Modulatory mechanisms controlling the NLRP3 inflammasome in inflammation: recent developments

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The protein NLRP3 has emerged as a central regulator in the inflammatory process, being implicated directly in hereditary cryopyrinopathies, and indirectly in diseases such as gout, Type 2 diabetes and atherosclerosis. NLRP3 is an important regulator of caspase-1, the enzyme that processes the immature form of IL-1β into the active protein. The control of NLRP3 has therefore become a focus of research with evidence for redox regulation, ubiquitination and regulation by miRNA-223, kinases and calcium all emerging as controllers of NLRP3. As our knowledge expands the prospect for precise pharmacological targeting of NLRP3 will improve and could lead to substantial clinical utility.

Introduction

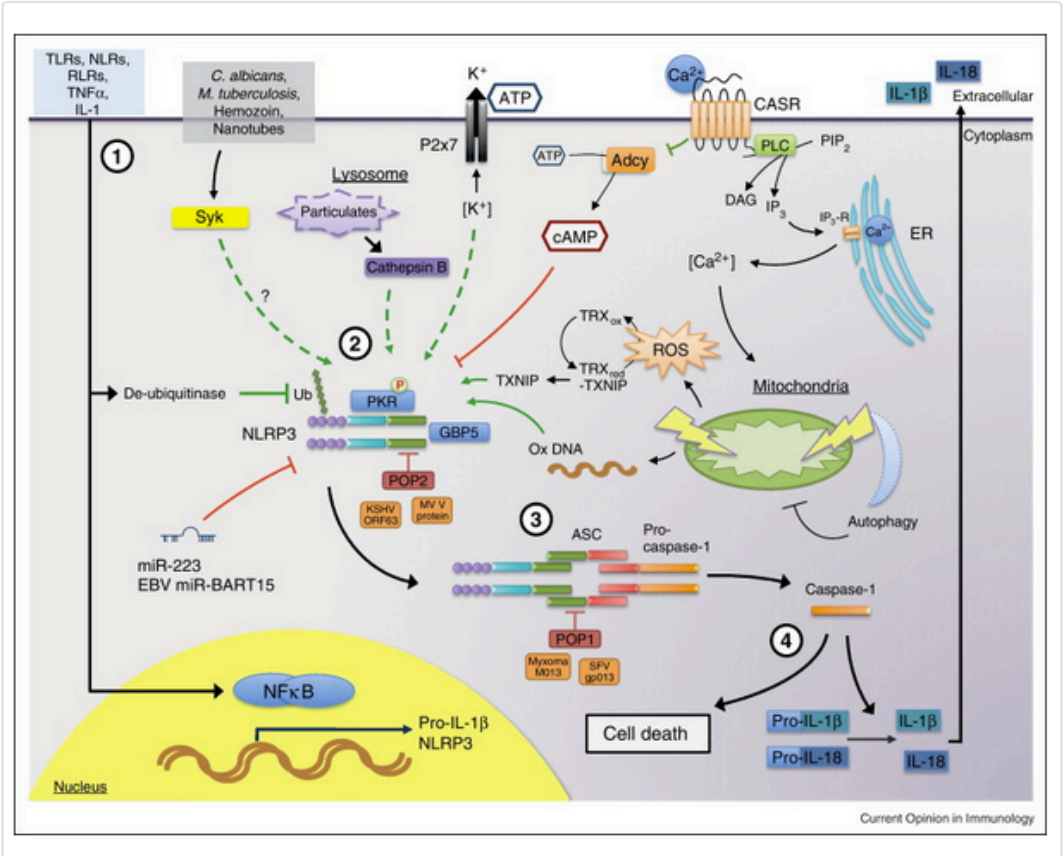
The pro-inflammatory cytokine IL-1β is the most studied of all cytokines because of its central role in the inflammatory process. IL-1β has also been implicated in many diseases, most recently in metabolic diseases such as gout, Type 2 diabetes (T2D) and atherosclerosis [1]. One reason for the recent focus on these particular diseases is the discovery of NLRP3, the key protein in the mechanism by which IL-1β production is regulated in macrophages [2]. IL-1β is initially made as a pro-form and is processed to the mature cytokine by caspase-1, which occurs in a complex with NLRP3 and ASC in a multi-protein complex termed the inflammasome [3]. The major function of NLRP3 is to sense phagocytosed material, and relay the signal to caspase-1 [4]. Diseases such as gout, T2D and atherosclerosis can be considered 'particle diseases' since there are roles for insoluble particles in their pathogenesis. Specifically, gout involves crystals of uric acid [5], T2D the amyloid protein Islet Amyloid Polypeptide [6] and atherosclerosis cholesterol crystals [7]. All of these have been shown to be phagocytosed by macrophages, activating NLRP3. NLRP3 has also been shown to be mutated in human in familial fevers termed cryopyrinopathies [8], and this discovery has allowed NLRP3 to move to centre stage as the key regulator of caspase-1 and IL-1β in human and generate considerable interest from the pharma sector [9•]. Attention has moved to the regulation of NLRP3, since dysregulation can obviously be seen as a potential cause of disease pathogenesis. Here we discuss recent insights into the modulation of NLRP3. Roles of reactive oxygen species (ROS), control by microRNA (miRNA), ubiquitination, phosphorylation and calcium are discussed. We also discuss the prospect of targeting NLRP3 with small molecule inhibitors, which could hold great promise clinically.

