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Title: Inflammasome inhibition under physiological and pharmacological conditions

Running title: Mechanisms of inflammasome inhibition

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Abstract

Inflammasomes are key regulators of the host response against microbial pathogens, in addition to limiting aberrant responses to sterile insults, as mediated by environmental agents such as toxins or nanoparticles, and also by endogenous danger signals such as monosodium urate, ATP and amyloid-β (1).

To date at least six different inflammasome signalling platforms have been reported (2, 3). This review focuses on the complex molecular machinery involved in

activation and regulation of the best characterised inflammasome, NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), and the development of molecular agents to modulate NLRP3 inflammasome function. Activation of the NLRP3 inflammasome induces inflammation via secretion of interleukin-1β (IL-1β) and interleukin-18 (IL-18) proinflammatory cytokines, with orchestration of pyroptotic cell death, to eliminate invading microbial pathogens. This field has gradually moved from an emphasis on monogenic autoinflammatory conditions, such as cryopyrin-associated periodic syndromes (CAPS), to the broad spectrum of innate immune-mediated disease. NLRP3 inflammasome activation is also linked to a range of common disorders in humans including type 2 diabetes (4), cystic fibrosis (5), myocardial infarction, Parkinson's disease, Alzheimer's disease (6) and cancers such as mesotheliomas and gliomas (7).

We describe how laboratory-based assessment of NLRP3 inflammasome activation is emerging as an integral part of the clinical evaluation and treatment of a range of undifferentiated systemic autoinflammatory disorders (uSAID) (8), where a DNA-based diagnosis has not been possible.

In addition, this review summarises the current literature on physiological inhibitors and features various pharmacological approaches that are currently being developed, with potential for clinical translation in autoinflammatory and immune-mediated conditions. We discuss the possibilities of rational drug design, based on detailed structural analyses, and some of the challenges in transferring exciting preliminary results from trials of small molecule inhibitors of the NLRP3 inflammasome, in animal models of disease, to the clinical situation in human pathology.

Introduction

The inflammasomes are large cytosolic multiprotein complexes composed of an initiator protein, such as a pattern recognition receptor, and an adaptor protein called ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)), which oligomerize to form an activating platform for an effector protein, pro-caspase-1. In addition to proinflammatory cytokine production and mediation of pyroptosis, inflammasomes also regulate the extracellular release of alarmins, such as high mobility group box 1 (HMGB1) protein, from both infected and activated immune cells, which promote cell proliferation and differentiation, with associated inflammation (9).

Inflammasome pathways

Hyperactivation of inflammasomes, either by genetic or environmental insult, may result in a wide range of pathological conditions, characterised by recurrent episodes of systemic and organ-specific inflammation (6). Despite the identification of a growing number of SAID disease genes, 40-60% of those with a typical SAID phenotype remain without a definitive diagnosis and, therefore, may be classified as having an uSAID (4). Inhibition of particular inflammasomes, or of the cytokines they release, have shown significant promise as treatments for both monogenic and uSAIDs, and this approach continues to be developed (10). The complex molecular mechanisms regulating the different inflammasomes means that it is key to identify the factors triggering inflammation in order to prescribe the optimal therapies. These complexities are a major cause of the variability in response currently associated

with therapies of various undifferentiated SAIDs, which may range from little or no effect to the development of severe adverse reactions (11).

In recent years, the discovery and classification of disorders as SAIDs, and an increased understanding of mechanisms of inflammasome activation (6, 12), have accelerated the development of therapeutics which target these mechanisms, such as IL-1 inhibitors, of which there are three currently being used in clinical practice. These are anakinra, a recombinant form of the receptor antagonist, IL-1Ra; rilonacept, a soluble chimeric Fc fusion protein of IL-1R1 and IL-1R3, and canakinumab, a monoclonal antibody that specifically neutralises IL-1β (13). These have all been shown to be highly effective for the treatment of NLRP3 inflammasome-related disorders, as well as in other conditions caused by activation of the NLRC4-inflammasome (14). Given their effectiveness in NLRP3 inhibition, further small molecule inhibitors of NLRP3 have been sought (15-17). The essential requirement of a structurally wild-type NACHT domain, however, is hindering their potential use in humans. According to the INFEVERS website [accessed 10/05/20] (18), 69/109 (63.3%) of pathogenic, or likely pathogenic, NLRP3 mutations are within this domain, which are predicted to impair inhibition by small molecules targeting the NLRP3 NACHT domain, such as MCC950 (19). Inhibition by these small molecules may still be effective, however, for individuals harbouring mutations elsewhere in the gene, such as the C-terminal region (20). These examples highlight the difficulties facing the clinician in identifying an effective therapeutic, and also underline the need to understand the underlying disease mechanism to choose the most appropriate therapeutic for each individual.

