both the NLRP3 inflammasome and caspase-8. These reports demonstrate both positive and negative regulatory functions of IAPs in caspase-1 activation and highlight the need of further studies to understand these processes.

In addition to IAPs, the LUBAC member SHARPIN also regulates caspase-1, although it is not clear how. Douglas *et al.* showed that SHARPIN-deficient mice presented an overactivation of the inflammasome in the skin and that this was mediated by caspase-1, as genetic ablation of caspase-1 and caspase-11 from SHARPIN-deficient mice significantly reduced skin inflammation. However, when they analysed inflammasome activation in SHARPIN-deficient bone marrow-derived macrophages, they observed a reduction in inflammasome activation compared with WT [51]. These results fit with that described by Gurung *et al.* [48] and highlight the fact that the same protein might be having different roles depending on the cell type. In the context of sepsis, it has been shown that in the lungs, SHARPIN interacts with caspase-1 preventing its activation and hence cleavage and maturation of IL-1 β and IL-18 in a LUBAC-independent manner [52]. Similar to Douglas *et al.*, in these study lungs from SHARPIN-deficient mouse presented higher levels of caspase-1 activation and IL-1 β release compared to WT. These studies highlight the importance of the different cellular environments for activity and function of E3 ligases.

Role of DUBs in canonical NLRP3 inflammasome activation

Deubiquitinases also contribute to NLRP3 inflammasome activation (Fig. 3). Involvement of DUBs as positive regulators of NLRP3 inflammasome was first identified when different DUB inhibitors G5, PR619, bAP15 and WP1130 were shown to block NLRP3-induced caspase-1 activation and IL-1 β release [20,53]. This was quickly followed by the work of Py *et al.* [54], who showed that BRCC3, a JAB1/MPN/Mov34 (JAMM) domain-containing Zn²⁺ metalloprotease DUB that forms part of the BRCC36 isopeptidase complex (BRISC) which specifically recognizes K63-polyubiquitin chains, was required for NLRP3 activation. This work suggested that BRCC3 directly interacted with NLRP3, and its presence was required for appropriate NLRP3 inflammasome formation. Recently, Ren *et al.* [55] showed that ABRO1, another

component of BRISC, is required for optimal NLRP3 activation and that ABRO1-deficient mice have impaired NLRP3 inflammasome responses. ABRO1 interacts with NLRP3 and promotes its deubiquitination allowing the formation of an active inflammasome complex. ABRO1 acts as a scaffold for the interaction of NLRP3 with BRCC3 and synergizes with BRCC3 to promote NLRP3 inflammasome activation by regulating NLRP3 deubiquitination. Furthermore, ABRO1 can deubiquitinate NLRP3 holding one of the mutations (A350V) that lead to its autoactivation and to Muckle–Wells syndrome, one the autoinflammatory CAPS (cryopyrin-associated periodic syndromes) [56]. This suggests that blockade of ABRO1/BRCC3 activity could prevent inflammasome activation in these patients.

Other DUBs that work as positive regulators of the NLRP3 inflammasome are USP7 and USP47, members of the ubiquitin-specific protease DUB family. Enzymatic activity of USP7 and USP47 increases after macrophages are exposed to inflammasome-activating signals (signal 2), suggesting that these signals regulate DUB activity at post-translational level [53]. This study showed that chemical inhibition of USP7 and USP47 blocked inflammasome formation and that although both DUBs contributed to inflammasome activation, its activation was majorly impaired when both USP7 and USP47 were not present. These results suggested a redundant or complementary behaviour of these DUBs in regulating inflammasome activation. Whether USP7 and USP47 directly interact with NLRP3 receptor or with other inflammasome component was not investigated.

Enzymatic activity of UCHL5 (from the ubiquitin C-terminal hydrolase family) is also increased after *Salmonella enterica* serovar Typhimurium infection, and UCHL5 silencing leads to impaired release of IL-1 β after infection [57]. Given that NLRC4 and NLRP3 can contribute to inflammasome activation after *Salmonella* infection, it is not clear to which inflammasome activation does UCHL5 contributes to.

So far, the only DUB described to negatively regulate the inflammasome is A20 (TNFAIP3) that belongs to the otubain protease family. A20 is a key negative regulator of the NF- κ B pathway, whose expression levels are upregulated by activation of this pathway as a negative feedback mechanism [58]. A20 has been implicated in several aspects of NLRP3 inflammasome regulation. A20-deficient macrophages exhibit spontaneous NLRP3 inflammasome activation in response to LPS alone and enhance inflammasome activation in response to soluble and particulate inflammasome activators compared to normal macrophages [59,60].

