Drugging the NLRP3 inflammasome: from signaling mechanisms to therapeutic targets

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Abstract

Diseases associated with chronic inflammation constitute a major health burden across the world. As central instigators of the inflammatory response to infection and tissue damage, inflammasomes – and the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome in particular - have emerged as key regulators in diverse rheumatic, metabolic and neurodegenerative diseases. Similarly to other inflammasome sensors, NLRP3 assembles a cytosolic innate immune complex that activates the cysteine protease caspase-1, which in turn cleaves Gasdermin D (GSDMD) to induce pyroptosis, a regulated mode of lytic cell death. Pyroptosis is highly inflammatory, partly because of the concomitant extracellular release of the inflammasome-dependent cytokines interleukin (IL)-1β and IL-18 along with a myriad of additional danger signals and intracellular antigens. Here, we discuss how NLRP3 and downstream inflammasome effectors such as GSDMD, ASC and NINJ1 have gained significant traction as therapeutic targets. We highlight the recent progress in developing small molecule and biologics inhibitors that are advancing into the clinic and serving to harness the broad therapeutic potential of modulating the NLRP3 inflammasome.

[H1] Introduction

Inflammation is the primary immune response to alert the body to an infection or tissue damage. The process starts within minutes or hours after a threat has been identified and is driven by innate immune cells that express cell surface or cytosolic Pattern Recognition Receptors (PRRs), which sense the presence of potentially harmful microbial agents and signs of tissue damage[1]. PRRs are viewed as an intrinsic component of the innate immune system and they directly or indirectly sense and respond to non-specific pathogen-associated molecular patterns (PAMPs), which are derived from microorganisms, and danger-associated molecular patterns (DAMPs), which are produced by or derived from damaged host cells[1].

PRRs are expressed by myeloid cell types such as tissue resident macrophages, dendritic cells, granulocytes, and marrow and blood monocytes, and also by non-hematopoietic cells at mucosal interfaces such as keratinocytes in the skin, and epithelial cells in the lung and intestines. They are strategically distributed to survey the extracellular and intracellular environments and comprise several different receptor families. Membrane-bound receptors such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are located on the cell surface or in endosomes. Cytosolic receptors such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), NACHT, LRR and PYD domains-containing proteins (NLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), and members of the tripartite motif (TRIM) receptors function to detect intracellular PAMPs or DAMPs[1]. Intracellular signaling cascades initiated by these receptor families largely converge on activation of key inflammatory transcription factors such as nuclear factor kappa B (NF-κB) and interferon regulatory factors (IRFs), resulting in the production of pro-inflammatory cytokines, chemokines and type I interferons (IFNs). Together, these signaling molecules shape the inflammatory response and coordinate downstream activation of the adaptive immune system[2]. Many of these secreted inflammatory mediators exit the cell via the classical ER–Golgi protein secretion pathway, but some, such as the proinflammatory cytokines interleukin (IL)-1β, IL-18, IL-1α, IL-33, IL-36 and high mobility group box 1 (HMGB1) lack the leader sequences required for conventional secretion through this route. They are passively released from lysed cells into the extracellular environment, although non-conventional secretion from live cells has also been proposed. Once in the extracellular environment, these inflammatory cytokines and DAMPs initiate and perpetuate an inflammatory response by recruiting and activating other immune cells. Among these inflammation-eliciting factors, IL-1 plays a central role because minute amounts are capable of inducing fever, activating lymphocytes, promoting the transmigration of leukocytes to the inflamed tissue and increasing inflammatory markers such as IL-6 and the acute phase reactants C-reactive protein (CRP) and serum amyloid A (SAA).

As the core signaling hubs driving IL-1ß activation and release, inflammasomes have emerged as critical multiprotein assemblies that promote inflammatory responses and support tissue repair after infection or tissue damage. Inflammasome complexes typically consist of a cytosolic PRR, the bipartite adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the inflammatory protease caspase-1[3,4]. The most well-studied PRRs that form inflammasomes include the NLR proteins NLRP1, NLRP3 and NLRC4; the NLRP1-related CARD8 sensor; the ALR protein AIM2; and the tripartite motif-containing (TRIM) family member Pyrin (**Figure 1**)[5]. Except for CARD8, which recruits caspase-1 directly, these inflammasome sensors oligomerize and recruit ASC upon sensing their respective ligands or upstream signals. ASC then undergoes prion-like polymerization to assemble a single, massive fibrillary structure known as the 'ASC speck', which binds and enables the proximity-induced activation of caspase-1[3,4]. Once active, caspase-1 cleaves pro-IL-1β and pro-IL-18 into the cognate inflammatory cytokines, as well as cleaving gasdermin D (GSDMD)[6-8]. GSDMD cleavage liberates the pore-forming N-terminal region (GSDMD NT) from the autoinhibitory C-terminal domain (GSDMD CT), after which GSDMD NT migrates into the plasma membrane to oligomerize into large protein pores that support unconventional protein secretion of bioactive IL-1β and IL-18, and induce ion fluxes across the plasma membrane[9-12]. GSDMD-induced plasma membrane perforation eventually leads to pyroptosis, a lytic mode of cell death that is characterized by osmotic swelling and plasma membrane rupture (PMR), releasing soluble intracellular DAMPs such as HMGB1 and lactate dehydrogenase (LDH) into the extracellular space[13]. Only recently, the cell-surface protein Nerve injury-induced protein 1 (NINJ1) has been demonstrated to drive PMR and extracellular release of HMGB1 and LDH downstream of GSDMD, a process which was long thought to be an uncontrolled and passive event[14].

Each inflammasome is named after its respective PRR, is activated in a stimulus-specific manner and exhibits a specific expression pattern (**Figure 1**). Human NLRP1 is the most prominent inflammasome sensor in human keratinocytes and lung epithelial cells, as reflected by gain-of-function mutations in the *NLRP1* gene that cause severe inflammatory and neoplastic syndromes which primarily affect the skin and respiratory tract[5]. The NLRC4 inflammasome is an important driver of intestinal inflammation in response to enteric pathogens as well as in patients with autoinflammatory diseases caused by activating mutations in *NLRC4*. Human CARD8 mediates inflammasome activation in monocytes, endothelial cells and resting lymphocytes, thus extending the relevance of inflammasome signaling and pyroptotic cell death to key cells of the vascular and adaptive immune system[5,15]. The cytosolic double-stranded (ds)DNA-sensing inflammasome sensor AIM2 is highly upregulated by type I IFNs in murine macrophages, and has been implicated in preclinical disease models of autoimmunity, atherosclerosis, neurodegeneration and cancer

immunotherapy[16-18]. However, the significance of AIM2 in sensing dsDNA in the human system is less clear[5]. Recent work showed that the synthetic dsDNA mimetic poly(dA:dT) is sensed by the NLRP1 inflammasome in human keratinocytes[19]. Moreover, AIM2 is abundantly expressed in human monocytes, yet cytosolic dsDNA detection by the cGAS–STING pathway in human monocytes promotes inflammasome activation via NLRP3[20]. The NLRP3 and Pyrin inflammasomes exert their function primarily in cells of the myeloid lineage, and activating point mutations in their coding regions cause prototypic autoinflammatory diseases (**Box 1**).

Although each of these inflammasome pathways induces inflammatory responses under specific conditions, the NLRP3 inflammasome in particular is involved in the pathogenesis of an exceptionally wide range of chronic inflammatory, metabolic and neurodegenerative diseases[3,4]. Moreover, NLRP3 has emerged as an actionable drug target, whereas pharmacological modulation of other inflammasome pathways awaits the discovery of potent and selective tool compounds. In parallel to continued progress in understanding NLRP3 biology, extensive drug discovery efforts in the past few years have given rise to several NLRP3-targeted drug candidates that are progressing through preclinical and clinical stages of development. This review will first discuss recent insights into NLRP3 inflammasome biology and the signaling mechanisms regulating its activation. We will then proceed to describe how fast-paced progress in understanding NLRP3 signaling is converging with structural biology and medicinal chemistry efforts to support the development of NLRP3 inhibitors and agonists. Additionally, we will highlight emerging alternative strategies for inflammasome blockade based on targeting the downstream inflammasome effectors ASC, GSDMD and the recently identified NINJ1.