Stratification of SAIDs

Stratification of SAIDs is one method which will contribute to more effective therapeutic choices. Many clinical trials of potential therapies have produced negative results, likely because of differing molecular pathways being involved in individuals with the same clinical diagnosis. In a recent study, de Jesus et al. undertook three independent screening methods in parallel to pinpoint disease pathology in patients with an uSAID (21). The authors managed to classify or diagnose 79% of cases (n=52/66). DNA sequencing was the most effective method, but this can often take time. The IFN-response-gene score, combined with cytokine analysis, may therefore be a more effective short term approach (22). Even with a known molecular pathology, targeting of a pathway may not lead to an effective therapeutic. The identification of caspase I as the final step of the inflammasome pathway led to many programs developing caspase I inhibitors; however, very few made it to clinic (23).

Another method used to diagnose and stratify patients with uSAIDs is the identification of disease-specific biomarkers. In order to be an effective biomarker, a molecule must be secreted into an accessible tissue (e.g. serum or urine), be specific to a disease (or class of diseases), be stable outside of the body and detectible using a suitable diagnostic method. A few obvious biomarkers of inflammation, such as serum IL-1ß levels, are already being used to diagnose inflammatory disorders, as well as to guide therapeutic decision making (24). The development of high-content, high-throughput systems, however, have enabled the screening of larger numbers of biomarkers in large sample cohorts (25, 26). The major limiting factor now facing diagnostic laboratories is the paucity of validated, reliable and detectible biomarkers. This ability to screen patients and identify not only

120 their disease, but also a therapy targeted to their specific disease, is the ultimate
121 goal for personalised medicine.

122 Stratification of SAIDs using a genetics approach is becoming more widely used,
123 with recent studies suggesting that a molecular diagnosis can be obtained in up to
124 60% of cases, allied to potential novel insights into the underlying conditions. Despite
125 this, there are limited options for many rare disorders, either due to a lack of effective
126 therapeutics or knowledge limitations regarding the effects of available therapeutics.
127 Identifying biomarkers that are clinically actionable, such as elevated IL-1ß, may,
128 therefore, be more useful for therapeutic decision making than identifying the
129 underlying pathogenic mutation(s).

130

131 **Physiological inhibitors of the NLRP3 inflammasome**

132 In addition to known pharmacological inhibitors, a number of physiological
133 inhibitors, or negative regulators, of the NLRP3 inflammasome have been
134 discovered. Further research into these physiological inhibitors is likely to drive future
135 drug development to modulate the various mechanisms leading to inflammasome
136 activation, rather than current pharmacological therapies that target the subsequent
137 downstream effects such as cytokine release. Known physiological NLRP3
138 inflammasome inhibitors have a range of functions, such as inhibition of NLRP3
139 translation or suppression of oligomerisation. While a brief overview of these
140 mechanisms is described below, a detailed breakdown can be found in
141 Supplementary Table 1.

142 A reduction in the level of intracellular NLRP3 is one mechanism whereby the
143 NLRP3 inflammasome can be inhibited. The 3'-UTR of *NLRP3* in particular contains

144 conserved regulatory sequences, that may be targeted by at least four different
145 micro-RNA's (miR's). When over-expressed, these miR's result in reduced NLRP3
146 protein production, inflammasome activation and IL-1β release. Furthermore,
147 transcriptomic analysis has revealed that miR-223 levels, in particular, are inversely
148 proportional to NLRP3 protein (27). In addition to miRs, the RNA binding protein,
149 tristetraprolin, functions by a similar mechanism to reduce NLRP3 expression.
150 Protein degradation is another mechanism by which intracellular NLRP3 levels can
151 be reduced. E3-ubiquitin ligases that promote ubiquitination of NLRP3, resulting in its
152 degradation by the proteasome or the phagosome, have also been identified as
153 effective negative regulators.

154 In the presence of NLRP3, further inhibition of inflammasome formation can be
155 mediated through suppression of oligomerisation. Several direct interactors of
156 NLRP3, such as HSP70 and Kir6.1, have been identified as key negative regulators,
157 by holding NLRP3 in an inactive state and thus unable to bind ASC (Supplementary
158 Table 1). One exception is Sirtuin-2 , an NAD-dependent deacetylase, which doesn't
159 bind NLRP3 directly, but instead uses microtubules to prevent NLRP3 and ASC
160 coming into close proximity (28). In addition to physical separation of NLRP3 and
161 ASC to prevent formation of the inflammasome, Sirtuin-2 can also deacetylate
162 NLRP3 to supress oligomerisation. Phosphorylation of Ser-295 by protein kinase A
163 (PKA) also impairs inflammasome assembly, by the loss of NLRP3 ATPase activity
164 (29). A family of inflamasome inhibitors, known as PYRIN domain-only proteins
165 (POPs) and CARD-only proteins (COPs) have also shown promise as inhibitors of
166 the inflammasome, by binding to NLRP3, or other regulatory proteins such as NF-
167 κB, to block its activation (30). While these proteins are endogenously expressed, it

remains unclear to what effect they respectively inhibit inflammation in unstimulated, inflammasome stimulated and mutation-containing cells.