Vande Valle *et al.* [60] showed that mice deficient in A20 (A20^{myel-KO}), which is a rheumatoid arthritis (RA) susceptibility gene, develop a spontaneous erosive polyarthritis that resembles rheumatoid arthritis in patients. This RA in mice was dependent on NLRP3 inflammasome and IL-1R signalling since A20-deficient macrophages presented enhanced NLRP3 inflammasome and caspase-1 activation, and IL-1 β processing and release. This work showed a role for A20 in the regulation of the inflammasome independent of its role in the priming step. The control of the inflammasome by A20 is not just limited to macrophages. Mice with microglia-specific A20 deletion (A20^{Cx3Cr1 KO}) present impaired microglial regulation and synaptic function. Injection of these mice with a sublethal LPS doses, known to induce neuroinflammation through microglia activation and expression of pro-inflammatory cytokines, led to severe hypothermia and increased mortality, compared to controls. This exacerbated inflammatory response was due to increased microglial NLRP3 inflammasome activation and IL-1 β release [61].

Duong *et al.* dissected the mechanisms of how this happens. They showed that RIPK3 is critical for A20-mediated control of NLRP3 inflammasome. A20 associates with caspase-1 and proIL-1 β in response to signal 1 (LPS), and NLRP3 activation by signal 2 further promotes these interactions preventing inflammasome activation. The authors showed that proIL-1 β interacts not only with caspase-1 and A20, but also with caspase-8, RIPK3 and RIPK1. This complex is associated with K63 polyubiquitination which increases in the absence of A20 in a RIPK3 manner. Finally, the authors showed that proIL-1 β itself is ubiquitinated at K133 and that this ubiquitination is required for cleavage [59]. This suggests that decrease in proIL-1 β ubiquitination by A20 prevents its cleavage explaining why in A20-deficient cells there is an increased IL-1 β release.

Whether the DUB activity of A20 is essential for its anti-inflammatory function is unclear [62]. A recent report has shown that A20 prevents inflammasome-dependent arthritis through its ZnF7 ubiquitin-binding domain. As just discussed [59–61], this work showed that A20-deficient mice (A20^{myc KO}) developed spontaneous arthritis that is critically dependent on RIPK1–RIPK3–MLKL-mediated necroptosis and that was accompanied by IL-1 β and TNF- α release. Further studies on BMDMs further revealed that IL-1 β release was mediated by RIPK3-dependent cell death (necroptosis) and that ZnF7 is critical for this function as LPS stimulation of BMDMs from mice with a mutated ZnF7 domain (A20^{mZnF7/mZnF7}) also induces the release of processed IL-1 β (although at lower levels compared with A20-knockout cells) [63].

Additional evidence of the involvement of DUBs in inflammasome activation has been described. Hwang *et al.* showed that HDAC6 (histone deacetylase 6) directly associates with NLRP3 through its ubiquitin-binding domain and that treatment with the pan-DUB inhibitor PR619 resulted in an elevation of this interaction and a decrease in NLRP3-dependent caspase-1 activation. The authors proposed that HDAC6 negatively regulates the NLRP3 inflammasome through its interaction with ubiquitinated NLRP3 and independently of its deacetylase activity [64] and that DUBs would be required to reduce this interaction and hence allow inflammasome activation. HDAC6 roles independent of its deacetylase activity have been described, one of them being ubiquitin-mediated protein degradation by either the proteasome or autophagy [65]. It is then possible that HDAC6 regulates NLRP3 by inducing its degradation by one of these mechanisms although this was not investigated in this study.

DUBs can also regulate ASC (Fig. 4A). Lee *et al.* [66] showed that the deubiquitinating enzyme, USP50, binds to and deubiquitinates lysine 63-linked polyubiquitin chains of ASC and that this is required for NLRP3 inflammasome activation, since USP50-deficient cells show impaired inflammasome activation. However, based on its predicted structure, USP50 is considered catalytically inactive because it lacks one of the canonical residues involved in catalysis [67]. Hence, whether the role of USP50 on ASC depends on the activity of the DUB or other functions remains to be demonstrated. Unlike for E3 ligases, there is no evidence yet of a role for DUBs in the direct regulation of caspase-1.

Control of other inflammasomes by the ubiquitin system

In addition to the canonical NLRP3 pathway, the ubiquitin system controls the activation of other inflammasomes although how this occurs is less understood.