[H1] The NLRP3 inflammasome

NLRP3 responds to a large and diverse set of pathophysiological and chemically unrelated stimuli, including high extracellular ATP concentrations, bacterial ionophores and pore-forming toxins, RNA viruses, and phagocytosed lysosome-damaging agents such as crystalline silica, cholesterol, and monosodium urate (MSU)[3,4]. Given this extraordinary diversity, NLRP3 stimuli are hypothesized to converge onto NLRP3 activation by eliciting a common second messenger that is sensed by NLRP3. Although the precise identity of such a common conduit for NLRP3 activation has remained elusive, most NLRP3-activating agents cause damage to the plasma membrane coinciding with a drop in cytosolic K^+ levels[21]. Calcium mobilization, chloride efflux, cytosolic translocation of oxidized dsDNA from damaged mitochondria and lysosome rupture are some of the mechanisms that have been proposed to act apically on NLRP3 activation. However, stimuli that have these various cellular effects are also likely to converge on plasma membrane

damage and K^+ efflux as the unifying signal leading to NLRP3 activation[21]. Nevertheless, NLRP3 activation also occurs in a K^+ efflux-independent manner and without plasma membrane damage in response to certain stimuli, such as in human monocytes stimulated with the TLR7 ligand imiquimod or extracellular lipopolysaccharide (LPS; a component of the outer membrane of Gram-negative bacteria that stimulates TLR4)[22,23]. NLRP3 might therefore have parallel activation mechanisms, or there might be a more downstream converging activation mechanism that remains to be discovered.

[H2] NLRP3 activation pathways

Although the molecular mechanisms driving NLRP3 activation remain to be fully elucidated, NLRP3 inflammasome activation is known to proceed through three distinct signaling pathways that are referred to as the canonical, non-canonical and alternative NLRP3 inflammasome pathways, respectively (**Figure 2**). Most stimuli engage the canonical NLRP3 inflammasome pathway (**Figure 2a**). NLRP3 activation through this pathway is generally viewed as a bi-phasic process that involves an initial priming phase followed by a subsequent activation step. Because basal NLRP3 expression levels are low in most naïve myeloid cells, the priming phase primarily serves to transcriptionally upregulate NLRP3 expression, usually via TLR-induced NF-κB–dependent signaling pathways[24]. Non-transcriptional NLRP3 priming mechanisms that reduce the threshold for NLRP3 inflammasome activation by phosphorylating or attaching other post-translational modifications to specific residues of NLRP3 have also been reported[25]. Additionally, recruitment of the mitotic serine/threonine kinase NEK7 has been implicated in priming NLRP3 for subsequent activation[26-28]. Interestingly, primary human blood monocytes and the human monocyte-like cell line THP-1 can circumvent the need for separate priming and activation steps, because NLRP3 inflammasome activation in these cells is already achieved by stimulation with extracellular LPS alone[23,29,30].

NLRP3 activation in the non-canonical inflammasome pathway occurs downstream of human caspases 4 and 5, and their murine orthologue caspase-11 (**Figure 2b**). In this pathway, the cytosolic presence of LPS elicits assembly of a cytosolic multi-protein scaffold in which members of the guanylate-binding protein (GBP) family coordinate the recruitment and activation of caspases 4, 5 or 11[31-33]. Caspase-4, 5 and 11 go on to cleave GSDMD, which induces plasma membrane pores that induce pyroptosis and in parallel activate the NLRP3 inflammasome by dissipating intracellular K^+ levels. A distinguishing feature of the non-canonical pathway is that caspases 4, 5 or 11 and GSDMD are sufficient for inducing pyroptosis. Whereas maturation of proIL-1ß and proIL-18 is relayed by the NLRP3 inflammasome in murine cells, caspase-4 can also cleave and activate proIL-18 in human epithelial cells[34].

Finally, a detailed genetic examination of NLRP3 activation in a human leukemia B cell line that has been transdifferentiated into monocyte/macrophage-like cells revealed an alternative NLRP3 inflammasome pathway (**Figure 2c**). Here, TRIF- and caspase-8-dependent activation of NLRP3 was proposed to enable caspase-1-mediated IL-1ß secretion from hyper-activated cells without eliciting pyroptosis or assembly of ASC specks[23].

[H2] Molecular mechanisms of NLRP3 activation

Recent structural studies have provided critical insights into how NLRP3 transitions from a closed, auto-inhibited conformation into an open and inflammasome-competent conformation[35-39]. NLRP3 is a relatively large protein composed of an amino-terminal Pyrin domain (PYD), a central NACHT domain preceded by a Fish-specific NACHT associated (FISNA) motif, and a stretch of 9 leucine-rich repeat (LRR) motifs at the carboxy terminus (**Figure 3a**). The PYD domain serves as the inflammasome effector domain responsible for the formation of NLRP3 nucleation seeds that initiate ASC polymerization[40]. The central NACHT domain of NLRP3 is composed of four subunits: a nucleotide binding domain (NBD), a helical domain (HD)1, a winged helix domain (WHD) and a second helical domain, HD2 (**Figure 3a**). The NBD subdomain of the NACHT contains extended Walker A and Walker B sites that accommodate ADP in the inactive, closed conformation and ATP (or dATP) in the active, open conformation of NLRP3.

When recombinant full-length human NLRP3 is analyzed by gel filtration, it segregates in two clearly distinct oligomeric states[41]. The smaller complex is largely devoid of ATPase activity, whereas NLRP3 in the high molecular weight entity displays nearly fourteen-fold increased ATP hydrolysis activity, which might support active NLRP3 in cycling back to the closed, ADP-bound state[41]. Cryo-EM analysis of the smaller, inactive complex revealed that inactive NLRP3 has ADP bound in its NBD, and folds as a pentamer of NLRP3 homodimers that altogether presents as a ring-like structure with the NACHT domains positioned at the polar sides and the intertwined LRR domains in the middle (**Figure 3b**)[37]. A cryo-EM structure of inactive human NLRP3 without the PYD shows that the construct assembles into a hexameric complex that preserves the arrangement of the NACHT and LRR domains in three-dimensional space[38]. A comparable arrangement of the NACHT and LRR regions has also been observed in the cryo-EM structure of murine full-length NLRP3 in which the inactive protein adopts a closed 12- to 16-mer double ring cage[35,38].

A number of interesting conclusions can be drawn from these inactive NLRP3 structures. First, the oligomeric ring of inactive NLRP3 is stabilized by an extensive network of hydrophobic interactions between the LRR and NACHT regions of adjacent NLRP3 subunits homodimers[35,37,38]. Second, the PYD domains are located in the central cavity of the ring, possibly to shield them against unintentional

recruitment of the inflammasome adaptor ASC. Finally, the inactive conformation prevents NLRP3 from binding ATP because the FISNA domain covers the NBD[36,37]. Furthermore, the spatial arrangement of the four subunits of the closed NACHT appears incompatible with ATP binding in the NBD. The structures show that the β-phosphate of ADP is engaged in strong interactions with His522 at the subdomain interface of the inactive NACHT to help stabilize the closed conformation. Therefore, conformational changes that partially open up the closed conformation of the NACHT are necessary before nucleotide exchange can occur at the NBD[36,37].

Consistent with previously suggested mechanistic models, a recent cryo-EM structure of active NLRP3 that is complexed with ATP confirms that NLRP3 activation involves pronounced conformational changes in the FISNA domain. Furthermore, the LRRs together with WHD and HD2 subdomains of the NACHT rotate away by approximately 85° compared to their position in the closed conformation (**Figure 3a**). These significant changes are required to open up the NBD for nucleotide exchange, and support the transition from the inactive ADP-bound state to the active ATP-bound conformation of NLRP3[39]. Notably, similar conformational changes were previously observed to occur when NLRC4 transitions from its inactive, ADP-bound state towards the active, ATP-bound conformation[42,43]. Another interesting observation from the active NLRP3 structure is that binding of NEK7 kinase to the LRR motifs of NLRP3 does not contribute substantially to stabilizing the flower-shaped disk structure of the active NLRP3 inflammasome complex[39]. Indeed, the active complex consists of 10 NLRP3 proteins subunits that are held together by FISNA-NACHT domains interactions near the center of the disk, whereas the LRR domains with bound NEK7 extend away from the disk's center (**Figure 3b**). This contrasts markedly with the cryo-EM structure of ADP-bound NLRP3 in complex with NEK7, in which the kinase domain of NEK7 is suggested to bridge an extended network of interactions involving the LRRs of adjacent NLRP3 subunits[44]. Together with functional studies showing that NEK7 is critical for NLRP3 activation in macrophages[26-28], these structural insights are consistent with NEK7 being a co-factor that chaperones NLRP3 in its transition from an ADP-bound inactive conformation to the ATP-bound active conformation that recruits ASC to initiate inflammasome signaling. However, NEK7 binding alone is not sufficient to elicit the conformational changes required for NLRP3 activation, because ADP was still bound in the NBD of the NLRP3-NEK7 co-complex (**Figure 3a**)[44]. Exactly how the necessary conformational changes are set in motion requires further research. Cellular K^+ efflux and the phosphorylation and ubiquitination status of NLRP3 have been put forward as potential mechanisms, but their precise roles remain unclear.