Redox regulation

A major recent area of interest has been the role of ROS in NLRP3 regulation but there is much controversy here and precisely how ROS might regulate NLRP3 is still unknown. An often-repeated finding is that antioxidants are potent inhibitors of NLRP3-dependent IL-1β production, which prompted a great deal of research on the role of ROS and oxidative stress in NLRP3 activation. Even though an involvement of redox signalling is generally accepted, many conflicting results have been reported. For example, ROS originating from the NADPH oxidase complex were originally found to be important [10]. However, macrophages from mice or humans with genetically defective NADPH oxidase had normal NLRP3 activation [11 and 12]. Subsequently, mitochondrial ROS were put forward as being essential for inflammasome activation, a finding that is supported by the co-localization of NLRP3 inflammasomes with mitochondria in the perinuclear space upon activation [13•].

Functionally, ROS were proposed to be exclusively involved in the 'priming' step of NLRP3 activation. Initially, ROS induction by TLRs was shown to be required for NFκB activation, which is the traditional priming signal and induces transcription of NLRP3 and pro-IL-1β [14]. Recently, a novel priming mechanism has been put forward, which results in the post-translational activation of NLRP3 by de-ubiquitination and also requires TLR-induced ROS, as described later [15••]. Despite these findings, two redox-related factors have also been suggested to be direct NLRP3-activating ligands. Firstly, thioredoxin-interacting protein (TXNIP) has been shown to dissociate from thioredoxin (TRX) upon oxidative stress, which allows it to directly bind to NLRP3 [16]. However, the involvement of TXNIP in NLRP3 activation in BMDMs could not be confirmed in other studies [6]. Secondly, Shimada et al. recently suggested oxidized mitochondrial DNA as a NLRP3 ligand [17••]. They find that upon inflammasome activation or during apoptosis, dysfunctional mitochondria can release oxidized DNA into the cytosol. In the presence of a priming stimulus, this leads to IL-1β production. This also ties in the intrinsic apoptotic pathway with inflammasome signalling, which share striking similarities at the level of the mitochondria (Figure 1).

Figure 1.



