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Activation and regulation of the inflammasomes

Eicke Latz[1,2,3,4], **T. Sam Xiao**[5], and **Andrea Stutz**[1]

[1]Institute of Innate Immunity, University Hospital, University of Bonn, Bonn 53127, Germany.

[2]Department of Medicine and Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester 01655, Massachusetts, USA.

[3]Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE), Bonn 53175, Germany.

[4]Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim NO-7491, Norway.

[5]Structural Immunobiology Unit, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Bethesda 20892 Maryland, USA.

Abstract

Inflammasomes are key signalling platforms that detect pathogenic microorganisms and sterile stressors, and that activate the highly pro-inflammatory cytokines interleukin-1 (IL-1) and IL-18. In this Review, we discuss the complex regulatory mechanisms that facilitate a balanced but effective inflammasome-mediated immune response, and we highlight the similarities to another molecular signalling platform — the apoptosome — that monitors cellular health. Extracellular regulatory mechanisms are discussed, as well as the intracellular control of inflammasome assembly, for example, via ion fluxes, free radicals and autophagy

A major role of the immune system is to maintain homeostatic tissue function. For example, sterile tissue damage that occurs after trauma needs to be detected and repaired. Pathogens that can invade and cause harm to tissues should be eliminated while our commensal microbiome must be tolerated, as it fulfils functions that are required for host survival. The innate immune system has a number of signalling receptors that recognize foreign molecular structures as well as self molecules that are altered, that have become too abundant or that emerge in areas normally devoid of these molecules[1,2].

Innate immune signalling receptors monitor the extracellular space as well as many subcellular compartments for signs of infection, damage or other cellular stressors. The inflammasomes are a group of multimeric protein complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and caspase 1. Inflammasome formation is triggered by a range of substances that emerge during infections, tissue damage or metabolic imbalances. Once the protein complexes have formed, the inflammasomes activate caspase 1, which proteolytically activates the pro-inflammatory cytokines interleukin-1 (IL-1)[3] and IL-18. In addition, inflammasome activation causes a rapid, pro-inflammatory form of cell death called pyroptosis[4].

Correspondence to: E.L. eicke.latz@uni-bonn.de.

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FURTHER INFORMATION

Eicke Latz's homepage: http://www.iii.uni-bonn.de/

With the discovery of pattern recognition receptors (PRRs), such as inflammasome sensor molecules, their signalling pathways and their ability to programme cellular immune responses, we are beginning to understand how the immune system protects the host at a molecular level. At the same time, we have learnt that immune dysregulation contributes to prevalent diseases in Western societies such as atherosclerosis, type 2 diabetes, cancer and neurodegenerative diseases. Thus, a fine balance must be maintained between the activation and inhibition of inflammation to allow the immune system to remove any sources of danger without causing harm to the host.

In this Review, we present an overview of the current understanding of inflammasome activation and regulation, and we discuss the recent findings about non- canonical processing of IL-1 . We also compare the structures and the regulation of inflammasomes with that of the apoptosome.

The inflammasomes and their co-receptors

Inflammasome components

At first glance, the inflammasomes are organized in a very simple manner: inflammasome sensor molecules (see below) connect to caspase 1 via ASC, which is an adaptor protein encoded by *PYCARD* that is common to all inflammasomes. ASC consists of two death-fold domains: one pyrin domain and one caspase activation and recruitment domain (CARD). ASC interacts with the upstream inflammasome sensor molecules via the pyrin domain[5]. This interaction triggers the assembly of ASC into a large protein speck consisting mainly of multimers of ASC dimers[6,7]. Using its CARD, ASC brings monomers of pro-caspase 1 into close proximity, which initiates caspase 1 self- cleavage and the formation of the active heterotetrameric caspase 1. Active caspase 1 proteolytically activates a number of proteins[8], including pro-IL-1 and pro-IL-18 (REFS 9,10), and induces their release via a non-classical secretion pathway[11].

The transcription of pro-IL-1 is induced by the activation of the transcription factor nuclear factor- B (NF- B), whereas pro-IL-18 is constitutively expressed and its expression is increased after cellular activation. Therefore, these potent pro-inflammatory cytokines are controlled by two checkpoints: transcription as well as maturation and release[11]. Caspase 1-mediated activation of members of the IL-1 cytokine family leads to the recruitment and the activation of other immune cells, such as neutrophils, at the site of infection and/or tissue damage.

Several inflammasome sensor molecules can trigger the formation of inflammasomes. Most of the inflammasomes that have been described to date contain a NOD-like receptor (NLR) sensor molecule, namely NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7, NLRP12 or NLRC4 (NOD-, LRR- and CARD-containing 4; also known as IPAF). The NLR proteins, with the exception of NLRP1, have a tripartite domain organization; they contain an amino-terminal death-fold domain (NLRPs contain a pyrin domain, whereas NLRC4 contains a CARD), a central NACHT nucleotide-binding domain and carboxy-terminal leucine-rich repeats (LRRs)[12]. The NACHT domain has ATPase activity and is thought to have a role in the oligomerization of the proteins, whereas the LRRs have regulatory functions and might be involved in ligand interaction. The death-fold domains of the NLR proteins interact with those of ASC and/or caspase 1. In addition to these domains, human NLRP1 contains a function-to-find domain (FIIND) and a C-terminal CARD. In the original description of the inflammasome, human NLRP1 was shown to recruit and to activate an additional inflammatory caspase, namely caspase 5, via its CARD[3].

NLRC4 and NLRP1 can both activate caspase 1 through their CARDs without recruiting ASC; however, the recruitment of ASC greatly enhances the formation of the complex and the processing of IL-1 [7,13–16]. Exactly how ASC is recruited to these inflammasomes remains unclear, as NLRC4 and mouse NLRP1B do not have pyrin domains. In a mammalian two-hybrid analysis, the CARD of NLRC4 was found to interact with the CARD of ASC[17]. We speculate that a CARD–CARD interaction between the NLR and ASC recruits a first layer of ASC, which in turn interacts with a second layer of ASC via pyrin–pyrin domain interactions.

Two other inflammasomes have been described that contain the PYHIN (pyrin and HIN domain-containing protein) family members absent in melanoma 2 (AIM2) and IFN inducible protein 16 (IFI16) rather than an NLR[18]. AIM2 consists of a pyrin domain to recruit ASC and a DNA-binding HIN domain, whereas IFI16 has one pyrin domain and two HIN domains for DNA binding. Retinoic acid-inducible gene I (RIG-I) protein is also thought to assemble an inflammasome with ASC and caspase 1 (REF. 19), possibly via its CARDs. However, for some of the inflammasome sensors (including NLRP6, NLRP12, RIG-I and IFI16), the potential to form inflammasomes has not been well established and other functions have been described for these molecules. Indeed, NLRP12 can function as a positive regulator of dendritic cell migration or as a negative regulator of non-canonical NF-B signalling[20,21], and NLRP6 can negatively regulate innate immunity[22]. RIG-I is widely known as a PRR that senses RNA and that signals via mitochondrial antiviral signalling protein (MAVS) to induce an interferon (IFN) response[2], and IFI16 has been suggested to be a DNA sensor that signals via the protein STING (stimulator of IFN genes; also known as TMEM173) to generate an IFN response[23].

Numerous activators of the inflammasomes and several different activation pathways have been described (reviewed in REF. 24). The PYHIN proteins and RIG-I recognize nucleic acids[18,19,25], whereas NLRC4 is activated by microbial proteinaceous ligands[26]. NLRP1 recognizes muramyl dipeptide, which is a bacterial peptidoglycan, and murine NLRP1B can also be activated by the lethal toxin from *Bacillus anthracis*[13,27]. Many triggers, including crystalline material, peptide aggregates and bacterial toxins, can stimulate NLRP3 (REF. 24). NLRP7, which is not expressed in mice, is activated by bacterial lipo-peptides[28], and the triggers for NLRP6 and NLRP12 remain to be identified[29–31].

In summary, the inflammasome sensor molecules can detect a broad range of molecular signatures to sense microorganisms and tissue stress. They are best known for triggering a robust inflammatory response via the activation of inflammatory caspases. However, it should also be noted that not all NLR molecules form inflammasomes and that other functions of NLRs, which are not as well understood, might be important contributors to an inflammatory response.

Cofactors for inflammasome activation

Some inflammasome sensor molecules require co-receptors to recognize their ligands or to be stabilized in their activated states. NLRP1 has been shown to bind directly to its ligand muramyl dipeptide *in vitro* and this was demonstrated to be sufficient to activate the assembly of an inflammasome. However, a requirement for the interaction of NLRP1 with nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which is another receptor for muramyl dipeptide, has been described. This suggests that NOD2 is a vital component of the NLRP1 inflammasome[32,33].

Recent studies by the groups of Vance[34] and Shao[35] have determined that the activation of NLRC4 also requires co- receptors[34,35]. In mice, NAIP (NLR family, apoptosis inhibitory protein) family proteins sense the proteinaceous NLRC4 activators and, in turn, activate the

assembly of the NLRC4 inflammasome. NAIP5 and NAIP6 have been shown to detect flagellin, and NAIP2 has been shown to sense the type III secretion system component PrgJ[34,35]. Only one NAIP orthologue has been found in humans (known as NAIP) and this is necessary to sense a type III secretion needle protein[35]; however, it remains to be determined whether and how human NLRC4 senses flagellin without another NAIP homologue. Some human cell lines (for example, U937) do not respond to flagel-lin[35]. However, studies using primary human cells that were infected with *Legionella pneumophilia* have determined that small interfering RNA (siRNA)-mediated knockdown of *NLRC4* results in enhanced bacterial growth only when *L. pneumophilia* expresses flagellin[36], which indicates that human NLRC4 might be able to sense flagellin. It is possible that NAIP could have dual specificity in humans and that it could recognize both a type III secretion needle protein[35] and flagellin[36]. Dual specificity could also be used by other inflammasome sensors that recognize a variety of structurally and chemically diverse activators.