The subcellular location of NLRP3 activation under physiological conditions has also been a matter of debate. Although early mechanistic models posited NLRP3 activation to occur at or near damaged

mitochondria, subsequent models favored the endoplasmic reticulum, mitochondria-associated endoplasmic reticulum membranes (MAMs) around the Golgi or the Golgi apparatus itself (reviewed by [45]). More recent models focus on the dispersion of the trans-Golgi network (TGN) — which occurs in response to various NLRP3 stimuli — as a putative site involved in NLRP3 activation[46,47]. These models suggest that inactive NLRP3 traffics to the TGN, where four consecutive lysine residues located in its FISNA domain interact with negatively charged phosphatidylinotisol 4-phosphate (PI4P). Concurrent with dispersion of the TGN into scattered vesicles induced by NLRP3-activating stimuli, inactive NLRP3 would undergo conformational changes and the dispersed NLRP3-containing TGN vesicles might traffic further along microtubules to the centrosome, where NLRP3 recruits NEK7 to prime the complex for subsequent ASC recruitment and inflammasome assembly (**Figure 2a**). However, the role of microtubules and the centrosome in NLRP3 activation is unclear because the microtubule polymerization inhibitor colchicine fails to block nigericin-induced activation of the NLRP3 inflammasome, although the same concentration inhibits microtubule-dependent Pyrin inflammasome activation in murine macrophages and human monocytes[48,49]. Furthermore, recent studies challenge the role of the dispersed TGN and suggest instead that NLRP3 is activated at endosomal vesicles that accumulate TGN markers due to disrupted endosome-TGN retrograde transport in response to various NLRP3-activating stimuli[50,51]. Without doubt, the precise molecular mechanisms and subcellular processes that prime NLRP3 for activation will continue to be a focus of investigation in coming years.

[H2] NLRP3 in inflammatory, metabolic and neurodegenerative diseases

NLRP3 inflammasome activation has been identified as a pathogenic mechanism in various cell-based and preclinical in vivo models of auto-inflammatory (**Box 1**), autoimmune, metabolic and neurodegenerative (**Box 2**) diseases (reviewed by [52]). For instance, NLRP3 is activated in human monocytes and murine macrophages exposed to oxidized low density lipoproteins and cholesterol crystals, which both are established vascular disease-associated factors. Activation of the NLRP3 inflammasome also occurs upon phagocytosis of MSU, associated with gout, and calcium pyrophosphate dehydrate crystals, associated with pseudogout. In neurodegenerative disease models, it is activated by protein fibrils and aggregates such as β-amyloid plaques (Aβ, associated with Alzheimer's disease), and tau fibrils (associated with Alzheimer's disease, Parkinson's disease and other tauopathies). It is also activated by aggregates of islet amyloid polypeptide (IAPP or amylin), which are associated with type 2 diabetes. The link between NLRP3 signaling and inflammatory, metabolic and neurodegenerative diseases is further strengthened by numerous reports showing that genetic and pharmacological inhibition of inflammasome signaling

suppresses disease pathology in patients (**Box 3**) and in mouse models of gout and pseudogout[53], atherosclerosis[54], nonalcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH)[55], type 2 diabetes[56], Alzheimer's disease[57], Parkinson's disease[58], multiple sclerosis[59], and SARS-CoV-2-induced lung injury[60], amongst many other diseases (reviewed by [52]).

While the aforementioned diseases are all complex and multi-factorial, disease symptoms in patients with cryopyrin-associated periodic fever syndromes (CAPS, **Box 1**) are centrally driven by aberrant NLRP3 activation as a consequence of gain-of-function mutations in *NLRP3*[61]. CAPS is characterized by systemic, cutaneous, musculoskeletal and central nervous system inflammation, encompassing three disorders of varying severity with Familial Cold Autoinflammatory Syndrome (FCAS) being the mildest; Muckle-Wells Syndrome (MWS) being of moderate severity; and Neonatal Onset Multisystem Inflammatory Disease (NOMID)/Chronic Infantile Neurological, Cutaneous and Articular Syndrome (CINCA) being the most severe (reviewed by [62]). A total of 264 disease-associated sequence variants of the *NLRP3* gene have been entered into Infevers - a manually curated reference database for variants in auto-inflammatory disease-associated genes[63] – out of which 123 variants are classified as 'pathogenic' or 'likely pathogenic' variants with strong genotype–phenotype association along the disease continuum (as of August 2023). When mapped onto NLRP3 cryo-EM structures, numerous pathogenic variants are found to reside at the interface between the FISNA and the NBD or within the NBD and the WHD-HD2 modules of the central NACHT domain. Such CAPS mutations are therefore thought to weaken NACHT subdomain interfaces in the inactive NLRP3 conformation or might help to stabilize the re-oriented FISNA domain in the active conformation of NLRP3[36,37,39]. Other CAPS-associated mutations distort interactions with the Walker B motif and diminish ATP hydrolysis activity[41]. Such mutations might therefore drive NLRP3 hyperactivity by prolonging ATP-residence time in the NBD and extending the time NLRP3 assumes an open, inflammasome-capable conformation[39,41].

[H1] Development of pharmacological NLRP3 inhibitors

The pathological involvement of NLRP3 inflammasome activation across many disease indications suggests that selective and potent NLRP3 inhibitors could have broad therapeutic potential. This has spurred the discovery, preclinical and clinical development of small-molecule NLRP3 inhibitors of distinct chemotypes, with early clinical studies currently underway to evaluate the safety and potency of the most advanced candidate therapies.

[H2] Sulfonylurea NLRP3 inhibitors

In 1997, before inflammasomes had been discovered, the anti-diabetic sulfonylurea drug glyburide (also named glibenclamide) was shown to block IL-1β release from human and murine macrophages stimulated with LPS and ATP[64]. In an effort to find structurally related compounds that are more potent than glyburide in blocking IL-1β release, Gabel and colleagues at Pfizer screened a focused library of structurally related diaryl-sulfonylureas and identified cytokine release inhibitory drugs (CRIDs) that inhibit IL-1β release with nanomolar potency[65,66]. It was not until 2009 that glyburide was discovered to selectively inhibit NLRP3-driven caspase-1 activation, IL-1ß secretion and pyroptosis, without modulating caspase-1 activation by the NLRC4 or NLRP1b inflammasomes, thus providing proof-of-concept for targeted pharmacological inhibition of NLRP3[67]. One of the CRID molecules, the Pfizer compound CRID3 (also known as MCC950 or CP-456773), was subsequently shown to inhibit NLRP3 inflammasome responses in human and murine cells with nanomolar potency[68]. CRID3 has been used in many experimental settings, and suppresses NLRP3-mediated pathology in numerous preclinical in vivo models, including the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, the APP/PS1 model of Alzheimer's disease, several in vivo models of Parkinson's disease, preclinical models of myocardial infarction, atherosclerosis, stroke, asthma and allergic airway inflammation, and inflammatory arthritis (reviewed by [52]). Given its remarkable selectivity and potency, CRID3 remains the gold standard tool compound for inhibiting NLRP3 in preclinical disease settings.