Current view of NLRP3 inflammasome activation. The initiating step in NLRP3 activation is a 'priming' signal (1) by pattern recognition receptors, most commonly Toll-like receptors that activate transcription of NLRP3 and pro-IL-1β through NFκB. At the same time, NLRP3 is activated non-transcriptionally by de-ubiquitination. NLRP3 levels can further be regulated by miR-223. Priming is followed by NLRP3 activation (2), the molecular mechanism of which is not yet fully understood. However, at least one of following three events is commonly required: potassium efflux, cathepsin B release from lysosomes and ROS production. Two direct ROS-related NLRP3 ligands have recently been proposed: TXNIP released from the redox system and oxidized mitochondrial DNA. Mitochondria and mitochondrial ROS seem to play an important role and can be influenced by calcium signalling; whether potassium efflux and lysosomal damage link into mitochondrial signalling as well is yet unknown. Autophagy negatively impacts NLRP3 activation, potentially by removing ROS-producing dysfunctional mitochondria. Furthermore, the second messenger cAMP can bind directly to NLRP3 and inhibit its activation. Extracellular calcium decreases cAMP through CASR signalling. Kinase signalling positively regulates NLRP3 activation through PKR that directly interacts with NLRP3, while Syk kinase activity is required for NLRP3 activation in response to specific ligands such as C. albicans. NLRP3 activation triggers the assembly of active inflammasome complexes containing ASC and pro-caspase-1 (3). Endogenous and viral Pyrin-only proteins (POPs) can interfere with inflammasome assembly, while GBP5 selectively promotes NLRP3 dependent ASC oligomerization. Inflammasome formation results in the processing and activation of caspase-1 (4). Caspase-1

cleaves the pro-forms of IL-1β and IL-18, releasing the mature cytokines and also induces cell death by pyroptosis.

Figure options

In contrast to oxidative stress, other reports surprisingly found that antioxidants are also essential for inflammasome activation. Macrophages deficient in the antioxidant enzyme SOD1, even though they have increased ROS, have decreased inflammasome activity because of oxidation of redox-sensitive cysteine residues on caspase-1 [18].

These seemingly contradictory findings might be reconciled by a more integrated view of the redox system, where an oxidative insult is always balanced by a cellular antioxidant response and vice versa. This is demonstrated by the fact that TLR stimuli lead to a biphasic redox response, starting off with an oxidative hit and followed by a rapid antioxidant response [19]. In addition, the baseline activation level of the redox system determines the potential for IL-1β production through NLRP3 [20•]. In contrast to primary monocytes, monocytic cell lines or cultured macrophages have a high baseline activation of the antioxidant system, leading to a stunted IL-1β response to NLRP3 stimuli. This highlights the fact that redox dependency is very much determined by the system it is studied in.

The importance of redox signalling could also explain findings that inhibition of autophagy increases IL-1β production by NLRP3 activators. Since dysfunctional organelles, including mitochondria, are removed by autophagosomes, inhibition of autophagy leads to an accumulation of ROS-producing mitochondria, which is accompanied by increased release of mitochondrial DNA [21]. However, autophagy is also involved in the removal of ubiquitinated inflammasomes [22] and pro-IL-1β in activated cells [23].

Regulation of NLRP3 expression by miR-223

Induction of NLRP3 expression by TLR ligands is necessary for optimal inflammasome activation, which is highlighted by the fact that constitutive NLRP3 overexpression is sufficient to induce IL-1β secretion to NLRP3 inflammasome activators [14 and 15••]. Clearly, regulation of NLRP3 levels offers an interesting mechanism to alter the inflammatory potential of immune cells and different cell functions could require a different threshold for NLRP3 activation. We and others recently identified miRNA-223 as a negative regulator of NLRP3 [24•• and 25••]. Interestingly, miR-223 is involved in hematopoietic differentiation and differentially expressed among myeloid cells [26]. Thus, it can fine-tune NLRP3 in a cell type-dependent and differentiation status-dependent manner in monocytes, macrophages, dendritic cells and especially granulocytes, which have very high miR-223 expression. Of note, miR-223 deficient mice exhibit phenotypes consistent with deregulated NLRP3, namely neutrophilia,

spontaneous lung inflammation and increased susceptibility to endotoxin challenge [27].

Viral regulation of NLRP3

Increasing evidence also suggests that many viruses have evolved mechanisms to dampen inflammasome signalling. Most mechanisms inhibit inflammasome assembly, caspase-1 activation or cytokine neutralization [28]. For example, KSHV ORF63 [29] and Measles virus V protein [30] can bind NLRP3 and prevent its activation. Several poxviruses express viral POPs, which interfere with ASC recruitment, such as shape fibroma virus gp013 [31] and myxoma virus M013 [32]. In addition to the endogenous miR-223, we also identified an EBV miRNA, miR-BART15, that can inhibit NLRP3 through the same target site as miR-223 [24••]. We found that miR-BART15 can be transferred from infected B cells to uninfected cells via exosomes. However, increasing evidence suggests that EBV can also directly infect myeloid cells [33], where it could affect NLRP3 expression. In general, dampening inflammasome signalling could be advantageous to viruses in order to escape pyrogenic effects of IL-1β, cell-mediated immunity by IL-18 and cell death of infected cells by pyroptosis.