Differential requirements for various NLRP3 activators

NLRP3 is unique among the NLRs in that its basal expression is not sufficient for inflammasome activation in resting cells[37,38]. Similarly to IL-1 generation, a two-checkpoint activation mechanism is in place for NLRP3 inflammasome activation; it requires a priming step (see below) and a second activation step that can be induced by various triggers. Given the broad range of NLRP3 activators[24], direct binding of these activators to NLRP3 seems to be unlikely. Indeed, as for NLRC4, it is possible that ligand specificity can be achieved via recruitment of different cofactors. There is evidence to support this theory, as the formation of the NLRP3 inflammasome in response to non-crystalline activators can be promoted by guanylate-binding protein 5 (GBP5)[39]. In addition, oxidized mitochondrial DNA, which is released into the cytosol under cellular stress and is a possible NLRP3 inflammasome activator, was recently reported to interact with NLRP3 (REF. 40) (BOX 1). However, whether NLRP3 directly interacts with oxidized mitochondrial DNA or whether a cofactor is required for this interaction remains unknown.

The activation of caspase 1 following the recognition of live Gram-negative bacteria depends on inflammatory pro-caspase 11 in mice (human orthologues are pro-caspase 4 and pro-caspase 5)[41], which results in `non-canonical NLRP3 inflammasome` activation. Pyroptosis in response to live Gram-negative bacteria but not in response to other inflammasome triggers is entirely dependent on caspase 11, whereas caspase 1 is dispensable for this process[41]. Therefore, in contrast to the standard two-checkpoint model of priming and activation, three signals are required for full NLRP3 inflammasome activation in response to Gram-negative bacteria[42,43]. First, a bacterial Toll-like receptor (TLR) activator leads to cellular priming and upregulation of NLRP3 and pro-IL-1 expression (the priming checkpoint in the standard model)[37,38]. The second ‘activation checkpoint’ can be divided into two parts in response to bacteria. One checkpoint is that bacterial mRNA from live bacteria (also known as vita-PAMPs)[44] activates NLRP3; the other checkpoint is that TLR4- and TRIF (TIR domain-containing adaptor protein inducing IFN -dependent signalling — which is triggered by bacterial lipopolysaccharide (LPS) — mediate the secretion of type I IFNs, inducing pro-caspase 11 expression and activation by triggering the IFN / receptor (IFNAR) (FIG. 1).

The mechanisms by which caspase 11 is required to activate caspase 1 in response to bacteria are still under investigation and the exact timing of the different signals is not yet clear. In the context of NLRC4 activation, caspase 11 was shown to interact with cofilin. This interaction regulates actin polymerization and triggers the fusion of bacteria-containing phagosomes with lysosomes. These events facilitate the release of flagellin into the

cytosol[45], where NAIP family proteins can interact with flagellin and induce the activation of the NLRC4 inflammasome. It is possible that a similar caspase 11-dependent release of bacterial mRNA from the phagolysosomal compartment could mediate the activation of the NLRP3 inflammasome.

There is also a debate about how pro-caspase 11 expression is induced. One study found that the expression completely depends on IFN signalling[42], whereas another study suggested that its expression could be induced independently of TRIF and IFN signalling[46]. Even if the expression of pro-caspase 11 could be induced independently of IFN signalling, caspase 11 activation in macrophages in response to *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection is dependent on IFNAR1 as well as signal tranducer and activator of transcription 1 (STAT1)[46]. Therefore, an IFN-inducible factor might be required for the activation of caspase 11 during *S.* Typhimurium infection[46].

Another recent study suggests a role for caspase 11 in defence against cytosolic bacteria that is independent of the known inflammasomes. These data have led to the development of a model in which a non-canonical caspase 11-containing inflammasome can be formed after cytoplasmic bacteria are sensed, which leads to pyroptosis but not to IL-1 release[47]. Further investigation is needed to determine when this complex is formed, under which circumstances caspase 11 activation will lead to NLRP3 activation and when it will cause cell death independently of the 'canonical' inflammasomes. A recent review discusses caspase 11 activation in more detail[48].

Non-inflammasome processing of IL-1β

The inflammasomes and IL-1β processing in vitro *and* in vivo

Studies that have been carried out *in vitro* using cells that are deficient in inflammasome components have undoubtedly been instrumental in identifying several inflammatory triggers as activators of inflammasomes. However, the contribution of inflammasomes to IL-1 activation *in vivo* has not, at times, been nearly as convincing. The reduction in inflammation in mice that are deficient in the inflammasome components ASC or caspase 1 can be much less pronounced than that in mice lacking the IL-1 receptor (IL-1R) or IL-1 . It is probable that inflammasome-independent IL-1 activation mechanisms are involved *in vivo* that could account for the observed differences between the *in vitro* and *in vivo* studies. For example, the *in vivo* responses to particulates (diesel exhaust particles or silica) and to *Mycobacterium tuberculosis* have substantial caspase 1-independent but IL-1 dependent components[49–51]. Indeed, a number of non-caspase proteases such as proteinase 3 have the ability to activate IL-1 in an inflammasome-independent manner[11].

In addition, IL-1 , which also activates IL-1R, could contribute to the inflammatory response *in vivo*. This could partly explain why IL-1R–deficient mice have a more severe phenotype than inflammasome component-deficient mice, in which only IL-1 production is affected. In contrast to IL-1 , IL-1 is constitutively expressed in many cell types and does not need to be processed for its biological activity; thus, biologically active IL-1 can be released during necrosis[11]. In addition, IL-1 can be found on the membrane of some cell types, where it is active[11]. IL-1 is also secreted in response to numerous NLRP3 inflammasome activators; however, its secretion is not strictly dependent on the inflammasome. Indeed, particulate activators of NLRP3, such as crystals, can induce IL-1 secretion in an NLRP3-independent manner[52]. Therefore, the discrepancies between the *in vivo* and *in vitro* data may be partially explained by inflammasome-independent processing of IL-1 and by the contribution of IL-1 , the secretion of which is regulated differently from that of IL-1 release.

The role of caspase 8 in IL-1β processing

One additional factor that can mediate IL-1 processing and activation is caspase 8 (FIG. 1). Recent studies indicate that caspase 8 might be able to cleave pro-IL-1 during immune responses. For example, following sensing of fungal components by the PRR dectin 1 that is expressed on human dendritic cells, signalling via the kinase SYK leads to the formation of a complex that is composed of caspase 8 and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1); this complex binds to ASC, which possibly recruits cleavage substrates[53]. The MALT1-ASC-caspase 8 complex directly mediates IL-1 maturation[53]. Thus, caspase 8 can be activated by a multiprotein complex that contains the canonical inflammasome adaptor ASC — this MALT1-ASC-caspase 8 inflammasome protein complex has therefore been termed a 'non- canonical caspase 8 inflammasome'. However, exactly how MALT1, caspase 8 and ASC interact is not understood, as these proteins all have different death-fold domains. In addition, NLRP3 has been implicated in dectin 1-mediated IL-1 release in mice[54] and it therefore remains to be established whether both of these mechanisms can function in parallel or whether they represent differences between the two species.

Other PRRs can also activate caspase 8. In the presence of the translation inhibitor cycloheximide, TRIF signalling that is downstream of TLR3 or TLR4 leads to pro-IL-1 processing by caspase 8 (REF. 55). Furthermore, in response to genotoxic stress, the `ripoptosome` assembles and activates caspase 8. The ripoptosome contains caspase 8, FAS-associated death domain protein (FADD) and receptor-interacting protein 1 (RIP1; also known as RIPK1), and forms spontaneously in response to loss or inhibition of the anti-apoptotic proteins X-linked inhibitor of apoptosis protein (XIAP), inhibitor of apoptosis protein 1 (IAP1; also known as BIRC3) and IAP2 (also known as BIRC2) (REF 56). The formation of this complex activates RIP3, which is necessary for the cleavage of pro-IL-1 by both the NLRP3-caspase 1 and the caspase 8 pathways[56] (FIG. 1). However, two other studies contradict these findings. One study showed that the NLRP3 inflamma some is more active in caspase 8-deficient dendritic cells, which indicates that caspase 8 might limit ripoptosome signalling that would otherwise trigger NLRP3–caspase 1 activation[57]. Another study found that IAP1, IAP2 and TNF receptor-associated factor 2 (TRAF2) are important for the non-degradative polyubiquitylation of caspase 1, which enhances its activity[58].

Other caspase 8-activating receptors of the tumour necrosis factor receptor (TNFR) family can also induce IL-1 activation. Indeed, activation of the TNFR family member CD95 (also known as FAS) can induce IL-1 processing that is independent of inflammasomes and of caspase 1 (REF. 59). In addition, it was recently shown that CD95 signalling mediates IL-1 and IL-18 processing through the activation of caspase 8 (REF. 60) (FIG. 1). Thus, more non-inflammasome pathways for the maturation of pro-IL-1 are emerging, and these pathways could contribute to the effects of IL-1 activation *in vivo*.