The NLRP3 inflammasome is highly sensitive to homeostatic changes in the cytosol, with changes in potassium ion concentrations able to trigger inflammation. Loss of proteins involved in the regulation of potassium ion efflux can, such as LPIN2, therefore result in NLRP3 inflammasome activation. This includes proteins that interact and regulate the ATP-induced opening of the P2X7 receptor channel, a key receptor for triggering NLRP3 inflammasome activation (31).

Failures in autophagy and mitophagy have both been linked with NLRP3 inflammasome activation. NLRP3 has been shown to bind key autophagy-associated proteins, such as unc-51 like autophagy activating kinase 1, which facilitate degradation of proteins, including NLRP3 itself. Mitochondrial defects, resulting in an increase in cytosolic reactive oxygen species (ROS), are also known to cause increased NLRP3 activation, with evidence of this process being reversed by the addition of anti-oxidants (32).

A key function of the NLRP3 inflammasome is the activation of pro-caspase-1 into its active form, which in turn cleaves cytokine precursors such as pro-IL-1β. A failure to recruit and activate pro-caspase-1 is, therefore, a mechanism by which the effects of NLRP3 inflammasome activation can be negated. Loss of the capase-1 inhibitor Flightless-1, or either of its known adaptor proteins, BCAP and LRRFIP2, have been shown to result in reduced levels of active caspase-1 and IL-1β, following NLRP3 inflammasome activation (33, 34).

Although targeting of negative regulators of the NLRP3 inflammasome, or of the cellular mechanisms they regulate, may be effective for disorders driven by

overactive NLRP3 inflammasomes, they may not be effective for all patients harbouring NLRP3 activating mutations. This is due to nature of the mutations altering protein binding sites, particularly around the NACHT domain (Fig. 1). Further research is therefore required to determine the effects of specific pathogenic variants on inflammasome inhibition.

Influence of CAPS mutations on NLRP3 inflammasome regulation

Gain-of-function mutations in *NLRP3* lead to CAPS, which encompass neonatal-onset multisystem inflammatory disease (NOMID), also known as CINCA (chronic infantile neurological cutaneous and articular syndrome), Muckle-Wells syndrome (MWS), and familial cold auto-inflammatory syndrome (FCAS). This group of disorders range in severity, but all are characterised by increased release of proinflammatory cytokines, particularly IL-1β and IL-18, leading to systemic inflammation (35). Whilst there is an extensive database of documented *NLRP3* sequence variants (18), many of which are confirmed to be related to disease states in humans, the mechanisms by which these mutations lead to pathologies are often largely overlooked.

CAPS mutations are known to disrupt various intrinsic processes that control NLRP3 regulation, for example post translational modifications, such as phosphorylation, ubiquitination and sumoylation, which have been widely reviewed (36-38). Phosphorylation has been established as a vital regulatory post-translational modification by which different signalling pathways can be recruited to both positively and negatively regulate NLRP3 activity (39). Mortimer et al. scrutinised PKA inhibition of NLRP3, which is instigated by the binding of prostaglandin E2 (PGE2) to

the EP4 prostanoid receptor, which in turn induces the cyclic adenylyl monophosphate (cAMP)-mediated stimulation of PKA (Fig. 2). This particular study showed that PKA directly inhibits the human NLRP3 inflammasome through disassembly of ASC oligomers, a process dependent on the PKA-mediated phosphorylation of Ser295 (29). This amino acid is located within the nucleotide-binding domain (NBD), and its modification therefore attenuates NLRP3 ATPase function. Furthermore, of the seven Ser295-adjacent CAPS mutations tested (I290M, M301V, G303S, E306K, Q308K, G309S or F311L) (Fig. 2), all but M301V were unresponsive to rapid cAMP-induced inhibition. Additionally, the inhibition of NLRP3-mediated cleavage of IL-1β by PGE2 required significantly higher (10- to 100- fold) concentrations of PGE2 in PBMCs from CAPS patients with mutations in the NBD domain compared to their WT equivalents. Notably, PBMCs from patients harbouring the Ser295-proximal MWS-related mutations, D303N and E313K, were less sensitive to inhibition of mature IL-1β release in response to physiologically relevant PGE2 concentrations (40). Together, these studies suggest a way in which these disease-related mutations may result in poorly regulated inflammasome activation.