NLRP1 inflammasome was the first NLR-forming inflammasome discovered [68], and it is unusual compared to other inflammasomes of the NLR family. Firstly, NLRP1 presents its CARD on the C terminus, and secondly, it presents a FIIND (function-to-find domain) which undergoes constitutive self-cleavage that results in two polypeptides that associate in a noncovalent manner and that are required for inflammasome formation [69]. The range of stimuli that trigger NLRP1 activation is limited, with the best characterized being anthrax lethal toxin (Le-Tx). Although processing of the NLRP1 N termini by

Le-Tx was known for a while, it was only recently that the mechanism of activation of NLRP1 has been revealed as a proteasome-dependent process also described as 'functional degradation' [69]. Le-Tx directly cleaves NLRP1 inducing the degradation of the NLRP1 N terminus polypeptide (based on the N-end rule [70]) freeing the NLRP1 C terminus polypeptide that contains the CARD domain to oligomerize, recruiting and activating caspase-1 [71,72]. This work was followed by the work of Xu *et al.*, where they identified the N-end rule ubiquitin ligase UBR2 as the one ubiquitinating NLRP1B to target it for proteasomal degradation in response to Le-Tx [73]. In addition to the N-end rule, it was also discovered that this mechanism of activation is not just limited to proteases and that other effectors that promote NLRP1 proteasomal degradation of the N terminus, such as *Shigella* E3 ligase IpaH7.8 or inhibition of serine dipeptidase DPP8 and DPP9, also lead to NLRP1 inflammasome activation [71,72]. What regulates NLRP1 self-cleavage remains unknown.

Regulation of AIM2 activation by the control of ASC levels by the autophagy pathway has been discussed above [41]. However, AIM2 can also be directly targeted for autophagy degradation in response to viral insults such as poly dA:dT or herpes simplex virus 1 (HVS-1) infection. Under these conditions, TRIM11 binds to AIM2 and undergoes self-ubiquitination at K458, allowing TRIM11 interaction with the autophagy cargo receptor p62 and consequently degrading AIM2 via autophagy [74].

How NLRP7 forms an inflammasome is poorly characterized. NLRP7 is ubiquitinated and degraded by lysosomal pathways in resting conditions. This occurs when ubiquitinated NLRP7 binds to STAM, a transducing adaptor molecule, which interacts with and recruits ubiquitinated cargo to the endosomal pathway for degradation in the lysosome. Bednash *et al.* [75] showed that the DUB STAMPBP (or AMSH) deubiquitinates NLRP7 rescuing this receptor from progressing to lysosome degradation and making it available for the formation of an active inflammasome.

NLRC4 inflammasome activation can also be controlled by ubiquitination, although very little is known about how this occurs. NLRC4 interacts with and is ubiquitinated by SUG-1 (protease regulatory subunit 8 homolog), and this ubiquitination allows for interaction with caspase-8 leading to its activation [76]. In fact, one of the point mutations in NLRC4 that cause autoinflammatory syndromes (H443P) leads to a stronger interaction with SUG-1 and enhanced caspase-8 activation and cell death explaining the mechanisms by

which this mutation leads to enhanced inflammation [77].

Control of interleukin-1 by the ubiquitin system

In addition to the inflammasome components discussed above, the ubiquitin system is key for the regulation of proIL-1 β levels and its activation and release in the inflammasome (Fig. 5). Pro-IL-1 β is ubiquitinated and degraded by the proteasome in BMDC (bone marrow-derived dendritic cells) and BMDM, as this degradation can be prevented by proteasome inhibitors [78]. In fact, the human papillomavirus takes advantage of this process for its survival. The HPV16 E6 oncoprotein, an E3 ubiquitin ligase (E6-AP), promotes IL-1 β ubiquitination that leads to a strong reduction of basal intracellular levels of proIL-1 β in a proteasome-dependent manner that is used by the virus as an innate immune escape mechanism [79].

The E2 ubiquitin-conjugating enzyme UBE2L3 is involved in proIL-1 β ubiquitination that results in its degradation in the proteasome as just described. UBE2L3 is an indirect target of caspase-1; hence, inflammasome assembly and caspase-1 activation lead to UBE2L3 depletion. This depletion will reduce proIL-1 β degradation, therefore increasing its availability to be processed and released as mature IL-1 β .

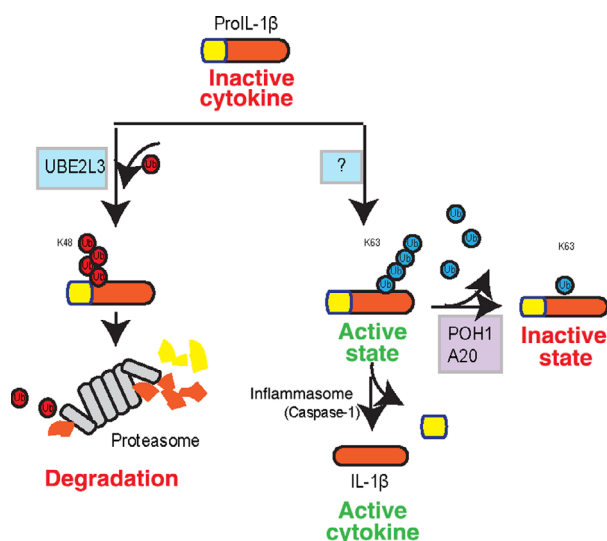


Fig. 5. Interleukin-1 β (IL-1 β) and its ability to be processed by the inflammasome are controlled by the ubiquitin system. ProIL-1 β levels are regulated by the proteasome after its ubiquitination by the E2-conjugating enzyme UBE2L3. K63 polyubiquitination of proIL-1 β at K133 is required for its processing by caspase-1. Deubiquitination by DUBs POH1 and A20 prevents its activation, independently of degradation.