Cell-extrinsic inflammasome regulation

Cell-autonomous regulatory feedback loops, as well as complex networks of direct cell–cell signals and indirect signals via messenger substances, regulate cellular activation. Many important immune responses can only be efficiently induced when two or more signals act on the responding cell in a timely manner — for example, as has been described for IL-1 maturation and NLRP3 activation. One level at which inflammasomes are regulated is at the level of expression of the individual inflammasome components[61].

Positive regulation and priming

Many innate immune signalling or cytokine receptors, such as the TNFR, activate transcription of *NLRP3* and thereby influence the susceptibility of immune cells to NLRP3 inflammasome triggers[37,38] (FIG. 2). In addition to the transcriptional regulation of *NLRP3*, deubiquitylation of NLRP3 has been identified as an early priming event, which only occurs in response to PRR stimulation, possibly involving the production of reactive oxygen species (ROS)[62]. NLRP3 deubiquitylation is mediated by the K63-specific deubiquitinase BRCC3 (REF. 63). Therefore, the NLRP3 activation threshold is regulated by both a fast-acting post-translational mechanism and a slower-acting transcriptional activation of *NLRP3* gene expression.

An early priming event has also been described for the AIM2 inflammasome, although the molecular mechanisms that are involved remain to be fully elucidated[64]. In addition, *AIM2* expression can be induced by IFN and IFN [65,66]. NLRC4 can only be activated by *S.* Typhimurium after a specific serine residue has been phosphorylated by protein kinase C (PKC encoded by *PRKCD*), which depends on the recognition of both flagellin and the type III secretion system. This indicates that PKC might be activated by PRR signalling[67]. Therefore, at least some inflammasomes are only fully capable of responding to danger signals in situations in which either pro-inflammatory cytokines are present or innate signalling molecules sense noxious signals.

A few molecules, such as `amyloid-β`, can induce both NLRP3 priming through TLR activation and NLRP3 inflammasome activation[68]. However, the response to aggregated amyloid- can be dramatically augmented when innate sensors or cytokine receptors become activated by additional stimuli. These priming mechanisms might be of great relevance in determining the magnitude of an inflammatory response to danger signals.

Conversely, genetic differences that influence the threshold of inflammasome activation might contribute to the development of chronic inflammatory or autoinflammatory diseases. It is known that about 60% of patients with `cryopyrin-associated periodic syndrome` (CAPS) carry activating mutations in the coding sequence of *NLRP3* itself or in other inflammasome components[69]. This indicates that inflammasome hyperactivity may be influenced by additional mechanisms. Indeed, single nucleotide polymorphisms in the promoter region of *NLRP3* were found in patients with CAPS[69]. In these patients, an increase in *NLRP3* expression as opposed to the presence of a more active protein (as a result of mutations in the coding sequence) could conceivably result in a lower threshold of NLRP3 inflammasome activation in response to danger signals and this could trigger the development of CAPS. Therefore, several mechanisms ensure that inflammasomes are not accidentally triggered and that an appropriately balanced immune response to danger signals can occur in a regulated manner.

Negative regulation of inflammasome activation

An inflammatory response should subside once it has carried out its function. For example, the release of proinflammatory cytokines such as IL-1 and IL-18 might not be required after an adaptive immune response has been initiated. Thus, it is not surprising that effector and memory $CD4^+$ T cells have the capacity to inhibit the activation of the NLRP1 and NLRP3 inflammasomes in a contact-dependent manner, possibly via TNFR superfamily molecules such as CD40 ligand (CD40L)[70]. In addition, T cell-derived IFN has been shown to downregulate the activity of NLRP3 via activation of inducible nitric oxide synthase (iNOS) in a mouse model of tuberculosis[71]; nitric oxide (NO) induces NLRP3 nitrosylation and thereby inhibits NLRP3 activity. However, it seems to be crucial that TLR

priming and IFN stimulation occur simultaneously, as IFN loses its inhibitory activity after sequential priming with IFN and a TLR stimulus[70,72].

In addition, type I IFNs can reduce IL-1 and IL-18 release by functioning at two levels. First, type I IFNs inhibit the production of the pro-forms of these cytokines; second, they repress the cleavage of the cytokine proforms by the NLRP1 and NLRP3 inflammasomes[72]. For IFN , the mechanism of NLRP3 inhibition might also involve the induction of NO[73]. Timing is also important for the inhibitory effect of IFN ; although LPS-induced IFN is necessary for caspase 11 expression and, thus, NLRP3 activation in response to bacteria[42] (see above), IFN blocks NLRP3 inflammasome activation when the cells sense it before they have been primed, for example, by a TLR stimulus[72]. It is worth noting that IFNs can have seemingly opposing effects on different inflammasomes. IFNs upregulate AIM2 expression but they downregulate IL-1 expression and inhibit the NLRP3 inflammasome. These effects could be adapted to generate optimal antiviral responses, in which excessive responses to danger signals could be harmful for the host.

Furthermore, the amount of *NLRP3* mRNA is tightly regulated by the `microRNA` miR-223, which leads to decreased NLRP3 protein levels and, thus, influences the threshold of NLRP3 activation[74,75]. Pro-inflammatory signals do not regulate miR-223; rather, miR-223 is expressed in a cell type-specific manner in innate immune cells — the lowest levels of expression are found in dendritic cells and the highest levels of expression are found in neutrophils. This might explain why dendritic cells have a comparatively low threshold for NLRP3 stimuli.

Taken together, these studies show that the inflammasome-mediated response can be adjusted by the activities of cell-extrinsic negative regulators and amplifiers. This ‘rheostat’-like fine-tuning facilitates the adjustment of inflammasome activation in response to danger signals and indicates that both systemic signals and the tissue context might influence the threshold of inflammasome activation (FIG. 2). Thus, priming and negative regulation might strengthen or limit the local response, respectively. In fact, it is conceivable that the effectiveness of certain anti-cytokine therapies that are used to treat chronic inflammatory diseases is partly mediated by alterations in the local responsiveness of inflammasomes to danger signals. Extrinsic regulators of inflammasome activity can also affect the overall responsiveness of the cell by influencing the cell-intrinsic regulatory mechanisms of inflammasome activation, which are described in the following section.

Cell-intrinsic inflammasome regulation

When the function of a cell is compromised or the survival of the cell poses a danger to the whole organism, it should be eliminated. As mentioned above, inflammasomes are localized in the cytosol and can be activated when microbial molecules are generated in this compartment. It is crucial that pathogens or protein aggregates that have been phagocytosed are subsequently neutralized in the phagolysosomal compartment. However, if the degradative function of lysosomes is defective or if the capacity of phagocytosis is overwhelmed, lysosomal damage occurs. This can activate the NLRP3 inflammasome, possibly involving the activity of lysosomal proteases[76]. In addition, the integrity of mitochondria is monitored by inflammasomes and apoptosomes, and mitochondrial stress can lead to inflammation and cell death (BOX 1). Indeed, inflammasomes (which induce pyroptosis through caspase 1 or caspase 11 activation) and apoptosomes (which activate caspase 9 in response to cytochrome *c* release from mitochondria) are two mechanisms by which compromised cells are eliminated. In order to be activated, the complexes need to oligomerize (BOX 2); the resulting different receptor complexes share many similarities in their regulation in response to cellular stress (discussed below).

Regulation by ion fluxes, oxidative state and autophagy

Much of a cell's energy is used to maintain ion gradients between the cytosol and the extracellular environment. Many enzymes and signalling pathways are regulated by dynamic changes in ion concentrations. In addition to its crucial role in cellular signalling, an intact ion gradient in healthy cells represents a mechanism that protects against cell death.

Indeed, the binding of cytochrome *c* that has been released from mitochondria to apoptotic protease-activating factor 1 (APAF1) and the subsequent assembly of the apoptosome only occurs when subphysiological K^+ concentrations are reached in compromised cells[77,78]. Similarly, activation of the NLRP1B and NLRP3 inflammasomes depends on low K^+ concentrations in intracellular compartments[79], and a low K^+ concentration promotes the assembly of the ASC speck[6]. In addition, high extracellular levels of K^+ can block IL-1 release after NLRC4 and AIM2 inflammasome formation[80,81], which indicates that low intracellular K^+ levels might also be required for the activation of these inflammasomes. However, the concentrations of K^+ that are necessary to block NLRC4 and AIM2 inflammasome formation are higher than those that are needed to block NLRP3 inflammasome formation. Interestingly, for NLRP7, a high extracellular K^+ concentration only slightly reduces IL-1 release[28]. Why some inflammasomes are more sensitive to high extracellular concentrations of K^+ than others is not understood. It was thought that the danger signal ATP and bacterial pore-forming toxins, which activate the NLRP3 inflammasome, could directly mediate K^+ efflux through the hemichannel pannexin 1 (REFS 82–84); however, pannexin 1-deficient mice do not show diminished caspase 1 activation[85]. Thus, the mechanisms by which several inflammasome triggers can induce K^+ efflux remain unclear.