Mechanism of action studies provided robust biochemical evidence that CRID3 reversibly binds the central NACHT domain of NLRP3 with high affinity[69-71]. Unexpectedly, photoaffinity labeling and functional studies in LPS-stimulated macrophages and transgenic mice suggested that a subset of CAPS-associated NLRP3 mutations substantially reduce the binding affinity and inhibitory potency of CRID3[71]. In accordance, the efficacy of CRID3 in inhibiting serum IL-1ß and IL-18 levels differed substantially in two *in vivo* mouse models of CAPS. High doses of CRID3 effectively suppress serum IL-1ß and IL-18 levels in LPS-challenged mice expressing the MWS-associated NLRP3^{A350V} mutation, whereas these inflammasome-dependent disease markers were refractory to CRID3 inhibition in animals expressing the FCAS-associated NLRP3^{L351P} mutation[71]. Consistent with this, a recent study showed that the half-maximal inhibitory concentration (IC_{50}) of CRID3 that is required to inhibit LPS-induced IL-1ß release from peripheral blood mononuclear cells (PBMCs) of CAPS patients is approximately 7 times higher than that required to inhibit IL-1ß secretion from PBMCs of healthy donors[72]. These findings suggest that a higher plasma concentration of CRID3-based therapies might be required to effectively treat disease symptoms in a subset of CAPS patients, compared to plasma concentrations required for diseases driven by wildtype NLRP3. Clearly, the development of orally available small molecule NLRP3 inhibitors remains important to expand treatment

options for CAPS patients, and it is encouraging to note that several CRID3-derived analogs with improved potency and pharmacological profiles are being clinically evaluated in CAPS patients (**Table 1**, ClinicalTrials.gov Identifier: NCT04086602, NCT05186051, NCT04868968). Moreover, developing CRID3-based therapies with increased potency against CAPS-associated NLRP3 mutations might be feasible now that the structures of active and inactive NLRP3 have been reported[36,37,39].

[H2] CRID3 inhibitory mechanism in atomic detail

A recently reported cryo-EM structure of full-length human NLRP3 complexed with CRID3 solved at a resolution of around 4.1 Å[37], and a crystal structure of the isolated NACHT domain complexed with a CRID3-derived analog NP3-146 at 2.8 Å resolution,[36] provide atomic insight into the binding pocket of CRID3. Both structures confirm previous reports that CRID3 binds the NACHT domain of NLRP3[69-71], and reveal the CRID3-binding pocket as a shallow, hydrophobic groove sitting at the interface of the four subunits of the closed NACHT domain (**Figure 3a**)[36,37]. Interestingly, the structures show that CRID3 does not interfere with the binding of ADP at the Walker A motif of the closed NACHT domain.

CRID3 consists of a central sulfonylurea that is linked to a hydrophobic tricyclic hexahydro-s-indacene ring on one side and an isopropyl furan moiety on the other side (**Table 2**). The recently solved NLRP3 structures reveal that the tricyclic hexahydro-s-indacene moiety of CRID3 (and the corresponding chloro-di-isopropylbenzene of the CRID3-analog NP3-146) occupies a hydrophobic cleft that is formed at the interface of the WHD, HD1 and HD2 subdomains of the closed NACHT and flanked by residue M661 of the extended LRR domain[36,37]. A similar hydrophobic pocket is not present in the NACHT of the previously solved crystal structure of NLRC4 in its inactive conformation[73], which probably contributes to the exquisite NLRP3-targeted specificity of CRID3[36]. The central sulfonylurea group of CRID3 is sandwiched between Arg351 and Arg578 of the NBD and the HD2 subdomains, respectively (**Figure 3c**). In addition, the urea group of CRID3 is engaged in a crucial hydrogen bond interaction involving Ala227 and Ala228 at the Walker A motif of the NBD. The compound's isopropyl furan group is solvent exposed, and does not appear to engage in key interactions with the protein. Thus, the CRID3-binding pocket at the subdomain interface of the closed NACHT, and the arrangement of key residues in the different NACHT subdomains that interact with CRID3, suggest that the inhibitor prevents NLRP3 activation by helping to stabilize the closed, inactive conformation of NLRP3 (**Figure 3c**).

Interestingly, the recently reported structure of active NLRP3 combined with structural modelling of the transition from the closed, inactive to the fully open, active conformation of NLRP3 indicate that the substantial spatial rearrangements in the NACHT that occur during activation completely destroy the

CRID3-binding pocket[36,37,39]. Accordingly, CRID3 has no influence on ATP hydrolysis by active NLRP3[41]. Furthermore, this implies that CRID3 cannot target NLRP3 in its active conformation, which suggests a satisfying molecular explanation for why some CAPS-associated NLRP3 mutations in and around the central NACHT render the mutant protein less prone to CRID3 inhibition[36,37,39,71].

Together, the aforementioned structures provide a detailed atomic model of the binding pocket of CRID3, and highlight structural features and atomic interactions between CRID3, ADP and core residues at the subdomain interface of the closed NLRP3 NACHT. They explain at atomic level how CRID3 potently inhibits NLRP3 activation by stabilizing the closed conformation and preventing its transition towards the open, inflammasome-competent active conformation[36,37]. These structural and mechanistic insights should prove invaluable for rational drug design and further improvement of next-generation NLRP3 inhibitors.

[H2] NLRP3 inhibitors in clinical development

CRID3 is widely used as a tool compound for inhibiting NLRP3 in preclinical disease models and its availability has been instrumental in helping to dissect the role of the NLRP3 inflammasome in a wide range of experimental settings (reviewed by [52]). However, its clinical development was allegedly halted by Pfizer due to signs of drug-induced liver injury with high CRID3 dosing in healthy volunteers[74]. The first off-target activity of CRID3 was only recently reported when CRID3 was shown to noncompetitively inhibit the esterase activity of carbonic anhydrase 2 with micromolar potency (IC_{50} = 11 µM)[75]. It is unclear whether this off-target inhibition accounts for the reported CRID3-induced liver injury in early human studies because carbonic anhydrase inhibitors are widely clinically used as diuretic medications and no side effects on the liver have been reported[76]. Regardless, the discovery of CRID3 as an NLRP3-targeted inhibitor spurred several companies to develop second-generation NLRP3 inhibitors with improved potency and pharmacological profiles (**Figure 4**), some of which have already entered clinical development (**Tables 1 and 2**). In addition to peripherally restricted compounds for the treatment of CAPS, autoimmune and metabolic diseases, new inhibitors that can pass the blood-brain barrier are also being pursued for the treatment of Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases of the central nervous system.

ZYIL1 (Zydus Lifesciences, formerly Cadila Healthcare) is the first NLRP3 inhibitor to successfully complete a proof-of-concept phase II clinical study in CAPS patients (NCT05186051). These patients experienced a rapid improvement in clinical parameters and achieved clinical remission within days after initial dosing. The inhibitor previously proved to be safe and well-tolerated in first-in-human single-

ascending dose (NCT04731324) and multiple-ascending dose (NCT04972188) phase I studies[77]. The chemical structure of ZYIL1 was recently disclosed as a CRID3 analogue in which the furan head is replaced by a substituted pyrrolidine ring (**Table 2;** [77]).

DFV890 (formerly IFM-2427) is a peripherally-restricted NLRP3 inhibitor that was discovered at IFM Therapeutics prior to its acquisition by Novartis[78]. DFV890 successfully completed phase I clinical trials and was recently evaluated in a phase II trial for COVID-19-associated pneumonia (NCT04382053), the results of which indicate that it failed to improve treatment efficacy beyond standard of care[79]. The inhibitor is currently under evaluation in phase II trials for FCAS (NCT04868968) and knee osteoarthritis (NCT04886258).

Ventyx Biosciences disclosed two NLRP3 inhibitors in its clinical pipeline: a peripherally-restricted NLRP3 inhibitor called VTX2735, and a CNS-penetrant NLRP3 inhibitor named VTX3232. Whereas VTX3232 has recently entered phase I human trials for neuroinflammatory diseases, VTX2735 successfully completed a phase I clinical trial, and has progressed into a phase II clinical trial to establish clinical proof-of-concept in CAPS patients[80].

Emlenoflast (formerly Inzomelid/MCC7840) and Selnoflast (formerly Somalix/RG6418/IZD334) are CRID3-derived analogues developed at Inflazome (now part of Roche) in which the isopropyl furan moiety of CRID3 has been replaced by substituted pyrazol, in the case of Emlenoflast, or by piperidine moieties in the case of Selnoflast ([81] and **Table 2**). These orally-available NLRP3 inhibitors successfully completed phase I safety and tolerability studies in healthy subjects, and showed promising clinical efficacy in adult patients with CAPS (Emlenoflast, NCT04015076; Selnoflast, NCT04086602). Emlenoflast is a brain-penetrant NLRP3 inhibitor that was designed to treat neurodegenerative diseases such as Alzheimer's and Parkinson's disease, but it is no longer part of Roche's clinical development pipeline[82]. Selnoflast is a peripherally-restricted NLRP3 inhibitor that is being developed to treat systemic inflammatory diseases, and the Roche development pipeline indicates that the inhibitor is currently in phase Ib human trials for ulcerative colitis and chronic obstructive pulmonary disease (COPD) (**Table 1**)[83].