Ubiquitination as a negative regulator of NLRP3

Inflammasome-mediated IL-1β production is a two-step process. A primary signal must activate NFκB to initiate pro IL-1β mRNA synthesis followed by a secondary signal that activates the inflammasome and IL-1β release [34]. For the NLRP3 inflammasome it was demonstrated that a primary signal such as TLR activation was also required to prime or 'license' NLRP3 itself. NLRP3 protein expression levels were shown to be a limiting step in inflammasome activation [14 and 35]. Two recent reports have demonstrated that LPS can rapidly prime NLRP3 in a manner distinct from transcriptional induction. In macrophages caspase-1 activation was shown to occur with simultaneous administration of LPS and the NLRP3 activator ATP, thus bypassing the transcriptional induction of NLRP3 that occurred after two hours of LPS stimulation [15•• and 36]. Juliana et al. further demonstrated that NLRP3 was basally ubiquitinated and that LPS stimulation reduces this ubiquitination in a manner that is dependent on mitochondrial ROS generation and results in NLRP3 activation. Interestingly, ATP could also induce de-ubiquitination of NLRP3 via a ROS-independent mechanism suggesting there are two de-ubiquitinating enzymes that regulate NLRP3 [15••]. Whether this mechanism is unique to LPS priming of NLRP3 remains to be examined.

A role for protein kinases in NLRP3 activation

The phosphorylation of NLRC4 on Ser533 mediated by PKCδ was recently shown to be essential for functional NLRC4 inflammasome formation [37], the first time that an activating covalent modification of any inflammasome has been reported.

Although phosphorylation of NLRP3 has not been directly demonstrated there is evidence to support a role for kinase signalling in its regulation. A recent study by Lu et al. has demonstrated that RNA-dependent protein kinase (PKR) is a broad inflammasome regulator. Activation of NLRP3, NLRP1, NLRC4 and AIM2 induced PKR phosphorylation and PKR deficiency attenuated caspase-1 activation in response to activation of all of these inflammasomes. Co-immunoprecipitation assays showed that PKR interacts with NLRP3 and furthermore in a cell free system activated PKR together with NLRP3, ASC and caspase-1 reconstituted a functional inflammasome [38•]. Previous work has also demonstrated a role for Syk tyrosine kinase activity in the activation of NLRP3 in response to a number of stimuli. Fungal infection by Candida albicans [39] the Plasmodium metabolite hemozoin [40], Mycobacterium tuberculosis infection [41] and carbon nanotubes [42] all activate NLRP3 and require Syk activity for effective inflammasome activation.

Pyrin domain interactions

NLRP3 has a characteristic tri-domain structure consisting of a C-terminal leucine rich repeat domain, a central nucleotide binding and oligomerization (NACHT) domain and an N-terminal Pyrin domain (PYD) [43]. The PYD domain of NLRP3 is crucial to its function as it interacts with the PYD domain of ASC, which mediates the activation of caspase-1 [2]. Two PYD only proteins (POPs) POP1 and POP2 that negatively regulate NLRP3 inflammasome activation in humans have been described. POP1 (also known as ASC2, ASCI, ASCL and PYDC1) binds to the PYD domain of ASC to which it is 64% identical and may disrupt the interaction of ASC with other proteins such as NLRP3 [44]. POP2 shows closer homology to the PYD domains of NLRP proteins than that of ASC. POP2 was shown to prevent the recruitment of ASC by NLRP3 [45]. A recent study by Shenoy et al. identified guanylate binding protein 5 (GBP5) as a selective regulator of the NLRP3 inflammasome. GBP5 interacts with the PYD domain of NLRP3 and it was shown that tetramers of GBP5 promote ASC oligomerization through NLRP3. Interestingly, GBP5 only played a role in NLRP3 activation in response live bacterial infection and soluble ligands such as ATP and nigericin but not crystalline substances such as MSU and alum [46••]. The crystal structure of the PYD of NLRP3 has been reported [47]. Interestingly, an unexpected disulphide bond between Cys-8 and Cys-108 of the NLRP3 PYD was identified. These Cys residues are evolutionarily conserved, suggesting that redox modifications could influence the structure of NLRP3 PYD [47]. Further structural studies should shed light on the conformational changes in NLRP3 and the mechanism of its activation.