In addition to the requirement for decreased K^+ levels, osmotic pressure regulates NLRP3 inflammasome activation[86]. Cells that have been subjected to hypotonic solutions undergo cellular swelling concomitant with a decrease in intracellular K^+ and Cl^- concentrations. Interestingly, cell swelling induces a regulatory volume decrease response through transient receptor potential cation channels (TRPM7 and TRPV2) that triggers intracellular Ca^{2+} mobilization. The mobilized Ca^{2+} has many molecular targets, including TGF activated kinase 1 (TAK1; also known as MAP3K7) (REF. 86). Although TAK1 might have a role in a PRR-dependent non-transcriptional priming pathway[87] — that is, in the signalling pathway that leads to the deubiquitylation, for example, of NLRP3 — further investigation into its role is required. Another transient receptor potential channel — TRPM2 — has also been implicated in NLRP3 activation in response to crystalline substances[88]. This channel senses intracellular ROS and responds by opening itself to facilitate Ca^{2+} influx into the cell; this is intriguing considering that both ion fluxes and the oxidative state (see below) have important roles in NLRP3 inflammasome activation.

Another regulator of Ca^{2+}, namely C/EPB-homologous protein (CHOP; also known as DDIT3), has been implicated in NLRP3 inflammasome activation[89]. However, the activation of CHOP following the induction of the unfolded protein response via inhibition of translation is insufficient for NLRP3 inflammasome activation[90] and, therefore, the exact role of CHOP in NLRP3 inflammasome activation is unclear. Two recent studies have implicated calcium-sensing G-protein coupled receptors (GPCRs) in the activation of NLRP3: calcium-sensing receptor (CASR) and GPRC6A signal via G_{i} and G_{q}, which inhibit adenylyl cyclase and activate phosphorlipase C, respectively[91,92]. Thus, sensing of extracellular Ca^{2+} leads to reduced cyclic AMP (cAMP) levels through adenylyl cyclase inhibition and to increased cytoplasmic Ca^{2+} concentrations via the activation of phospholipase C. It is worth noting that the GPCR platelet activating factor receptor can also stimulate inflammasome formation[92], which indicates that other GPCR pathways could control inflammasome activation. The role of cAMP in inflammasome activation remains

unclear as one study has reported that cAMP can directly inhibit NLRP3 (REF. 91), whereas another study reported that cAMP levels had no direct influence on inflammasome activation[92]. Taken together, these studies show that NLRP3 activation is regulated by the ion content of the cell (FIG. 3); however, the molecular targets and the mechanisms by which ion fluxes regulate NLRP3 remain to be fully elucidated.

The redox state of the cell is another important indicator of the viability of cells, and many signalling pathways are influenced by changes in the redox state. In particular, ROS facilitate the assembly of the apoptosome in several ways. For example, oxidative modifications to caspase 9 enable its recruitment to the apoptosome[93]. By contrast, nitrosylation of caspase 9 inhibits its function[94]. Similarly, caspase 1 can be inhibited by nitrosylation[95], which suggests that modification by reactive molecules is a general mechanism for the regulation of caspase activity. The exact role of the redox state in NLRP3 inflammasome activation remains controversial. Originally, it was thought that ROS produced by NADPH oxidases following the phagocytosis of crystals activate the NLRP3 inflammasome[96–98]. However, studies that have been carried out either using cells that were deficient in NADPH protein components or using cells from patients with defective NADPH oxidase subunits have challenged this idea by showing that these patients have normal, or even increased, caspase 1 activity[99–101]. In addition, ROS are produced by stressed mitochondria and these mitochondria-derived ROS have been implicated in the activation of the NLRP3 inflammasome[102]. However, inhibitors of ROS that have been used in previous studies can inhibit NLRP3 priming rather than the actual activation of the inflammasome[103]. As ROS are involved in several signalling pathways, defining the exact roles of ROS in NLRP3 priming and activation remains challenging.

Furthermore, autophagy can regulate both apoptosis and inflammasome assembly, and, depending on the conditions, can be either pro- or anti-apoptotic[104]. For inflammasome activation and IL-1 release, autophagy is a negative regulator: mice that are deficient in autophagy-related protein 16-like 1 (ATG16L1) — an essential component of the autophagy machinery — show higher IL-1 levels in response to stimulation. This indicates that autophagy limits IL-1 activation or release[105]. The mechanisms by which autophagy regulates inflammasome activation are still under debate. One hypothesis suggests that autophagy is involved in the removal of ubiquitylated inflammasomes[106] or pro-IL-1 molecules[107]. An alternative or additional mechanism could be that autophagy removes damaged mitochondria, which suggests that autophagy could prevent the release of mitochondrial ROS and DNA into the cytoplasm and thereby limit NLRP3 inflammasome formation[102].

These studies show that inflammasomes are tightly regulated by intracellular ion concentrations (FIG. 3), by the redox state of the cell and by the nutritional situation of the cell. It seems that inflammasome activation is regulated at multiple levels and that several requirements need to be met for full inflammasome activation to occur.

Regulation by interacting host proteins

In addition to general cellular conditions such as ion concentrations and the nutritional state, specific regulatory proteins have evolved that regulate inflammasome formation (TABLE 1). The formation of the inflammasome can be controlled both at the level of inflammasome sensor molecules and, further downstream, at the level of ASC and caspase 1 interaction. As mentioned above, inflammasomes form large protein aggregates and there are two levels at which death-fold domains are used to recruit effector inflammasome components. First, ASC is recruited to the inflammasome receptors by homotypic pyrin domain interactions. Next, in a second death-fold domain interaction, ASC binds to and activates pro-caspase 1

via its CARD. Studies have elucidated inflammasome regulation at the level of death-fold domain interactions and regulatory proteins.

Caspase activity during cell death is regulated by CARD-containing proteins such as CARD8, which has also been shown to be a binding partner of NLRP3 (REF. 108). CARD8 has multiple functions in regulating apoptosis, one of which is to directly bind to pro-caspase 9 and to suppress its activation[109]. Similarly, mouse caspase 12, which is a paralogue of caspase 1, interacts with caspase 1 to reduce its activity[110]. A polymorphism in the caspase 12 (*CASP12*) gene in humans leads to either a truncated protein or to a full-length protein (caspase 12L)[111]. Similarly to mouse caspase 12, caspase 12L reduces cytokine production in response to LPS[111], which — at high doses — can activate the NLRP3 inflammasome and thus caspase 1 without the need for a second signal. Therefore, caspase 12L and mouse caspase 12 probably function as decoy proteins for caspase 1, thereby limiting its activation. CARD-only proteins (COPs) can also inhibit caspase 1 activation[112]. CARD18 (also known as ICEBERG) and CARD16 (also known as pseudo-ICE and COP1) were the first 'decoy' COPs to be described[113]. Through their CARD–CARD interaction, these two inhibitors sequester pro-caspase 1 and inhibit its activation by the inflammasome. CARD17 (also known as INCA), which is another decoy protein, is upregulated by IFN to suppress IL-1 generation[114]. Thus, proteins containing a CARD can sequester caspase 1, thereby blocking the formation of a functional inflammasome complex.

An additional level of fine-tuning and regulation of the inflammasomes can be achieved by interfering with the pyrin–pyrin interaction in the inflammasomes. Several pyrin domain-only proteins (POPs) and other pyrin-containing proteins have been characterized. POP1 (also known as PYDC1 and ASC2) and POP2 (also known as PYDC2) inhibit the interactions between the inflammasome sensor molecules and ASC[115–117]. However, COPs and POPs have not been identified in the mouse genome, which indicates that a more complex regulatory system might be present in humans. The fact that COPs and POPs have not been identified in the mouse genome makes the study of these proteins difficult, and many of the COPs and POPs have only been investigated using overexpression studies and not using genetic deletion or RNA interference.

Alternative splicing of ASC can provide an additional level of regulation by giving rise to ASC variants that negatively regulate the assembly of the inflammasomes[118]. Furthermore, NLRP10 (also known as PYNOD) was suggested to negatively regulate inflammasomes by sequestering ASC[119,120], but deletion of *Nlrp10* in mice did not confirm this hypothesis[121,122]. NLRP7 has been suggested to be a negative regulator of inflammasomes; however, more recent findings indicate that NLRP7 might assemble an inflammasome in response to microbial acylated lipopeptides[28,123].

The role of pyrin, which is a protein that is encoded by *MEFV* (Mediterranean fever gene), in inflammasome regulation also remains unclear. One study found that *Mefv* deletion in mice lead to increased IL-1 release without influencing caspase 1 activity or inflammasome assembly. These findings suggested that pyrin can inhibit IL-1 release downstream of the inflammasomes[124]. However, another study found that short-term IL-1 release was not impaired after *Mefv* gene deletion and that IL-1 was only inhibited after several days of stimulation[125]. In addition, mice carrying *Mefv* mutations that have been identified in patients with familial Mediterranean fever (FMF) showed ASC-dependent but NLRP3-independent release of IL-1 [125]. Therefore, whether pyrin is an inhibitor at the level of IL-1 release or whether it forms a 'pyrin inflammasome' remains to be determined.