The Roche subsidiary Genentech acquired NAFLD/NASH-focused Jecure Therapeutics in 2018, and obtained rights to Jecure's CRID3-based NLRP3 inhibitor RG6338[78]. However, Roche recently announced that it has removed RG6338 from its clinical pipeline[84].

NodThera's NLRP3 inhibitor NT-0796 was announced by the company to be safe and well-tolerated in phase I studies[85]. It is targeted towards neuroinflammatory diseases, and was reported to effectively reach the central nervous system in humans. The company recently added another brain-penetrant NLRP3 inhibitor NT-0527 to its pipeline, and is advancing this inhibitor through Investigational New Drug (IND)-

enabling studies. NodThera also announced positive interim results from a single-ascending dose phase I study in which the peripherally-restricted NLRP3 inhibitor NT-0249 is dosed once daily. No chemical structures for these three clinical candidates have been publically disclosed.

Dapansutrile (also known as OLT1177 or 3-methylsulfonylpropionitrile; Olatec Therapeutics; **Table 2**) proved to be safe and well-tolerated in healthy volunteers (**Table 1**), and is being assessed in phase II studies for Schnitzler's Syndrome (NCT03595371) and in patients with moderate COVID-19 symptoms that show evidence of early cytokine release syndrome (NCT04540120). It previously completed phase II studies for osteoarthritis (NCT01768975) and a phase IIa study for acute gout (**Table 1,** EudraCT number 2016-000943-14). In the gout study[86], dapansutrile treatment led to marked joint pain reduction in all dose groups, but additional studies are required to optimize the dosing regimen in this inflammatory disease. Dapansutrile was reported to inhibit the ATPase activity of NLRP3 and suppress downstream inflammasome responses[87]. As a nitrile derivative, the inhibitor might target the NACHT by forming covalent adducts with thiols, alcohols or amine groups, but further analysis is required to better understand its selectivity profile and action mechanism.

Tranilast is a substituted anthranilic acid (**Table 2**) that is clinically licensed for treating asthma, and keloid and hypertrophic scars in South Korea and Japan (reviewed in [88]). Its discovery by scientists at Kissei Pharmaceuticals in 1976 by far outdates that of the inflammasome in 2002[89]. Tranilast interacts with the NLRP3 NACHT and inhibits NLRP3 inflammasome activation in macrophages treated with LPS and nigericin without modulating TLR-induced priming or activation of the NLRC4 and AIM2 inflammasomes[90]. Its mechanism of action remains unclear, but in light of its electrophilic character the inhibitor is likely to covalently attach to nucleophilic surface residues of NLRP3. Among other disease models, tranilast has shown protective effects in mouse models of gout, type 2 diabetes and MWS[90]. However, the anti-inflammatory mechanism of this tryptophan derivative is likely to involve modulation of additional inflammatory pathways beyond targeting NLRP3, because the compound was suggested to suppress activation of NF-κB, MAPK and PKC-dependent signaling pathways (reviewed in [88]).

The experimental anti-tumor drug RRx-001 (ABDNAZ; **Table 2**), which is in phase III studies for small cell lung cancer (SCLC) (NCT03699956), is proposed to act by covalently binding to NLRP3, thereby preventing recruitment of NEK7[91]. RRx-001 was effective in mouse models of NLRP3-associated inflammatory conditions such as LPS-induced systemic inflammation, dextran sodium sulphate-induced colitis and the EAE model of multiple sclerosis. However, NLRP3 is not the sole target of this reactive dinitroazetidine compound, and a polypharmacological mechanism that includes the generation of reactive oxygen species and indirect oxidation of nucleic acids has been put forward[92].

In addition to the aforementioned clinical-grade inhibitors that target NLRP3 directly, a phase I clinical trial to explore the safety and tolerability of HT-6184 has recently been completed (**Table 1**, NCT05447546). Although its developer, Halia Therapeutics, positions the compound as a novel allosteric NEK7 modulator that targets both NLRP3 priming and inflammasome assembly, the structure of HT-6184 and preclinical data remain to be disclosed.

[H2] Next-generation CRID3-based inhibitors in preclinical development

Complementing the aforementioned clinical stage NLRP3 inhibitors, several next-generation CRID3-based NLRP3 inhibitors have been discovered and are at earlier stages of development. Consistent with insights from the recent CRID3-complexed NLRP3 structures discussed above, an early structure–activity relationship (SAR) analysis of a series of CRID3 metabolites and additional CRID3-like molecules revealed that the hexahydro-s-indacene tricyclic moiety and the central sulfonylurea group are critical for CRID3 bioactivity[93,94]. Probably due to their acidic center, sulfonylureas like CRID3 bind significantly to plasma proteins, which reduces their bioactivity and effective plasma concentrations in vivo[95,96]. Although the CRID3 sulfonylurea moiety has a central role in NLRP3 inhibition, CRID3 analogues with structural modifications of this moiety that retain the low nanomolar potency of the parental molecule have also been discovered[97,98]. Zydus Lifesciences reported a series of N-cyano-sulfoximineurea derivatives of CRID3, of which compound 15 (ZY19800) proved equipotent to CRID3 in both *in vitro* and *in vivo* settings (**Table 2**)[97]. Interestingly, NodThera reported that replacement of the sulfonylurea with an ester-substituted urea resulted in another series of CRID3 analogues (**Table 2**, compound 44 and 45) that are equipotent to CRID3 in inhibiting IL-1β secretion from *ex vivo*-stimulated PBMCs, but display significantly improved IC_{50} values in a whole blood assay. This improvement was attributed to neutralization of the sulfonylurea-associated negative charges and associated plasma protein binding[98]. More recently, NodThera succeeded in replacing the central sulfonylurea group of CRID3 with a thiocarbonyl group, giving rise to a novel chemotype of CRID3-related NLRP3 inhibitors[99]. An exemplar from these series is compound NDT-30805 (**Table 2**), which displays a two-fold improved potency compared to CRID3 in PBMCs and whole blood assays[99]. However, the binding pocket and pharmacophores of NDT-30805 and CRID3 are likely to be conserved, considering that they share the tricyclic hexahydro-s-indacene ring, and *in silico* modelling studies suggest that the thiocarbonyl and CRID3's sulfonylurea could engage in similar hydrogen bonds with residues of NLRP3's NACHT in the vicinity of the hydrophobic binding pocket[99].

Substitutions of the hexahydro-s-indacene tricyclic ring of CRID3 that do not substantially affect CRID3 inhibitory activity have also been described. For instance, replacing the hydrophobic tricycle moiety

with substituted benzenes led to the discovery of an equally effective inhibitor (**Table 2**, compound 4b), which significantly reduced inflammation and mechanical hyperalgesia in an *in vivo* mouse model of gout[100].

Finally, the isopropyl furan moiety of CRID3 is the most amenable to substitution. This is fully consistent with the aforementioned structural data showing that the compound's isopropyl furan group is solvent exposed and does not contribute to interactions with the NLRP3 binding pocket. This has led to a range of structural analogs in which the metabolically reactive furan moiety of CRID3 is replaced by a variety of substituents ranging from smaller scaffolds to larger bio-isosteric heterocyclic ring systems with significant impact on inhibitory potency[93,101]. For example, the furan moiety of MCC950 can be exchanged for a thiazole, generating a thiazolo-alkenyl sulfonylurea derivative (**Table 2**, compound 7) that maintains activity[101]. Likewise, an aniline analogue of CRID3 (**Table 2**, compound 8b) reaches IC_{50} values that are comparable to that of the parent compound[93].

[H2] Next-generation glyburide-based NLRP3 inhibitors

In addition to the aforementioned CRID3-based inhibitors, NLRP3 inhibitors that use glyburide as a starting point have also been reported; JC121 and its methylated (JC124) and hydroxylated (JC171) analogs are examples (**Table 2**). These benzene sulfonamide analogues lack the cyclohexylurea moiety that is responsible for glyburide's anti-diabetic activity. This novel glyburide-based class of inhibitors inhibited NLRP3 activation in macrophages with single-digit micromolar potency, and showed promising activity in mouse models of Alzheimer's disease and acute myocardial infarction (**Table 2**, and [102-105]). Furthermore, the inhibitory potency of this series was substantially improved with substitutions of the sulfonamide moiety of JC124, giving rise to inhibitors that target NLRP3 activation in macrophages with nanomolar potencies without interfering with flagellin-induced activation of the NLRC4 inflammasome or dsDNA-induced AIM2 activation (**Table 2**, compounds 14 and 17)[102]. Additionally, a series of nanomolar potency sulfonamide inhibitors that more potently penetrate the blood–brain barrier for NLRP3 inflammasome blockade in the central nervous system have been described (**Table 2**, compounds 19 and YQ128)[106,107]. Further development of this chemical scaffold might provide a chemical pathway towards clinical-grade NLRP3 inhibitors.