In the absence of signal 2-driven caspase-1 activation, UBE2L3 turns off the highly inflammatory and potentially dangerous proIL-1 β cytokine by reducing its expression levels [80].

Mouse proIL-1 β is K48 and K63 polyubiquitinated at K133 (also conserved in humans), and ubiquitination of this site supports its proteolytic cleavage. A20 can deubiquitinate proIL-1 β hence preventing its activation and adding to the negative regulatory role of this DUB. ProIL-1 β ubiquitination might support its cleavage by encouraging higher order oligomerization of inflammasome components [59]. In line with this work, Zhang *et al.* [81] have shown that POH1, a DUB that forms part of the 19S subunit of the proteasome, interacts with and deubiquitinates proIL-1 β by decreasing the K63-linked polyubiquitin chains, as well as decreasing the efficacy of proIL-1 β cleavage. This supports the need of proIL-1 β polyubiquitination for caspase-1-mediated cleavage.

Conclusion

Post-translational modifications by the ubiquitin system play a fundamental role in the control of the inflammatory response, and in particular in the activation of the inflammasome, which has been the main focus of this review. In the last few years, we have seen much progress in our understanding of how ubiquitin controls inflammasome activation, in particular the NLRP3 inflammasome. Mechanistic insight has come from *in vitro* and *in vivo* studies taking advantage of the use of cellular and mouse models deficient in particular components of the ubiquitin system, as well as of the increasing availability of tools such as selective ubiquitin chain antibodies, tandem ubiquitin-binding entities or activity-based ubiquitin probes [82]. However, we still lack crucial knowledge on several issues. For instance, we do not understand how different poly-Ub chains contribute to NLRP3 conformational changes that shift it to an active state and how deubiquitination of this receptor does allow for inflammasome assembly. Similarly, we do not understand how ubiquitin contributes to the activation of the non-canonical or alternative NLRP3 activation pathways.

Crosstalk between the ubiquitin system and inflammasome can be bidirectional as shown by the work of Eldridge *et al.* [80] that showed that caspase-1 can cleave and inactivate the E2 Ub-conjugating enzyme UBE2L3. It is then possible that other members of the ubiquitin system are also regulated by inflammasome activation, modulating not only inflammasome activity but a wider aspect of the inflammatory response. For instance, the DUBs USP10 and VCIP135 have been identified as

caspase-1 substrates, suggesting that caspase-1 might have additional roles as a regulator of the ubiquitin system during inflammatory episodes [83].

While this review has focused on ubiquitin, there are ubiquitin-like proteins such as SUMO or NEDD8 that can also regulate the inflammatory responses [84]. It has been shown that NLRP3 desumoylation by the SUMO-specific proteases SENP6 and SENP7 promotes NLRP3 activation [85]. Crosstalk between the SUMO and ubiquitin pathways has recently been reported, and future research will be necessary to understand how these modifications regulate inflammasome activation [86].

Although not discussed here, pathogens can hijack components of the ubiquitin system or produce proteins with very similar functions, such as viral DUBs or bacterial E3 ligases, to manipulate the host for their own benefit [84,87]. These result in manipulation of the host ubiquitin system by the pathogen as a mechanism for pathogens to escape from or delay the effects of the inflammatory response. This also includes control of inflammasome activation [88,89]. Despite the importance of the noncanonical NLRP3, NLRC4 and AIM2 inflammasomes in responses to infection, very little is known about how pathogens and the ubiquitin system control activation of these inflammasomes and this could constitute a potential area of future research [90].

Targeting the ubiquitin system for therapeutic purposes is of great interest, in particular after the success of the proteasome inhibitor bortezomib in myeloma treatment [91]. However, there is still much we need to learn about the ubiquitin system before we can successfully target it to treat inflammatory disease. We need to better understand how E1-E2-E3 and DUBs are regulated, especially when present in protein complexes whose composition can vary depending on their location in the cell, cell type or in response to different cellular challenges, for example in disease or during ageing [92]. Hence, understanding the spatial-temporal regulation of these enzymes in health and disease is key, especially if we want to target these in the future. Finally, there are still many DUBs whose function is unknown and new ones that have just been discovered. This highlights how this is an area of research that is still growing and that there are exciting times ahead for new discoveries on the ubiquitin system.

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Conflict of interest

The authors declare no conflict of interest.

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