In addition to death domain interactions targeting the formation of the inflammasome complex, regulation can occur at the level of the inflammasome sensor molecules. A recent report linked the inhibition of translation with NLRP3 inflammasome activation[126]. Protein synthesis could be inhibited by the phosphorylation of the initiation factor eukaryotic translation initiation factor 2 subunit- (EIF2 ; also known as EIF2S1). This phosphorylation event could be mediated by different kinases that sense endoplasmic reticulum (ER) stress, or by the presence of heavy metals or double-stranded RNA (dsRNA). Protein kinase R (PKR; also known as EIF2AK), which is activated by dsRNA and can phosphorylate EIF2 , has been suggested to be involved in NLRP3, NLRP1, NLRC4 and AIM2 inflammasome activation, and this was found to be dependent on its kinase activity[127]. However, another study concluded that PKR does not have any role in inflammasome activation[128]. The reasons for these seemingly contradictory results could be attributed to differences in mouse strain backgrounds and will need to be further evaluated. In another study, ER stress was shown to activate the NLRP3 inflammasome[90] and, although the classical unfolded protein response was shown to activate EIF2 phosphorylation (consistent with the above observations), this pathway was not found to be necessary[90]. The authors proposed that another, not yet characterized, ER stress pathway activates the NLRP3 inflammasome[90]. Finally, heat shock proteins (HSPs) also have important roles in the regulation of cell death, and it has been shown that HSP90 and the co-chaperone ubiquitin ligase-associated protein SGT1 are required for NLRP3 activation (REF. 129).

Taken together, these studies show that the activation of inflammasomes can be regulated at different levels and that many proteins contribute to the overall response of these important signalling platforms. Elucidating the exact function of the different components seems to be challenging. Overexpression could considerably perturb the finely balanced thresholds of inflammasome activation, and it is possible that inflammasomes respond differently depending on culture conditions and/or genetic background.

Conclusion and future perspectives

In this Review, we summarize our rapidly evolving understanding of how inflammasomes are activated and we highlight the many mechanisms that are involved in their regulation. Although we have gained detailed knowledge about many aspects of inflammasome activation, several important questions remain unanswered or poorly understood.

One main conceptual question that remains unanswered is whether inflammasome sensors can directly interact with their triggers and, thus, whether they represent true receptor molecules. AIM2 can tightly interact with DNA and has recently been crystallized with its ligand; it therefore qualifies as a receptor molecule[130]. The NLR inflammasome sensors all have LRR domains that, for example, in TLRs, directly recognize lipids, nucleic acids or proteins. However, whether the NLR inflammasome sensor molecules can directly recognize their various triggers or whether they use accessory host proteins for this process remains a matter of debate. In the case of NLRC4, additional host molecules (that is, NAIPs) have been suggested to function as the direct receptors for the ligands[34,35]. Therefore, NLR inflammasome sensors could, in fact, function as adaptor molecules rather than receptors.

In addition, post-translational modifications of NLRs, such as phosphorylation[67], ubiquitylation[62,63] and even proteolysis[131–133], have been suggested to be necessary for the activation of certain NLR sensors. Therefore, the modification of NLRs by host enzymes could be crucial for their activation and, at the same time, could represent a novel target for pharmacological intervention strategies. Future research should focus on the identification of host molecules that function upstream of the NLRs. In the case of NLRP3, the elucidation of

the unknown factors that influence its activation represents a particular challenge, as these factors could influence both the priming and activation events. Therefore, combinatorial proteomic and genomic approaches, as well as overexpression systems in which priming is not required, could be instrumental.

Another important challenge will be to more precisely define the contribution of inflammasomes to the inflammatory response *in vivo*. The ability of a particular trigger to activate inflammasomes and IL-1 family cytokines *in vitro* does not necessarily indicate that inflammasomes fully control the inflammatory response to the respective trigger *in vivo*. It is becoming increasingly evident that the activation of inflammas-ome-independent IL-1 can substantially contribute to tissue inflammation. In acute inflammatory situations, in which neutrophils have important roles, inflammasomes can be functionally redundant in the activation of IL-1 and thus might only partially contribute to the inflammatory tissue response. Caspase 8, which has recently been implicated in IL-1 activation, could be one of the factors that contributes to inflammasome-independent responses *in vivo*. As many pathways can trigger caspase 8 activation, the relative contributions of caspase 1 and caspase 8 to IL-1 production and inflammation *in vivo* need to be carefully investigated in future studies. Furthermore, caspase 8 is known to mediate cell death; hence, it will be important to better understand the consequences of the different forms of cell death in the inflammatory tissue response.

Moreover, in this Review we compare the activation and regulation of inflammasomes to that of the apoptosome, which is another multimolecular caspase-activating platform. We note with interest that, in addition to the way in which the effector proteins of these platforms are tightly controlled, the activity of the triggers can be modulated; for example, cytochrome *c* can be modified to be more or less pro-apoptotic. Indeed, apoptosome activation by cytochrome *c* is only possible after cytochrome *c* has incorporated a haem group in the mitochondrion[134]. By contrast, nitrosylation of cytochrome *c* inhibits its ability to induce the formation of the apoptosome[135]. We predict that similar regulatory mechanisms are in place for the triggers of inflammasomes.

The importance of self-proteolysis or proteolysis by the trigger anthrax lethal toxin in the activation of NLRP1 is still not fully understood[131–133,136]. It is possible that proteolysis of inflammasome sensor molecules could lead to multiple effects that have not yet been defined. The quest for the exact details of inflammasome activation is ongoing; it seems probable that signals emanating from mitochondria and lysosomes will provide important insights. In addition, we will probably learn from comparing these potential regulatory mechanisms with those that govern apoptosome and ripoptosome activation. The identification of upstream mechanisms and the discrimination between priming and activation could be instrumental in developing novel therapies on the basis of the specific inhibition of individual inflammasomes (BOX 3). Given their broad roles in mediating inflammatory pathologies, inflammasome inhibitors could become effective therapies many prevalent and emerging inflammatory diseases.

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Glossary

ASC	An adaptor protein that was originally found to form protein precipitates in apoptotic cells that are termed protein specks
Pyroptosis	A rapid form of cell death following caspase 1 activation which shares characteristics with both apoptosis (such as DNA fragmentation) and necrosis (such as cell swelling and rupture)
Apoptosome	A large multimeric protein complex of apoptotic protease-activating factor 1 (APAF1) that recognizes cytochrome *c* release from damaged mitochondria and activates caspase 9
Death-fold domains	Commonly found in proteins that are involved in cell death pathways and in inflammasomes. The four main death-fold domains — the pyrin domain caspase activation and recruitment domain (CARD), death domain and death effector domain — associate with each other through homotypic interactions
NOD-like receptor	(NLR). A protein that contains amino-terminal pyrin caspase-recruitment domains or other signalling domains, followed by a NACHT domain and carboxy-terminal leucine-rich repeats. Some NLR proteins are involved in forming inflammasomes.
Non-canonical NLRP3 inflammasome	An inflammasome-like complex containing NLRP3 (NOD-, LRR-and pyrin domain-containing 3), the adaptor protein ASC, caspase 1 and caspase 11. The term ‘non-canonical inflammasome’ is used loosely to describe an inflammasome-like complex that does not conform to the three ‘canonical’ components of a canonical inflammasome: an inflammasome sensor molecule ASC and caspase 1. Two other non-canonical inflammasomes have also been described so far: one containing dectin 1, MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1), ASC and caspase 8 (termed the non-canonical caspase 8 inflammasome), and a caspase 11-activating platform that has not yet been fully described
Ripoptosome	A cytosolic multiprotein complex that induces cell death following genotoxic stress or depletion of inhibitor of apoptosis protein (IAP). The core ripoptosome contains receptor-interacting protein 1 (RIP1) FAS-associated death domain protein (FADD) and caspase 8, but it can also recruit other proteins such as RIP3
Amyloid- □	An endogenous peptide that is generated by proteases in the brain. It is prone to , aggregation and plaque formation. Amyloid- □plaques are a hallmark of Alzheimer’s disease and can activate the NLRP3 (NOD- LRR- and pyrin domain-containing 3) inflammasome
Cryopyrin-associated periodic syndrome	(CAPS). Characterized by NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome hyperactivity and the excessive release of interleukin-1 □, which leads to an autoinflammatory disease phenotype with periodic fever episodes, urticaria and often severe arthritis
MicroRNA	Small endogenous RNA molecules that can recruit the RNA-induced silencing complex to an mRNA, leading to inhibition of translation or to degradation of the mRNA

Autophagy	A homeostatic process during which cellular components are recycled through the lysosomal compartment. Its main triggers include nutrient starvation defective organelles and infection

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Box 1|Sensing of mitochondrial stress

Mitochondria are required to maintain cellular energy levels and so their health is crucial for cell viability. Mitochondrial dysfunction can trigger cell autonomous pathways that lead to apoptosis or to an inflammatory response. One of the molecules that signals that there is mitochondrial dysfunction is cytochrome *c*, which is an inner mitochondrial membrane protein and an essential component of the electron transport chain. After cytochrome *c* has leaked into the cytosol, it binds to apoptotic protease-activating factor 1 (APAF1), which is a scaffold protein for the activation of the initiator caspase 9, forming the apoptosome (REF. 137).

This intrinsic pathway of apoptosis is regulated by B cell lymphoma 2 (BCL-2) proteins[138]. Pro-apoptotic members of the family (such as BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist/killer (BAK)) promote cytochrome *c* release from the mitochondria, whereas the anti-apoptotic family members (such as BCL-2 and BCL-XL) inhibit cytochrome *c* release[138]. Interestingly, these anti-apoptotic proteins also regulate the NLRP1 (NOD-, LRR- and pyrin domain-containing 1) inflammasome. BCL-2 and BCL-XL bind to NLRP1 via their loop domains, thereby suppressing the ability of NLRP1 to bind to ATP and to oligomerize[139,140].