[H2] NLRP3 inhibitors with distinct chemotypes

Inhibitors of NLRP3 that do not rely on the glyburide and CRID3 scaffolds have been discovered, suggesting that the chemical space for development of next-generation drugs could be significantly expanded. For

example, CY-09 is a substituted benzoic acid inhibitor that selectively inhibits NLRP3 signaling *ex vivo* with micromolar potency (**Table 2**), and protected mice from MSU-induced peritonitis as well as from MWS-associated neonatal lethality[108]. Although its binding pocket remains undefined, CY-09 physically binds the NACHT domain of NLRP3 and does not interfere with ATP hydrolysis by recombinantly purified NLRP3[41,108].

In addition to the synthetic inhibitors described above, many natural compounds with anti-NLRP3 inflammasome properties have been discovered. Natural compounds offer a rich chemical diversity and poly-pharmacology, but their less stringent selectivity profile can sometimes hamper progression to in-human studies. Erianin (**Table 2**) is a natural bibenzyl derivative isolated from the orchid species *Dendrobium chrysotoxum Lindl.* It targets NLRP3 with nanomolar potency by forming a covalent adduct with the NACHT domain, and has been attributed with anti-tumor, anti-microbial and anti-angiogenic activities that likely involve inflammasome-independent activities[109].

Oridonin (**Table 2**) is a natural diterpenoid derivative extracted from the herb *Rabdosia rubescens* and was reported to inhibit recruitment of NEK7 by covalently modifying Cys279 in the NLRP3 NACHT domain[110]. In addition to targeting NLRP3, oridonin exhibits a variety of pharmacological actions, including inhibition of NF-κB, MAPK and AMP-activated protein kinase (AMPK) signaling pathways that contribute to its reported anti-inflammatory and anti-tumor activities[111]. The compound showed beneficial effects in mouse models of peritonitis, gouty arthritis and type 2 diabetes, but it is unclear to what extent this is due to NLRP3 blockade. A novel series of synthetic oridonin analogues with increased potency against NLRP3 and improved selectively have recently been discovered[112]. The most potent representative from this series (**compound E6; Table 2**) was shown to target IL-1ß secretion in LPS-induced THP-1 cells with an IC_{50} of 0.45 micromolar without modulating TLR signaling or activating the NLRC4 and AIM2 inflammasomes.

[H2] NLRP3 agonism in cancer therapy

Inducing pyroptosis to help overcome the immunosuppressive microenvironment of solid tumors and increase the efficacy of immunotherapies has gained significant traction in recent years[113,114]. The activity of the NLRP3 agonistic imidazoquinoline BMS-986299 in this regard was evaluated in a phase I clinical trial in patients with advanced solid tumors, alone or in combination with the immune checkpoint inhibitors Nivolumab and Ipilimumab (NCT03444753). The study aimed to explore the extent to which NLRP3 activation contributes to eliciting an anti-tumor immune response and to promoting tumor immune cell infiltration. Although the mechanisms involved remain unclear, intra-tumoral NLRP3 activation in combination with checkpoint inhibition showed modest clinical activity. However, a significant proportion of the patients that received the combination therapy presented with mild to severe (grade 3 and 4)

treatment-related adverse events, including hepatitis, nephritis, colitis and diabetic ketoacidosis[115]. Therefore, a better understanding of the mechanisms underlying the toxicity associated with NLRP3 agonism is urgently needed to overcome this setback and further explore the clinical potential of NLRP3 activation in cancer therapy.

[H1] Emerging strategies for modulating multiple inflammasomes

[H2] ASC targeting

ASC has a general role as an inflammasome adaptor that bridges the interaction between human inflammasome sensors (aside from CARD8) and the CARD-containing procaspase-1 zymogen. Although NLRC4 can recruit caspase-1 and trigger pyroptosis in the absence of ASC, expression of ASC enhances NLRC4-induced caspase-1 activation. ASC specks are assembled in the cytosol of inflammasome-activated cells and then released extracellularly during pyroptosis, along with other soluble factors. Notably, the extracellular pool of ASC specks propagates an inflammatory response, partly by promoting further IL-1β maturation in the extracellular space and partly by propagating inflammasome activation in the cytosol of cells that internalize the extracellular ASC specks[116,117]. Consequently, ASC specks have been detected in serum of CAPS patients, and ASC-targeted autoantibodies were present in the serum of patients with various autoimmune diseases. Biological therapeutics (biologics) to neutralize extracellular ASC are being explored based on the concept that extracellular ASC specks contribute to propagation of inflammasome-mediated inflammatory pathology. Zyversa Therapeutics is developing a humanized antibody named IC-100 that targets human ASC (**Figure 4**) and exhibits therapeutic potential in the EAE mouse model of multiple sclerosis[118]. Other investigators have developed a camelid-derived ASC-neutralizing single chain antibody (VHH_{ASC} nanobody) to target and disintegrate extracellular ASC specks. VHH_{ASC} could neutralize extracellular inflammatory functions of ASC specks, while preserving the ability of intracellular ASC specks to promote IL-1β release[119]. Moreover, systemically administered VHH_{ASC} dampened inflammatory pathology in mouse models of gout and antigen-induced arthritis.

In parallel to the strategies with ASC-targeted biologics, small molecule compounds libraries have been screened for inhibitors that target ASC functions in both the extracellular and intracellular compartments. This has led to the discovery of a small molecule ASC inhibitor MM01 that impairs ASC oligomerization and inhibits activation of caspase-1 by several inflammasome pathways with micromolar potency in cellular assays[120]. *In silico* structural modelling suggests that MM01 binds to the surface of ASC CARD and sterically hinders the CARD-CARD interactions required for ASC speck assembly and recruitment of procaspase-1. MM01 also suppressed inflammatory read-outs in the uric acid crystal-induced peritonitis model[120].

Considering the general role of ASC in bridging caspase-1 activation by NLRP3 and other inflammasome sensors, small molecule- and biologics-based ASC-targeting strategies have the potential to broadly suppress inflammasome activity across a range of human diseases.

[H2] GSDMD as a therapeutic target

GSDMD is an inflammasome effector that, in addition to ASC, has been the focus of intense investigation in recent years for its potential as a broad therapeutic target given it is required in multiple inflammasome pathways. GSDMD is a member of the gasdermin family of pore-forming proteins and serves as the primary executioner of pyroptosis in inflammasome pathways (reviewed by [121]). Whereas IL-1β is selectively activated by caspase-1, GSDMD is proteolytically activated by caspase-1 in canonical inflammasomes or by caspases 11, 4 or 5 in the non-canonical inflammasome pathway (**Figure 2**). After its cleavage, the GSDMD NT translocates and homo-oligomerizes in the plasma membrane to assemble into large β-barrel pores that allow extracellular release of IL-1ß and IL-18 and promote pyroptosis (**Figure 2**)[121]. A detailed analysis of GSDMD pore formation using high-resolution atomic force microscopy suggests that GSDMD NT first inserts into the cytoplasmic leaflet of the plasma membrane by binding to microdomains enriched for specific phospholipids (PI4P and PI(4,5)P2), and then oligomerizes into dynamic intermediate structures that give rise to the fully opened ring-shaped transmembrane pore[122]. With an inner diameter of approximately 21 nm, these pores are sufficiently large to allow passage of small DAMPs such as ATP and mature IL-1β and IL-18, but not of larger DAMPs such as HMGB1[14,123]. Recent work further suggests that GSDMD NT can perforate the mitochondrial membrane by binding to the mitochondria-enriched phospholipid cardiolipin, thereby inducing the release of mitochondrial DAMPs such as mitochondrial dsDNA (mtDNA) and ROS into the cytosol[124-126]. Consistent with this model, a single cell time-lapse analysis of pyroptotic macrophages revealed that GSDMD-dependent mitochondrial depolarization occurs prior to plasma membrane rupture[127]. Interestingly, mitochondrial GSDMD pore formation and release of mtDNA into the cytosol of LPS-transfected macrophages requires LPS binding to the nuclear orphan receptor Nur77, which subsequently recruits mtDNA and NLRP3 to induce caspase-1 maturation and IL-1ß secretion [124]. In other studies, excessive mitochondrial fragmentation and ROS production due to expression of the Parkinson's disease-associated *G2019S* allele of *LRRK2* led to GSDMD-dependent release of mtDNA and ROS into the cytosol of macrophages[126]. These events subsequently caused pyroptosis mediated by NLRP3 and AIM2 to be rerouted to RIPK1/RIPK3-mediated necroptosis, thereby dampening secreted IL-1ß levels[126]. Finally, GSDMD NT-mediated mtDNA release was also reported in endothelial cells, where it

impaired endothelial recovery after inflammatory injury by activating the cGAS/STING dsDNA-sensing pathway[125].