In addition to PKA, Ser295 is phosphorylated by protein kinase D (PKD). A study by Zhang et al. showed that NLRP3 inflammasome activators cause mitochondria-associated endoplasmic reticulum membranes to localise to the Golgi, and that NLRP3 is recruited to these membranes. Associated with this is an accumulation of diacylglycerol, which recruits PKD, and its phosphorylation of Ser295 is both necessary and sufficient for NLRP3 inflammasome activation (Fig. 3) (41). This study concluded that PKD inhibition can block NLRP3 activity in PBMCs taken from patients exhibiting uncontrolled inflammasome activation. However, the mutations tested, T436N and R260W, are further away from Ser295 than those previously

examined (Fig. 3) (29), and, as such, testing whether CAPS-related mutations proximal to Ser295 are sensitive to PKD inhibition would be valuable. As previously noted, it is also possible that Ser295 phosphorylation plays multiple roles regarding NLRP3 inflammasome activation, and that a process of sequential phosphorylation and dephosphorylation may exist.

Bruton tyrosine kinase (BTK) has also been proposed to play a role in NLRP3 inflammasome regulation. BTK inhibition reduces NLRP3-mediated IL-1β release from MWS PBMCs (42), although the precise role of BTK with respect to the NLRP3 inflammasome is still under debate, due to evidence that BTK deficiency leads to exacerbated inflammasome activation (43). BTK-mediated phosphorylation of multiple NLRP3 tyrosines (Y136, Y140, Y143 and Y168) (Fig. 4) has been implicated as a molecular switch of inflammasome activity by weakening phosphatidylinositol 4-phosphate (PtdIns4P) interactions, resulting in shorter retention to the Golgi with enhanced NLRP3 inflammasome oligomerisation and increased IL-1β secretion (Fig. 4) (44). When mapped onto the NLRP3-NEK7 cryogenic electron microscopy (cryo-EM) structure (45), Y168 is in the vicinity of several likely pathogenic CAPS mutations, as well as a putative ADP molecule, and thus may influence nucleotide binding (Fig. 4) (44). This may provide valuable insights into a further mechanism impacting NLRP3 inflammasome regulation.

In addition to impacting phosphorylation-mediated NLRP3 regulation, CAPS mutations may affect sumoylation. Lys689, specifically, has been identified as a site of both ubiquitination and sumoylation in human NLRP3 (Fig. 5) (46, 47). Sumoylation negatively regulates NLRP3 at multiple sites, and mutation of Lys689 results in hyperactivation of NLRP3, which phenocopies that seen in CAPS patients (47). Although the introduction of single disease-related mutations (R260W, E690K

and E692K) did not reduce the overall level of NLRP3 sumoylation, it was suggested in this study that the similarity in phenotype between the K689R mutant and disease-related E690K and E692K mutants may be due to their location in the predicted SUMO motif surrounding K689, leading to disrupted inflammasome regulation (47).

Besides post-translational modifications, interactions between NLRP3 and other proteins are also influenced. Caspase recruitment domain-containing protein 8 (CARD8), also known as Cardinal, is one such protein. CARD8, a member of the CARD family, consists of an N-terminal function to find (FIIND) domain and a C-terminal CARD domain, and genetic alterations in *CARD8* have been associated with numerous inflammatory diseases (48). CARD8 is a negative regulator of NLRP3, which inhibits IL-1β release by interacting with the NACHT domain (49, 50). A frameshift variant of CARD8, which results in loss of the FIIND and CARD domains, failed to interact with the NACHT domain of NLRP3, and has been implicated in the pathogenesis of a childhood syndrome called periodic fever with aphthous stomatitis, pharyngitis, and cervical adenitis (51). Similarly, the disease-associated NLRP3 mutants R260W, D303N, N477K and H312P all abolish the interaction between CARD8 and NLRP3, and, as a result, IL-1β secretion is not reduced following CARD8 overexpression, as seen with the WT form. It has been postulated that changes in local structure caused by these polymorphisms may disrupt certain interactions between NACHT and CARD8 (50). Additionally, loss of CARD8 binding is associated with diminished NLRP3 serine phosphorylation (52), suggesting this as an additional mechanism by which CAPS mutations, in disrupting CARD8-NLRP3 interactions, cause disease states.