Mitochondrial stress can also be sensed by the NLRP3 inflammasome. Indeed, excessive production of reactive oxygen species (ROS)[102] and release of oxidized mitochondrial DNA[40] from stressed mitochondria have been suggested to activate the NLRP3 inflammasome. In addition, impaired mitochondrial homeostasis leads to changes in metabolites that are crucial for the function of the cell, such as the coenzyme NAD^+ (REF 141). Low NAD^+ levels inactivate the α-tubulin deacetylase sirtuin 2 (REF 142), which results in the accumulation of acetylated α-tubulin. Acetylated α-tubulin regulates the transport of mitochondria and helps the formation of an efficient interaction between the adaptor protein ASC and NLRP3 (REF 143). Furthermore, the RIG-I-like receptor (RLR) adaptor molecule mitochondrial antiviral signalling protein (MAVS), which is localized on the outer mitochondrial membrane, is required for optimal NLRP3 activation[144].

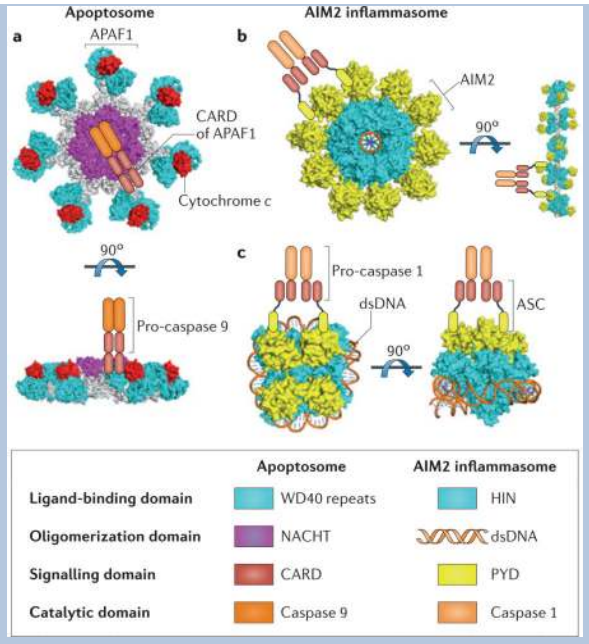
Another intriguing mechanism by which lipopolysaccharide-activated macrophages can increase their output of interleukin-1 β (IL-1 β) is through a substantial increase in levels of the tricarboxylic acid cycle intermediate succinate[145]. This metabolite stabilizes the transcription factor hypoxia-inducible factor-1 α, which leads to more sustained IL-1 β transcription[145]. Therefore, monitoring of mitochondrial stress by apoptosomes and inflammasomes seems to directly link mitochondrial health and activity to cell death and inflammation.

Box 2|Oligomerization of death-inducing molecular platforms

Receptors of the death-inducing molecular platforms are activated by ligands such as cytochrome *c* for the apoptosome component apoptotic protease-activating factor 1 (APAF1), and double-stranded DNA (dsDNA) for the absent in melanoma 2 (AIM2) inflammasome. By contrast, the NLRP1 (NOD-, LRR- and pyrin domain-containing 1) inflammasome can be activated by self-proteolysis in its function-to-find (FIIND) domain[131–133], which might be relevant to its proteolytic activation by the anthrax lethal toxin[136]. The cleaved fragments remain associated with each other but the proteolytic event enables NLRP1 to recruit the adaptor protein ASC and caspase 1. For the other receptors, ligand binding is thought to induce conformational changes, promoting oligomerization of the receptors into signalling platforms that recruit adaptors and activate effector caspases. Oligomerization of APAF1 and NOD-like receptors (NLRs) is mediated by their NACHT domains, which bind and hydrolyse deoxyadenosine triphosphate (dATP) or ATP[146,147]. However, AIM2 seems to use the multivalent ligand dsDNA as its oligomerization platform[130]. Both receptor oligomerization and ligand binding might be modulated by the regulatory domains of the receptors, including the WD40 repeats of APAF1, the HIN domain of AIM2 and the leucine-rich repeats (LRRs) of the NLRs. Two examples of the death-inducing platforms are shown in the box figure.

The apoptosome consists of seven APAF1 molecules that are activated by cytochrome *c*; this then recruits pro-caspase 9 molecules[137,148,149] (see figure, part **a**). Six- or eight-fold symmetry of the apoptosomes has also been observed, which suggests that there are variable oligomerization states[137,148,149]. Two hypothetical models of the AIM2 inflammasome highlight the positioning of the activating DNA either at the centre (see figure, part b) or the periphery (see figure, part **c**) of the complex. It is possible that the physiological AIM2 inflammasome may contain characteristics of both models, and the oligomerization states can be variable owing to the sequence-independent nature of the DNA-binding. For clarity, only one caspase dimer is shown for each oligomeric platform. According to a recent model, the pyrin domain (PYD) of ASC can interact with itself in a helical manner to form filamentous structures[5], which might mediate ASC pyroptosome formation[6].

CARD, caspase activation and recruitment domain.



Box 3|Pharmacological interference of inflammasome activation

The fact that the NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome in particular is under the control of numerous regulatory mechanisms provides several opportunities for therapeutic intervention. It is conceivable that the biological effectiveness of certain anti-inflammatory agents is at least partly due to their ability to interfere with NLRP3 activation. Recently, the mechanism of action of certain anti-inflammatory therapies that have been in use for years has been partly ascribed to the inhibition of NLRP3. For example, the therapeutic application of interferon- (IFN) is effective for most but not all patients with multiple sclerosis. IFN-mediated downregulation of NLRP3 inflammasome responses might be of particular relevance in this central nervous system pathology, which has been linked to inflammasome activation[150]. Interestingly, a recent study has documented that IFN therapy can only reduce the pathology in mouse experimental autoimmune encephalomyelitis models that are dependent on NLRP3, which indicates that IFN can actively reduce NLRP3-mediated brain inflammation *in vivo*[151].

In addition, a number of small-molecule compounds have been shown to inhibit NLRP3 activation. For example, glyburide, which is a pharmaceutical compound that is used in the treatment of type 2 diabetes, has been shown to directly inhibit the NLRP3 inflammasome, albeit only at high doses[152]. Furthermore, the anti-inflammatory compound parthenolide, which is naturally present in the plant *Tanacetum parthenium* (also known as feverfew) that is used in herbal remedies, and the nuclear factor- B (NF- B) inhibitory compound Bay 11–7082 can both interfere with the ATPase activity of NLRP3, thereby hindering inflammasome activation[153]. The NLRP3 inflammasome is also inhibited by the TGF -activated kinase 1 (TAK1) inhibitor 5Z-7-oxozeaenol and its related compounds[87]. Future investments in the identification of small molecules that target these important intracellular signalling platforms or their upstream regulators could generate a novel class of highly effective anti-inflammatory therapeutics.