The in vivo role of GSDMD has been extensively examined in preclinical disease models. GSDMD deletion proved highly beneficial in mouse models of endotoxic shock[6], CAPS[128], Familial Mediterranean Fever (FMF)[129], NAFLD/NASH[130], colitis[131] and multiple sclerosis[132]. Therefore GSDMD is viewed as an appealing disease target for pharmacological inhibition. Disulfiram (Antabuse**; Table 3**), an FDA-approved medication for the treatment of alcoholism, inhibits human GSDMD by covalently modifying the redox-sensitive thiol group of Cys191 (Cys192 in murine GSDMD), which prevents GSDMD NT from oligomerizing and forming membrane pores (**Figure 4**)[133]. Disulfiram protected mice from LPS-induced endotoxic shock and showed activity in the EAE model of multiple sclerosis[134]. Similarly, necrosulfonamide (NSA; **Table 3**) targets the critical Cys191 in GSDMD NT and demonstrated efficacy in LPS-induced endotoxemia[135]. GSDMD is also inhibited by dimethyl fumarate (DMF, Tecfidera; **Table 3**), a licensed drug for the treatment of multiple sclerosis and psoriasis that succinates Cys191 as well as others cysteine residues[136]. Dimethyl fumarate protected mice from endotoxic shock and reduced inflammation in mouse models of multiple sclerosis and FMF[136].

Despite the promise in animal models, the inherently promiscuous nature of these thiol-reactive inhibitors means they are challenging starting points for developing potent and selective GSDMD-targeted inhibitors. Disulfiram reacts with and inhibits the active site cysteine of caspases 1 and 11, in parallel to inhibiting NF-κB signaling and proteasome activity[137]. As an alkylating compound, necrosulfonamide inhibits the necroptosis effector molecule mixed lineage kinase domain-like pseudokinase (MLKL)[138]. Dimethyl fumarate, in turn, dampens immune responses in myeloid and lymphoid cells by inactivating the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[139]. However, a compound called C202-2729 (**Table 3**) that was identified in an in silico screen of 100.000 ChemDiv compounds is reported to display a more stringent selectivity profile[140]. C202-2729 inhibited GSDMD pore formation *in vitro* in a liposome leakage assay with micromolar potency (IC_{50} = 32 micromolar) without modulating caspase-1 activity. The inhibitor also prevented GSDMD-driven pore formation downstream of caspase-1-dependent GSDMD cleavage in stimulated macrophages[140]. C202-2729 is thought to non-covalently interact with the Tyr54 and Lys235 residues in cleaved GSDMD NT that are critical for its uptake in the plasma membrane and for oligomerization, respectively[140]. Notably, human GSDMD-targeting nanobodies that sterically hinder GSDMD NT oligomerization and pore formation by binding to the same region have recently been discovered[141,142]. Surprisingly, extracellular administration of these GSDMD-targeted nanobodies inhibited inflammasome-induced IL-1β secretion and pyroptosis of primary human macrophages, suggesting that

initial GSDMD pore formation allows the nanobodies to enter the cytosol and then halt further GSDMD pore assembly, which allows membrane repair[141]. Although high doses (at the micromolar level) were necessary to inhibit IL-1β production and pyroptosis, these initial findings in ex vivo-cultured human macrophages offer an interesting proof-of-concept that GSDMD-targeting nanobodies could have therapeutic potential.

X-ray crystallography and cryo-EM structures of gasdermin family members have contributed significantly to understanding the molecular basis of GSDMD auto-inhibition and the interfaces involved in lipid-binding and oligomerization of GSDMD NT. A cryo-EM structure of the human GSDMD pore indicates that it has a 31-fold to 34-fold symmetry, in which each GSDMD NT unit features a large transmembrane β-barrel and a globular domain facing the cytosol. Crystal structures of full length murine and human GSDMD revealed that GSDMD CT inhibits translocation into the plasma membrane by masking the lipid-interacting surface of GSDMD NT[143]. Caspase-mediated cleavage and removal of GSDMD CT results in large conformational changes to GSDMD NT that expose its lipid binding moiety to support plasma membrane translocation, followed by oligomerization and assembly of the full transmembrane GSDMD NT pore (reviewed by [121]). Notably, co-crystallization of GSDMD and the protease domains of human caspase-1 or caspase-4/11 revealed the unexpected existence of a hydrophobic surface patch in GSDMD CT that interacts with a conserved exosite, which is a binding site remote from the caspase's active site pocket. Together with the long-known substrate recognition interface near the caspase cleavage site in the linker region of GSDMD, the exosite is critical for determining substrate recognition and caspase-mediated GSDMD cleavage[144,145]. In addition to clarifying how inflammatory caspases interact with GSDMD, these structural insights should inspire novel pharmacological strategies that target the caspase's exosite or its interaction patch in GSDMD CT.

Conceptually novel strategies to suppress GSDMD activation have also been discovered by studying microbial virulence mechanisms. The Gram-negative intracellular pathogen *Shigella flexneri* causes bacterial dysentery, and evades pyroptosis of host cells by modifying caspases 4 and 11 with ADP-riboxanation, leading to their inactivation[146]. This virulence mechanism might be harnessed for therapeutic use if chemical strategies could be developed to selectively ADP-riboxanate inflammatory caspases. This human pathogen also expresses the ubiquitin ligase IpaH7.8 that selectively targets human, but not murine, GSDMD for proteasomal degradation[147]. Interestingly, the GSDMD-neutralizing nanobodies discussed above target a similar binding site as IpaH7.8[142]. Furthermore, the SARS-CoV-2 nucleocapsid interacts with the linker region in GSDMD, hindering caspase-1-mediated GSDMD cleavage[148]. In addition, the viral protease 3C of Enterovirus 71, which has been linked to hand-foot-and-mouth disease in young

children, cleaves GSDMD, yielding a shorter inactive N-terminal GSDMD fragment[149]. Yet another ingenious strategy to suppress GSDMD pores is deployed by *Mycobacterium tuberculosis*, the causative agent of tuberculosis in humans. *M. tuberculosis* expresses a phospholipid phosphatase named PtpB that dephosphorylates PI4P and PI(4,5)P2 in the host cell plasma membrane. By changing the phospholipid composition of the plasma membrane, PtpB effectively blocks translocation of cleaved GSDMD NT to the plasma membrane and downstream pore formation[150]. Consistent with pathogen-induced suppression of pyroptosis playing a key role in early stages of tuberculosis, *in vivo* infection with a PtpB-inactivated *M. tuberculosis* strain resulted in markedly lower bacterial burdens compared to mice that had been infected with wildtype bacteria[150]. The mechanism of action of mycobacterial PtpB shares striking resemblance to that of the small molecule GSDMD inhibitor C202-2729. Overall, the rich diversity in microbial virulence mechanisms that target GSDMD not only attests to the central role of pyroptosis in host defense, but also suggests novel strategies for therapeutic inhibition of GSDMD pores in inflammatory diseases.

Considerable evidence points to crosstalk between pyroptosis and apoptosis signaling in myeloid cells[8,151-156]. When GSDMD pore formation is defective, caspase-1 and caspase-8 initiate an apoptotic signaling cascade that limits extracellular release of inflammatory cytokines and DAMPs while ensuring clearance of inflammasome-activated cells through efferocytosis mechanisms. Consistent with this, GSDMD ablation completely protected against chronic inflammatory pathology in an *in vivo* model of FMF[129]. GSDMD deletion also abolished systemic inflammatory disease in a mouse model of CAPS[157]. However, serum levels of IL-1β and IL-18 increased in a Gasdermin E (GSDME)-dependent manner when GSDMD-deficient CAPS mice were additionally challenged with LPS or TNF[158]. These findings are consistent with the notion that caspase-3-mediated cleavage of GSDME can act redundantly with GSDMD in promoting the release of IL-1β and IL-18 when exposed to hyperinflammatory cues[158,159]. In summary, GSDMD inhibition has emerged as a promising strategy for treating acute and chronic inflammatory diseases, although evidence exists that redundant mechanisms are in place to safeguard IL-1ß release under conditions of overwhelming inflammation.