NIMA-related kinase 7 (NEK7) is a highly conserved serine/threonine kinase which acts as a regulator of mitotic processes (53) that has been identified as a mediator of

291 the NLRP3 inflammasome (54-56). This regulation occurs independently of its kinase
292 activity (54, 55), but is dependent on interactions between NEK7 and the central
293 NOD domain and leucine-rich repeats (LRRs) of NLRP3. This was initially
294 determined by using deletion mutants, followed by the cryo-EM structure of NLRP3,
295 which showed the C-terminal lobe of NEK7 nestled against the curved LRR and
296 globular NACHT domains of NLRP3. Although mutations in the LRR domain are less
297 frequently reported, this region is implicated several pathologies; NLRP3 harbouring
298 the NOMID-related missense mutations, G755A and G755R (57, 58), in the LRR
299 domain bind to NEK7 with a higher affinity than the WT protein, whereas the
300 hypomorphic missense NLRP3 mutant, D946G, associates less strongly (Fig. 6)
301 (55). Additionally, oridonin, the bioactive component of *Rabdosia rubescens,* which is
302 often used in traditional Chinese medicine to treat inflammatory disorders, has
303 recently been identified as directly targeting NLRP3 (16). Oridonin binds covalently
304 to Cys279 in the NACHT domain, thereby blocking the NLRP3/NEK7 interaction and
305 preventing formation of the inflammasome complex [29]. Together, this indicates that
306 some CAPS mutations could cause amplified inflammatory responses by stabilising
307 the binding between NLRP3 and NEK7, and that a fine-tuned binding affinity
308 between these proteins is vital for normal NLRP3 inflammasome function.

309 Most CAPS mutations, as identified in the Infevers database (18), can be found in
310 the NACHT domain, surrounding the ADP binding site (59) (Fig. 1). Within the
311 NACHT domain are found both a Walker A motif, which mediates ATP binding, and a
312 Walker B motif, responsible for ATP hydrolysis (60). These two sites are vital to
313 efficient inflammasome function, since ATP binding is required for both the normal
314 functioning of WT NLRP3, and for the constitutive activity of disease-associated
315 mutants (61). This has been further highlighted by the fact that NLRP3 inhibitors bind

316 to these motifs; MCC950 directly binds to the Walker B motif (62) and CY-09 to the
317 Walker A motif (17), thereby preventing NLRP3 inflammasome function.
318 Determination of NLRP3 by cryo-EM has allowed a series of validated CAPS
319 mutations to be mapped onto the structure and examined in greater detail. Residues
320 D303 and E304, both associated with NOMID/MWS in humans (63, 64), are located
321 on the Walker B motif of the ATPase, and, as such, may directly influence 'sensing'
322 of the nucleotide-binding state. It seems likely that these mutations affect nucleotide
323 binding and hydrolysis and, consequently, NLRP3 inflammasome activity, although
324 current studies have not investigated the effects of NLRP3 mutations on their affinity
325 for nucleotide binding.

326

327 **Inflammasomes and structures of their components**

328 To date, several inflammasomes structures and their integral components have
329 been determined. The NLRC4 inflammasome structure has arguably been the most
330 thoroughly explored, with high resolution cryo-EM (65) and crystal (66) structures in
331 their inactive and active states, in addition to a cryo-EM structure of the NLRC4
332 CARD filament (67). However, recent efforts have also resulted in the publication of
333 a cryo-EM structure of inactive human NLRP3, in complex with NEK7 (45). This
334 represents a potentially huge leap forward in our ability to investigate the most
335 studied of the inflammasomes, both in terms of its intrinsic function and for future
336 inhibitor design.

337 The greater availability of structural information has aided the understanding of the
338 functional roles of the NLRP3 domains, for example the N-terminal pyrin domain
339 (PYD). This plays a vital role in mediating NLRP3 inflammasome assembly via high-

affinity PYD/PYD interactions between NLRP3 and ASC (68), the latter also forming a CARD/CARD interaction with caspase-1 (69). A crystal structure of the NLRP3 PYD revealed a canonical 6-helical bundle structural fold, similar to other PYDs, and the charged peripheral surface, which largely facilitates the NLRP3-ASC interaction by charge complementarity (70). One notable aspect of the structure was a highly conserved disulfide bond present between the PYD and the nucleotide-binding site domain (70). This bond has been suggested to be involved in NLRP3 inflammasome sensitivity to altered redox states, mediated by ROS (71), highlighting a potential regulatory mechanism.

The central NACHT domain possesses ATPase activity, and comprises an NBD, helical domain 1 (HD1), winged helix domain (WHD) and helical domain 2 (HD2). The recently determined NLRP3 cryo-EM structure featured the globular NACHT domain with ADP bound to the NBD (45). This structure gave some valuable insights, including into the CAPS mutations primarily localised to the NACHT domain as described above (18). Mapping of the validated pathogenic NLRP3 mutations showed that the majority surround the ADP-binding site (Fig. 1), potentially affecting the inactive conformation of NLRP3 and thereby enhancing inflammasome activation (45). In addition, when modelled onto the previously determined NLRC4 oligomer structure (66), a 90° rotation of the NBD and HD1 regions relative to the WHD, HD2 and LRR, likely necessary for oligomerisation and activation, was observed (45).