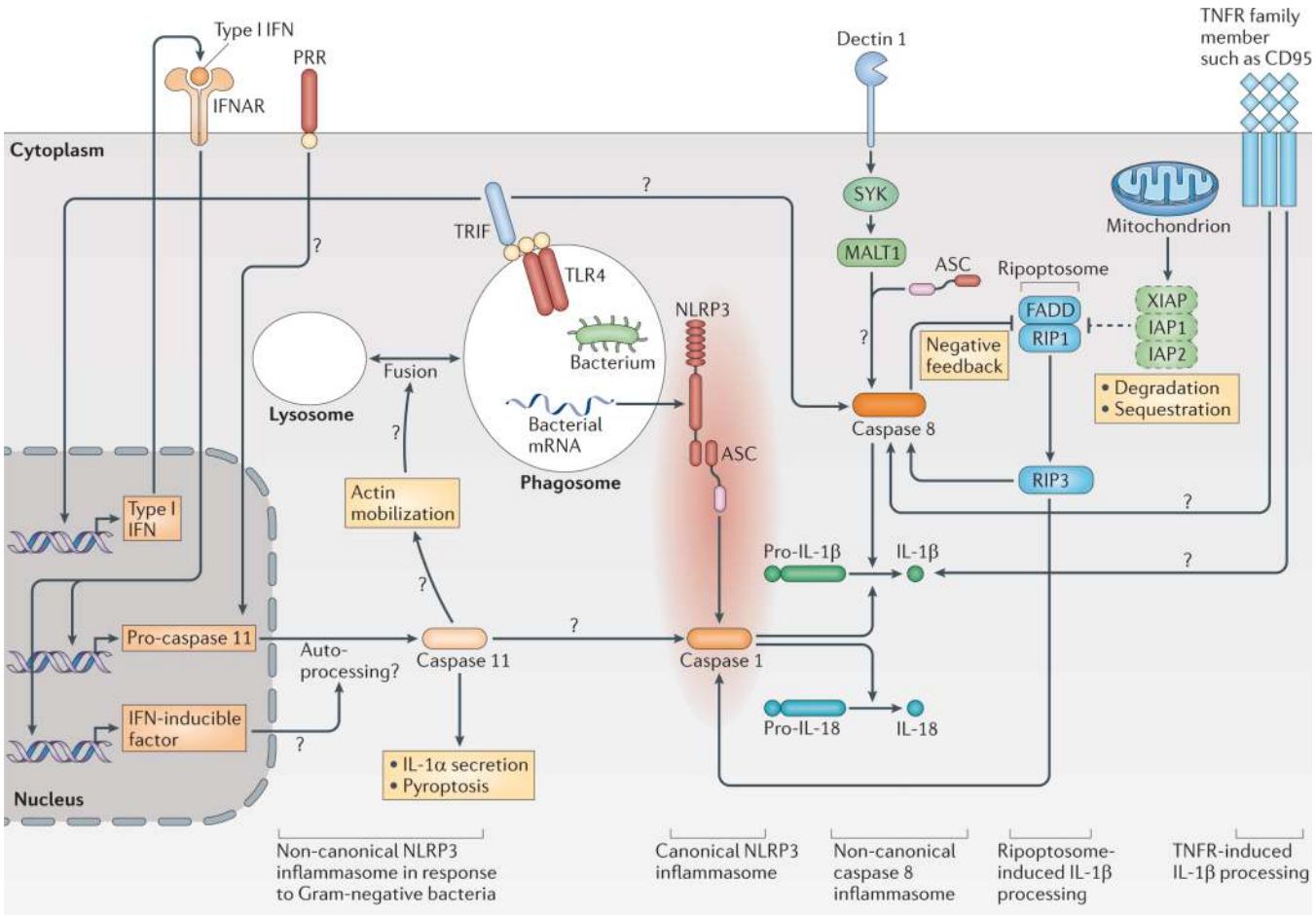


Figure 1. Canonical and non-canonical activation of IL-1

NLRP3 (NOD-, LRR- and pyrin domain-containing 3) needs additional cofactors for the processing of interleukin-1 (IL-1) in response to Gram-negative bacteria; this path way has been termed the non-canonical NLRP3 inflammasome. Toll-like receptor 4 (TLR4) signalling via TIR domain-containing adaptor protein inducing IFN (TRIF) induces the secretion of type I interferons (IFNs), which lead to the activation of caspase 11 via autocrine signalling through the IFN / receptor (IFNAR), possibly involving additional (not yet known) IFN-inducible factors. Caspase 11 is necessary for the activation of caspase 1 and has an independent role in IL-1 secretion and pyroptosis in response to Gram-negative bacteria. A possible mechanism for caspase 11 action might be its role in actin mobilization via cofilin, which might lead to increased fusion of lysosomes to phagosomes and, potentially, to increased leakage of bacterial mRNA (also termed vita-PAMPs) to the cytoplasm to activate NLRP3 (not shown). A platform consisting of mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), caspase 8 and the adaptor protein ASC (termed the non-canonical caspase 8 inflammasome) is formed in response to stimulation of dectin 1. Caspase 8 might also be activated by TLRs using the signalling adaptor TRIF in the presence of cycloheximide. In addition, the formation of the ripoptosome is triggered by the loss of inhibitor of apoptosis proteins (IAPs) with concurrent TLR stimulation. The ripoptosome — consisting of FAS-associated death domain protein (FADD) and receptor-interacting protein 1 (RIP1) — activates caspase 8 via RIP3. However, caspase 8 can also limit ripoptosome action on NLRP3. Members of the tumour necrosis factor receptor (TNFR) family might also induce pro-IL-1 cleavage, as has been

shown for CD95 (also known as FAS). Question marks show pathways that are still speculative. PRR, pattern recognition receptor; XIAP, X-linked inhibitor of apoptosis protein.

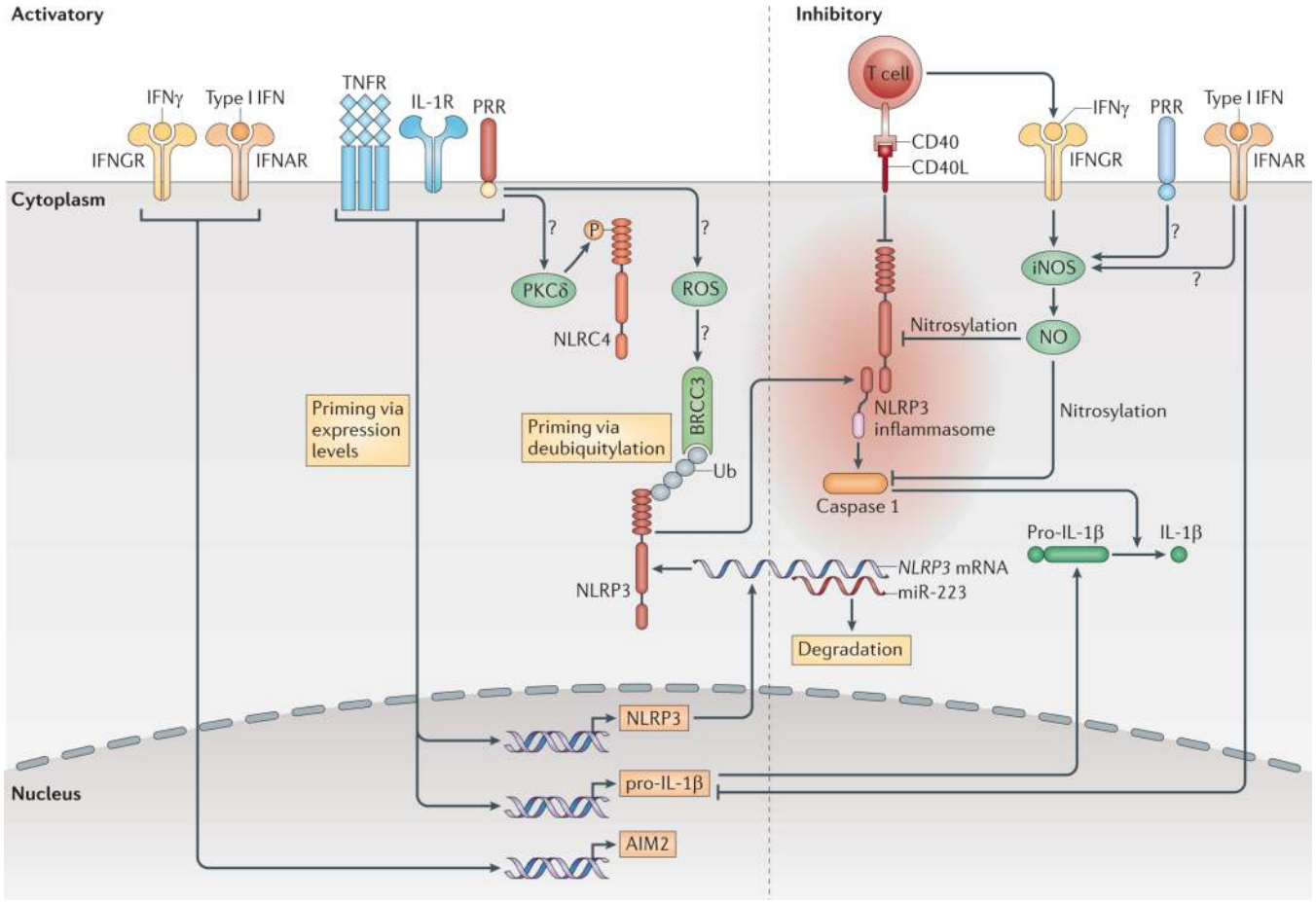


Figure 2. Extracellular signals regulate the inflammasomes

Pro-interleukin-1 (pro-IL-1) and NLRP3 (NOD-, LRR-and pyrin domain-containing 3) expression are induced by transcriptionally active pattern recognition receptors (PRRs) or by cytokine receptors. Furthermore, NLRP3 deubiquitylation by the K63-specific deubiquitinase BRCC3 is c rucial for its activation. Direct contact with mature or memory T cells inhibits the inflammasomes, probably via tumour necrosis factor receptor (TNFR) superfamily interactions. Type I interferons (IFNs) inhibit the transcription of pro-IL-1 , but also upregulate the expression of absent in melanoma 2 (AIM2). Both type I IFNs and IFN inhibit NLRP3 through the induction of nitric oxide (NO) via inducible nitric oxide synthase (iNOS), possibly with the requirement of concomitant priming by PRRs. Ub shows ubiquitylated proteins. Question marks show pathways that are still speculative. CD40L, CD40 ligand; IFNAR, interferon- / receptor; IFNGR, interferon- receptor; IL-1R, IL-1 receptor; miR-223, microRNA-223; NLRC4, NOD-, LRR- and CARD-containing 4; ROS, reactive oxygen species; PKC , protein kinase C