Calcium signalling

Intracellular Ca2+ store release was previously implicated in NLRP3 dependent IL-1β secretion [48]. A report by Murakami et al. has shown that multiple NLRP3 activators induce Ca2+ signalling. Depletion of endoplasmic reticulum (ER) Ca2+ stores and inhibition of extracellular Ca2+ influx both attenuated ATP stimulated

NLRP3 activation. Furthermore, pharmacological inhibitors of key Ca2+ signalling mediators such as phospholipase C attenuated IL-1β release. Mechanistically, Ca2+ signalling was found to promote mitochondrial damage and therefore NLRP3 activation [49••]. More recently it has been demonstrated that the calcium-sensing receptor (CASR) activates the NLRP3 inflammasome through PLC signalling. In addition it was shown that activation of CASR by extracellular Ca2+ results in the inhibition of adenylate cyclase and a reduction in cyclic AMP (cAMP) levels. It was found that cAMP binds NLRP3 and negatively regulates its activation [50••].

Pharmacological manipulation of inflammasome activity

The current best treatments for inflammasome disorders target the main product of inflammasome activity, IL-1β [51]. Anti-IL-1β biologicals such as the recombinant IL-1 receptor antagonist Anakinra are clinically successful anti-IL-1 therapies [9•]. Other strategies for treating IL-1 related disease such as developing P2X7 receptor antagonists and caspase-1 inhibitors have also been explored and tested in clinical trials [9• and 52].

Several previously characterized small-molecule inhibitors have more recently also been shown to affect NLRP3 inflammasome function. Glyburide is a sulfonylurea drug used in the treatment of T2D, where it acts by inhibiting potassium channels in pancreatic β cells. Glyburide inhibits IL-1β production in response to multiple NLRP3 stimuli but not NLRC4 or NLRP1 activation. The inhibitory activity of glyburide was not dependent on potassium channels or NLRP3 ATPase activity. Interestingly, glyburide did not inhibit temperature-induced IL-1β release from monocytes of familial cold autoinflammatory syndrome (FCAS) patients, suggesting it does not directly inhibit NLRP3 but upstream signalling [53•]. We have also found that a compound termed CRID3 can act on or close to NLRP3 and block IL-1β production [54].

Parthenolide is a sesquiterpene lactone that has multiple anti-inflammatory properties. Separate to its effects on NFκB activation parthenolide has now also been shown to inhibit caspase-1 and NLRP3. Parthenolide inhibited caspase-1 activation in response to NLRP3, NLRP1 and NLRC4 stimulation. The authors suggest this is a result of alkylation of caspase-1 on a number of Cys residues. Parthenolide also directly inhibits NLRP3 by inhibiting its ATPase activity that is required for activation [55]. Bay 11-7082 is another NFκB inhibitor that was also found to specifically inhibit NLRP3. Bay 11-7082 inhibits the ATPase activity of NLRP3 suggesting that this may be the mechanism of inhibition [55].

Currently available inhibitors of inflammasome function have either not been clinically successful or have multiple targets. The development of small molecule

inhibitors that directly target the NLRP3 inflammasome could provide a cheaper and less invasive therapy for IL-1β and inflammasome-related diseases.

Conclusions

Our understanding of the precise biochemical control of the NLRP3 inflammasome is still sparse. We still have no clear mechanism for NLRP3 activation by ROS. However the recent insights into control by miR-223, viral manipulation, role of protein kinases and calcium represent an excellent start in the effort to understand how NLRP3 is controlled. Given the importance of NLRP3 for inflammatory diseases, more information on its modulation will help our understanding of NLRP3 in disease and its potential manipulation therapeutically.

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