The LRR domain has previously been implicated in the recognition of stimuli, mediation of protein-protein interactions and maintaining the inflammasome in an inactive state (72, 73). The deubiquitinase, BRCC3, has been shown to mediate a regulatory mechanism by which ubiquitinated NLRP3 remains inactive until priming signals initiate BRCC3-mediated deubiquitination of the LRR domain, allowing

NLRP3 to oligomerise (74). However, more recent structural and functional studies have raised questions concerning the role played by the LRR domain in the NLRP3 inflammasome.

LRR domains vary between inflammasomes; in NLRP3 this domain consists of 12 repeats encircling NEK7. The main protein-protein interactions exist between NEK7 and the LRR and NACHT domains of NLRP3, and mutagenesis identified the interactions between the LRR and NACHT HD2 domains in NLRP3, and the first half of the C-lobe of NEK7 to be the most essential (45). Indeed, NEK7 with the second half of the C-lobe removed, which interacts with the NACHT domain, still binds to NLRP3 (54), highlighting the importance of the LRR domain in this interaction. Additionally, of the two major NLRP3 isoforms identified in humans, the variant lacking exon 5 resulting in a truncated LRR domain, exhibits a loss of activity due to a lack of NLRP3-NEK7 interaction (75).

At first glance, there is clear evidence for the LRR domain playing an important role in NLRP3-NEK7 interactions, which are known to be important due to the essential role NEK7 plays in inflammasome function (54, 55). However, other studies have shown that this domain is not required for NLRP3 activation. NLRP3 mutants lacking LRRs are constitutively active when expressed in THP-1 (76) or HEK293T (77) cells. Furthermore, of the more than 20 NLRP3 variants produced by Hafner-Bratković et al., macrophages expressing NLRP3 lacking all LRRs responded to canonical stimuli to a comparable degree to that seen in the WT protein (78). Together, these studies indicate that further investigation is required to delineate the role of the LRR domain in NLRP3 regulation, and that a structure of NLRP3 in the active state would be invaluable.

NLRP3 inflammasome inhibitors – design and target identification

Current small molecule inhibitors of the NLRP3 inflammasome were largely discovered prior to publication of the NLRP3/NEK7 cryo-EM structure (45), limiting the opportunities for rational drug design. Some inhibitors have been discovered by elucidating the mechanisms by which existing medicines work, for example oridonin, the major active constituent of many traditional Chinese medicines, directly targets the NLRP3 inflammasome (16). Similarly, tranilast was originally recognised as an anti-allergic agent (79) but was later found to bind the NACHT domain directly (80). Screening of compound libraries has also proven effective when discovering novel inhibitors. For example, 3,4-methylenedioxy-β-nitrostyrene (MNS) was found through the screening of a library of 160 kinase inhibitors (81), and CY-09 resulted from optimisation of the structure of C172, an inhibitor identified through screening of an in-house bioactive compound library (17). Both Bay 11-7082, along with several structurally related vinyl sulfone compounds, and BOT-4-one, were discovered via screening NF-κB inhibitors (82, 83), and MCC950 (initially named CP412,245, then CRID3), arguably the best characterised NLRP3 inhibitor, was found in a rational screen for inhibitors of IL-1β production consisting of pharmacological agents known to affect ion transport activity. This study identified a series of diarylsulfonylurea-containing compounds structurally related to glyburide, including MCC950, which was later found to inhibit NLRP3 specifically (84).

Whilst these methods have delivered a wide range of antagonists, the NLRP3 structure, with advancement in our understanding of inflammasome structure that this represents, has opened up avenues for the rational design of NLRP3

modulators. Existing inhibitor binding sites have also become clearer; for example, the NACHT domain is the site of action for many well-known inhibitors including MCC950, which was shown in a pair of associated studies to interact with the NACHT domain Walker B motif (19, 85). Therefore, this information could signify an important advancement in rational drug design, which is becoming more common with the increasing availability of protein structures (86). As the NLRP3 structure was recently determined at the time of publication (45), it is too early to say whether this approach will be adopted for the development of NLRP3 inflammasome inhibitors. Few published studies have explored the structure of NLRP3, or related proteins, as a basis for virtual screening. Abdullaha et al. have produced a homology model of NLRP3, based on the crystal structure of the NLRC4 inflammasome (66), and used this to dock and develop compounds based around a 2-aryl quinazolin-4(3H)-one scaffold (87). In contrast, Pal et al. used the structures of the human NLRP3 PYD (70) and the human ASC pyrin domain filament (88) to design α-helical stapled peptides capable of targeting the ASC PYD and disrupting ASC filament formation (89). Whilst this represents the beginning of a potentially burgeoning field of structure-based NLRP3 inflammasome inhibitor design, whether this approach will be widely adopted is yet to be seen.