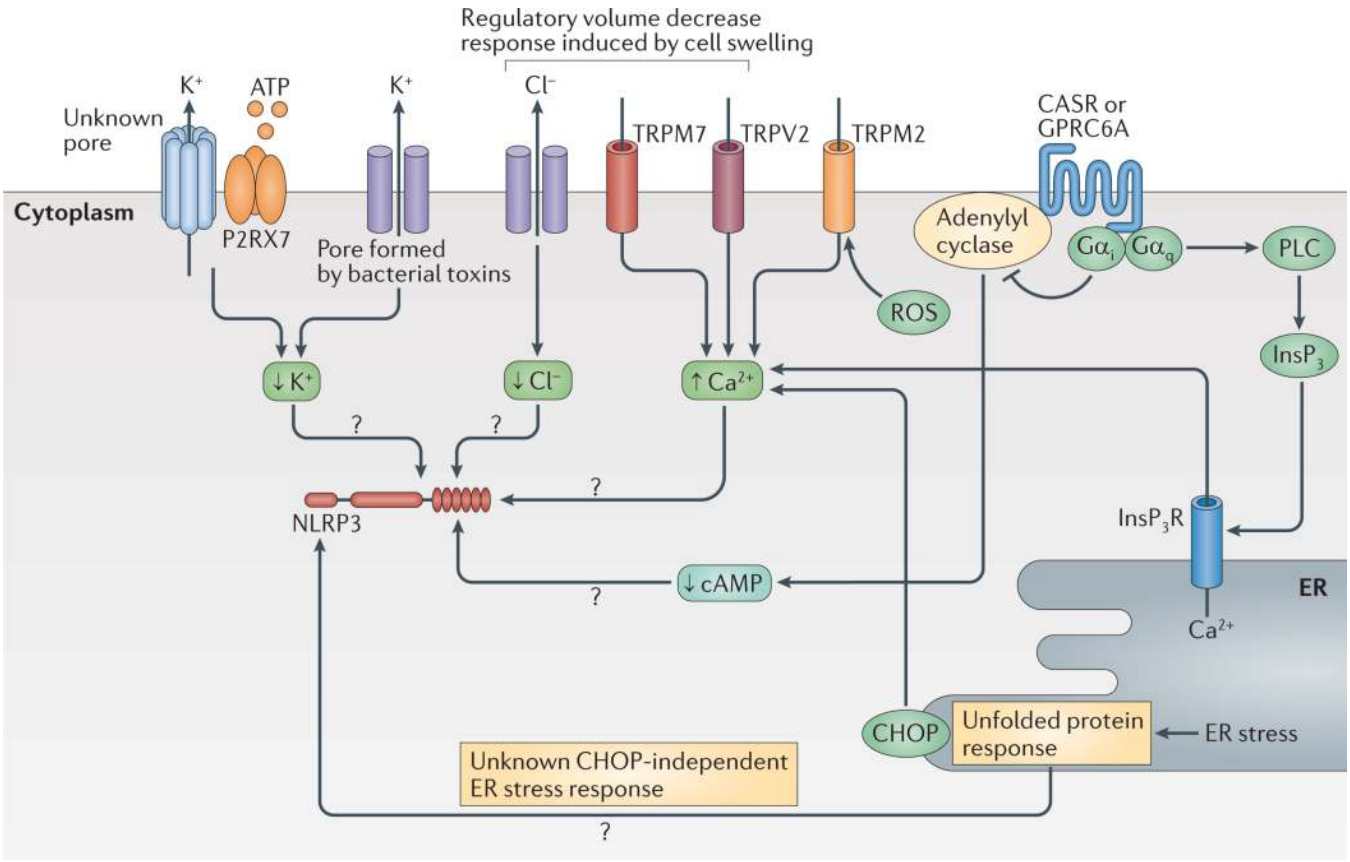


Figure 3. Ion fluxes and cell stress regulate the NLRP3 inflammasome

K^+ and Cl^- efflux, as well as Ca^{2+} mobilization, have a role in the regulation of NLRP3 (NOD-, LRR- and pyrin domain-containing 3). K^+ efflux is either achieved directly by pore-forming toxins such as nigericin or indirectly — for example, via the purinergic receptor for ATP P2RX7. Cl^- and Ca^{2+} ion fluxes can be regulated during the regulatory volume decrease response by transient receptor potential receptors (TRPV2 or TRPM7). In addition, reactive oxygen species (ROS) can activate TRPM2 for Ca^{2+} influx. Ca^{2+} is also regulated by the unfolded protein response via C/EPB-homologous protein (CHOP). The unfolded protein response is also implicated in other pathways that regulate NLRP3. G-protein coupled receptors (GPCRs) such as calcium-sensing receptor (CASR) and GPRC6A regulate both Ca^{2+} levels and cyclic AMP (cAMP) levels via activation of phospholipase C (PLC) or inhibition of adenylyl cyclase, respectively. High cAMP levels might directly inhibit NLRP3 (not shown). Inositol-1,4,5-trisphosphate ($InsP_3$) that is generated by PLC leads to release of Ca^{2+} from the endoplasmic reticulum (ER). How Ca^{2+} influences NLRP3 activation is not fully understood but it has many molecular targets. Question marks show pathways that are still speculative. $InsP_3R$, $InsP_3$ receptor.

Table 1

Regulatory proteins of the inflammasomes

Regulatory protein	Inflammasome component targeted	Mechanism of action	Sources of experimental evidence	Refs
Accessory NLR proteins				
NOD2	NLRP1	Enhances NLRP1 inflammasome formation	Knockout mice and biochemical interaction	32,33
NAIP2 *	NLRC4	Necessary for recognition of the type III secretion system by the NLRC4 inflammasome	RNAi and biochemical interaction	34,35
NAIP5 or NAIP6 *	NLRC4	Necessary for recognition of flagellin by the NLRC4 inflammasome	RNAi and biochemical interaction	34,35
NAIP‡	NLRC4	Necessary for recognition of flagellin and/or the type III secretion needle protein by the NLRC4 inflammasome	RNAi, biochemical interaction and overexpression	35,36
CARD-containing regulatory proteins				
Caspase 12 and caspase 12L‡	Caspase 1	Inhibits caspase 1 activity, probably by sequestration	Knockout mice and biochemical Interaction	110,111
CARD18‡ (also known as ICEBERG)	Caspase 1	Inhibits caspase 1 activity, probably by sequestration	Biochemical interaction and Overexpression	113
CARD16‡ (also known as COP1)	Caspase 1	Inhibits caspase 1 activity, probably by sequestration	Biochemical interaction and Overexpression	113
CARD17‡ (also known as INCA)	Caspase 1	Inhibits caspase 1 activity, probably by sequestration	Biochemical interaction and Overexpression	114
PYD-containing regulatory proteins				
POP1‡	ASC	Inhibits inflammasome assembly, probably by sequestration	Biochemical interaction and Overexpression	115
POP2‡	ASC	Inhibits inflammasome assembly, probably by sequestration	Biochemical interaction and Overexpression	116,117
ASC splice variant	ASC§ or caspase 1§	Inhibits inflammasome assembly, probably by sequestration	Biochemical interaction, co-localization and overexpression	118
NLRP10§	ASC§	• Inhibits inflammasome assembly, probably by sequestration§ • Role in adaptive immunity§	• Inhibitor: knock-in mice, biochemical evidence and overexpression • Adaptive immunity: knockout mice	119–122
NLRP7‡§	ASC§	• Inhibits inflammasome assembly by sequestration§ • Activates the formation of the NLRP7 inflammasome§	• Inhibitor: overexpression and biochemical evidence • Inflammasome: RNAi and biochemical evidence	28,123
Pyrin§	ASC§ or IL-1 §§	• Inhibits IL-1 release§	• Inhibitor: Knockout mice • Activator: knock-in mice of FMF-mutated pyrin	124,125

Regulatory protein	Inflammasome component targeted	Mechanism of action	Sources of experimental evidence	Refs
Accessory NLR proteins				
		• FMF-associated pyrin activates the formation of an inflammasome[§]		
Other regulators				
GBP5	NLRP3	Enhances NLRP3 inflammasome formation in response to non-crystalline activators	Knockout mice and biochemical evidence	39
BRCC3	NLRP3	Deubiquitylates NLRP3 as a priming step	RNAi and biochemical evidence	63
PKC □	NLRC4	Phosphorylates NLRC4 as a priming step	Knockout mice and biochemical evidence	67
BCL-2	NLRP1	Inhibits NLRP1 inflammasome activation by inhibiting nucleotide binding	Knockout mice, biochemical evidence and overexpression	139,140
BCL-XL	NLRP1	Inhibits NLRP1 inflammasome activation by inhibiting nucleotide binding	Biochemical evidence and overexpression	139,140
IAPs[§]	Caspase 1	Increases the activity of caspase 1 by monoubiquitylation	Knockout mice (for IAP1 or IAP2) and biochemical evidence	58
IAPs[§]	NLRP3	Loss of IAPs leads to activation of the ripoptosome, which is necessary for caspase 1 activation	IAP1, IAP2 and XIAP triple-knockout mouse	56
PKR	NLRP1, NLRC4, AIM2 and NLRP3	• Activated by interacting with the inflammasome sensor molecules[§] • Does not have an effect[§]	• Activator: knockout mice (kinase domain knocked out) and biochemical evidence • No role: knockout mice (kinase and RNA-binding domain knocked out)	127, 128
Other regulators (cont.)				
HSP90	NLRP3	Necessary for activation, possibly owing to its chaperone function	Biochemical evidence and inhibitor of HSP90	129
SGT1	NLRP3	Necessary for activation, possibly owing to its chaperone function	RNAi and biochemical evidence	129

AIM2, absent in melanoma 2; BCL, B cell lymphoma; CARD, caspase activation and recruitment domain; FMF, familial Mediterranean fever; GBP5, guanylate-binding protein 5; HSP90, heat shock protein 90; IAP, inhibitor of apoptosis protein; IL-1 □ interleukin-1 □ NAIP, NLR family, apoptosis inhibitory protein; NLR, NOD-like receptor; NLRC4, NOD-, LRR- and CARD-containing 4; NLRP, NOD-, LRR- and pyrin domain-containing; NOD2, nucleotide-binding oligomerization domain-containing protein 2; PKC □ protein kinase C □ PKR, protein kinase R; POP, pyrin domain-only protein; PYD, pyrin domain; RNAi, RNA interference; XIAP, X-linked IAP.

[*] Expressed only in mice.

[‡] Expressed only in humans.

[§] Two contradictory studies have been published about this interactor.