NLRP3 inhibitors in clinical trials

As the NLRP3 inflammasome is directly involved in the pathogenesis of many different diseases, inhibitors of NLRP3 activation are being rapidly developed for a wide range of human disorders, including autoinflammatory, metabolic,

436 cardiovascular and neurodegenerative conditions. NLRP3 inhibitors currently
437 undergoing clinical trials are described below and summarised in Table 1.

438 Diacerein is an anthraquinone, an aromatic organic compound approved in Europe
439 and in the USA for treatment of osteoarthritis (90). In a model of gout, it was shown
440 to suppress protein expression and activity of caspase-1, as well as formation of
441 NLRP3 inflammasome complexes, as visualised by confocal microscopy (90). Its
442 ointment formulation, AC-203, has also received FDA approval for the treatment of
443 generalised severe epidermolysis bullosa simplex (91). This genetic condition is a
444 blistering skin disease with neutrophilic inflammation, associated with mutations in
445 the *KRT5* or *KRT14* genes, encoding keratin 5 and keratin 14 proteins respectively.
446 Diacerein is currently being tested in phase II clinical trials for epidermolysis bullosa
447 simplex and ocular surface disease (clinicaltrials.gov identifiers NCT03389308 and
448 NCT04351100, respectively).

449 Dapansutrile (OLT1177) is a β-sulfonyl nitrite compound that inhibits NLRP3
450 ATPase activity and NLRP3-dependent ASC specks aggregation (92). Dapansutrile
451 has been shown to reduce acute gout pain and inflammatory biomarkers in a phase
452 IIA trial (93). It is currently being developed in phases I/II for systolic heart failure and
453 Schnitzler's syndrome (clinicaltrials.gov identifiers NCT03534297 and
454 NCT03595371, respectively).

455 Inzomelid is an oral, brain-penetrant inhibitor of NLRP3-containing inflammasomes;
456 a phase I trial has been completed and it has alleviated symptoms in one CAPS
457 patient (94). A phase II study for CAPS is being planned (94). The brain-penetrant
458 nature of this inhibitor raises the possibility that this selective, small molecule

inhibitor of the NLRP3 inflammasome will soon be tested in neurodegenerative disorders.

Tranilast is a trytophan analog that inhibits NLRP3-induced ASC speck formation, and IgE-induced histamine release from mast cells. It is in clinical used in Japan, China and South Korea, to treat asthma, allergic conjunctivitis and hypertrophic scars (95). Affinity precipitation assays of biotinylated tranilast have showed that this compound directly binds to NLRP3 and thereby impedes its oligomerisation (80). A phase II study of tranilast in CAPS is currently recruiting, and trials are also planned for sarcoidosis, scleredema diabeticorum and mucinoses (clinicaltrials.gov identifiers NCT03923140, NCT03528070, NCT03512873 and NCT03490708, respectively).

No human clinical trial is currently registered for MCC950, to the best of our knowledge. Of note, a phase II study has been conducted in rheumatoid arthritis but was interrupted because of liver enzyme elevation in the treatment group (96).

Although not directly interacting with NLRP3, the most promising agent that modulates innate immune activation in neurological disorders is masitinib, a tyrosine kinase inhibitor targeting c-KIT (97). C-KIT is a receptor tyrosine kinase constitutively expressed by mast cells and macrophages, and intriguingly, mastinib has also been revealed to act as an NLRP3 activator in BMDMs (98). Masitinib also inhibits other significant kinases, such as Lyn and Fyn, in microglial cells and macrophages (97, 99). Mastinib slowed the progression of lateral amyotrophic sclerosis in a randomised placebo-controlled study (99) and has also been reported to delay the evolution of primary progressive and non-active secondary progressive multiple sclerosis (100). A phase III study is currently ongoing in Alzheimer's disease, with an interim analysis showing a positive trend in one of the treatment arms (101). Defining

the cell-specific impact of masitinib on the human NLRP3 inflammasome is likely to increase our understanding of neurodegenerative and neuroinflammatory disorders in the near future.

Future directions

NLRP3 inflammasome activation is linked to a much wider range of human diseases than autoinflammation, namely cardiovascular, neurodegenerative, neuroinflammatory and oncologic conditions. In CAPS, a given NLRP3 inhibitor may only work on a small proportion of patients and treatment will probably be tailored to each mutation. In neurological disorders, NLRP3 inhibitors could be revolutionary, since few treatments are currently effective, although blood-brain barrier penetration and cell-specific targeting of inflammation will be decisive to tailoring therapy and reducing side effects. NLRP3 inhibition in cancer is in its infancy but holds great potential. Indeed, arsenic compounds, already approved in promyelocytic leukemia, are direct inhibitors of NLRP3 (102). Another example is acute lymphoblastic leukemia, where glucocorticoid resistance is caused by NLRP3 overactivation, and is reversed by caspase-1 inhibitors (103). In the field of polygenic inflammatory diseases, NLRP3 inhibitors will compete with disease-modifying anti-rheumatic drugs (DMARDs), many of which, such as methotrexate (104), hydroxychloroquine (105), or ciclosporin (106) are indirect inhibitors of NLRP3. Conversely, glucocorticoids induce NLRP3 overexpression (107), which suggests that NLRP3 inhibitors may act synergistically and could, potentially, solve the problem of glucocorticoid resistance in inflammatory and haematologic diseases. Altogether, this highlights the potential

benefits to human health of fully understanding the mechanisms by which the NLRP3 inflammasome is negatively regulated.

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Immunome project consortium for Autoinflammatory Disorders (ImmunAID)

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1: The NLRP3/NEK7 complex domains and related CAPS mutations

A 3D cartoon representation of NLRP3 in complex with NEK7 is shown on the left, with the pyrin domain omitted from NLRP3 and the N-lobe from NEK7. Domains are colour-coded as outlined in the 2D depiction on the right, with domains not represented show in grey. Validated disease-causing CAPS mutations are shown as yellow sticks and are described in further detail in Supplementary Table 2.

Figure 2: PKA-mediated NLRP3 inhibition pathway and the relevant CAPS mutations

The protein kinase A (PKA) signalling pathway is shown on the left. Activation of the EP4 receptor by prostaglandin E2 (PGE2) binding causes the Gαs subunit to dissociate from the receptor. Gαs, in turn, induces adenylate cyclase (AC) activity, which mediates the synthesis of ATP into cyclic adenosine monophosphate (cAMP), which subsequently activates protein kinase A (PKA), leading to the PKA-mediated phosphorylation of Ser295. This phosphorylation is disrupted by the NLRP3 mutations shown in the white box. The location of CAPS-related amino acids is shown on the right, in both the whole receptor (top) and a magnified view (bottom), with yellow sticks representing mutated residues and blue sticks showing phosphorylated residues.

Figure 3: PKD-mediated NLRP3 activation pathway and the relevant CAPS mutations

The protein kinase D (PKD) signalling pathway is shown on the left. Mitochondria-associated endoplasmic reticulum membranes (MAMs) localise adjacent to Golgi membranes in response to inflammasome activators, which is related to enhanced diacylglycerol (DAG) production at the Golgi. DAG, in turn, binds to PKD, which phosphorylates NLRP3 proteins retained at MAMs adjacent to the Golgi at Ser295. This phosphorylation allows NLRP3 to dissociate from the MAMs and assemble into the active inflammasome. The location of CAPS-related amino acids is shown on the right, in both the whole receptor (top) and a magnified view (bottom), with yellow sticks representing mutated residues and blue sticks showing phosphorylated residues.

Figure 4: BTK-mediated NLRP3 modification pathway and the relevant CAPS mutations

The bruton tyrosine kinase (BTK) signalling pathway is shown on the left. BTK is rapidly activated by NLRP3 inflammasome activators, and modifies four tyrosines in NLRP3 (Y136, Y140, Y143 and Y168). This alteration weakens the interactions between NLRP3 and phosphatidylinositol-4-phosphate (PtdIns4P) at the Golgi membrane, leading to dispersion and subsequent oligomerisation of NLRP3. The location of CAPS-related amino acids is shown on the right, in both the whole receptor (top) and a magnified view (bottom), with yellow sticks representing mutated residues and blue sticks showing phosphorylated residues.

Figure 5: CAPS mutations affecting NLRP3 sumoylation

878 Residues implicated in CAPS disruption of NLRP3 sumoylation are shown in the
879 protein as a whole (top) and as a magnified view (bottom), with yellow sticks
880 representing mutated residues and blue sticks showing the sumoylated residue.

881

882 **Figure 6: CAPS mutations affecting NLRP3/NEK7 interactions**

883 Residues implicated in CAPS disruption of NLRP3/NEK7 interactions are shown in
884 the protein as a whole (top) and as a magnified view (bottom), with yellow sticks
885 representing mutated residues.

886

887 **Table 1: NLRP3 inflammasome inhibitors in clinical trials**