[H2] Preventing pyroptotic cell lysis by targeting the cell-surface protein NINJ1

Pyroptotic cell lysis has been considered a passive process following GSDMD pore formation. However, the integral plasma membrane protein NINJ1 has recently been discovered to have a critical role in PMR during pyroptosis and post-apoptotic secondary necrosis[14]. Consequently, NINJ1 drives the extracellular release of DAMPs such as HMGB1 that are too large to pass through GSDMD pores[14]. NINJ1 is a small monomeric protein that consists of two extracellular-facing amphipathic α-helices followed by two

transmembrane helices. Super-resolution imaging studies revealed that NINJ1 creates large gaps in the plasma membrane of pyroptotic cells by auto-oligomerizing into clusters of highly branched filaments that range in size from 500 nm to micrometers (**Figure 2**)[160]. Recent cryo-EM studies further demonstrated that the externally exposed α-helices insert into the plasma membrane upon NINJ1 activation to support its polymerization in a fence-like array[160]. The amphipathic nature of NINJ1's α-helices allows its filaments to cap membrane edges at a scission and thus enable rupture of the plasma membrane. Although further research is required to understand what triggers the conformational changes leading to NINJ1 polymerization, plausible mechanisms might involve sensing of local alterations in physicochemical properties of the plasma membrane such as the local curvature of the lipid bilayer or changes in its phospholipid composition.

Given that NINJ1 is a key mediator of PMR associated with pyroptosis and post-apoptotic secondary necrosis, targeting NINJ1 might provide therapeutic benefit in inflammatory diseases where excessive cell lysis and DAMP release contributes to pathology. Consistent with this hypothesis, genetic NINJ1 ablation is beneficial in preclinical models of pulmonary fibrosis, multiple sclerosis and acute hepatitis[161-163]. Importantly, the loss of neuron-associated NINJ1 expression was shown to cause repetitive and anxiety-like behavior in NINJ1 knockout mice[164]. This suggests that NINJ1-targeted pharmacological agents should ideally be restricted to the periphery. The therapeutic potential of peripheral modulation of NINJ1 activity is nicely illustrated by a recently reported NINJ1-neutralizing monoclonal antibody named 'clone D1' that potently inhibits the assembly of NINJ1 polymers and attenuated cell lysis and liver injury in mouse models of acute hepatitis[163]. The substantial therapeutic effect of interfering with NINJ1-driven PMR was unexpected and highlights an exciting novel strategy for exploring the contribution of larger DAMPs to the pathogenesis of various inflammatory diseases in future investigations.

[H1] Concluding remarks

Ever since the discovery of the inflammasome over 20 years ago[89], our understanding of inflammasome pathways and their roles in various diseases has expanded at a thrilling pace. These discoveries have opened up new research fields to understand the mechanistic role of inflammasomes in infectious, inflammatory, metabolic and neoplastic diseases and have broken new ground in translational research. Exciting clinical findings from the seminal CANTOS trial (**Box 3**) suggest that IL-1-targeted therapies that are licensed for rheumatoid arthritis and specific inflammasomopathies could be effective in many more chronic diseases than we could have anticipated.

As inflammasome research has matured in recent years, a multi-disciplinary community of researchers has joined the field. Consequently, remarkable progress in understanding NLRP3 regulation has emerged, with structural biology studies conferring atomic-level insight into activation mechanisms of NLRP3 and identifying the binding pocket of CRID3-based inhibitors. In parallel, significant efforts are underway in the academic community and biopharmaceutical companies towards developing next-generation targeted NLRP3 inhibitors with optimized potency and pharmacological profiles, some of which have already progressed into clinical studies for various diseases. In addition to its core role in inflammasome signaling, NLRP3 has also been attributed with inflammasome-independent roles in supporting the polarization of T helper 2 (T_H2) cells and alternatively activated (M2) macrophages, cells which are centrally involved in the pathophysiology of asthma[165,166]. Moreover, as a co-factor of the transcription factor IRF4, NLRP3 has been implicated in modulating the expression and secretion of IL-33, thereby contributing to atopic dermatitis-associated inflammation[167]. It will be interesting to explore the extent to which NLRP3-targeted inhibitors also suppress these inflammasome-independent roles of NLRP3 to alleviate asthma and atopic dermatitis symptoms.

Therapeutic strategies that aim to simultaneously inhibit multiple inflammasome pathways are also under development, with drug candidates targeting ASC, GSDMD and NINJ1 spearheading these efforts. These emerging inflammasome targets offer distinctive mechanisms of action that can influence the safety and efficacy profiles of potential therapies because ASC, GSDMD and NINJ1 operate downstream of several inflammasome sensors, yet their selective inhibition only perturbs specific facets of downstream inflammasome responses. Now that the development of clinical-grade caspase-1 inhibitors has largely been abandoned[168], the strategies discussed above to neutralize the activities of ASC, GSDMD and NINJ1 across inflammasome pathways might help rejuvenate efforts to harness the therapeutic potential of broad inflammasome blockade. The role of GSDMD has been explored in many disease models across therapeutic areas, and the structural mechanisms of GSDMD autoinhibition, activation and pore formation have also come into the limelight. This combination of approaches has provided a deep understanding of the molecular basis of GSDMD activation and its role in human disease. Undoubtedly, this knowledge will also be applied for rational drug design of GSDMD-targeted therapeutics in the future. Grounded in many important discoveries over the past twenty years, inflammasome research has finally come of age and we anticipate exciting clinical breakthroughs in the next 20 years.

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This is the first in vivo evidence that GSDMD blockade can be effective in a chronic autoinflammatory disease model.

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This important paper describes the first large-scale clinical trial demonstrating that IL-1β inhibition confers protection against atherosclerotic disease.

Acknowledgments

We apologize to colleagues whose work was not cited because of space constraints. This work was supported by the Fund for Scientific Research (FWO)-Flanders grant G017121N and European Research Council Grant 101101075 (PyroScreen) to M.L.

AUTHOR CONTRIBUTIONS

THE AUTHORS CONTRIBUTED EQUALLY TO ALL ASPECTS OF THE ARTICLE.

PEER REVIEW INFORMATION

NATURE REVIEWS DRUG DISCOVERY THANKS [REFEREE#1 NAME], [REFEREE#2 NAME] AND THE OTHER, ANONYMOUS, REVIEWER(S) FOR THEIR CONTRIBUTION TO THE PEER REVIEW OF THIS WORK.

Competing Interests

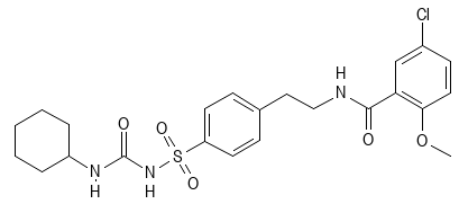
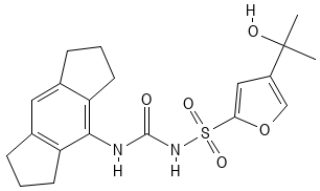
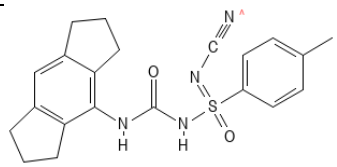
ML serves as a scientific consultant for Ventyx Biosciences and Novo Nordisk. LVW declares no conflict of interest.

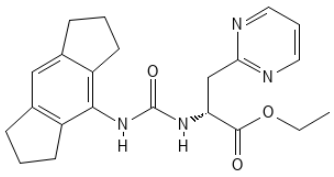
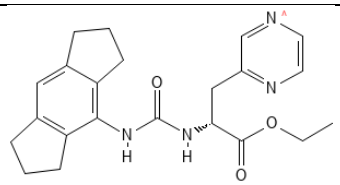
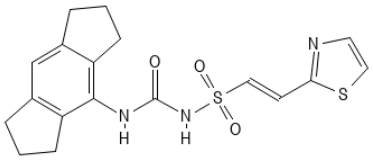
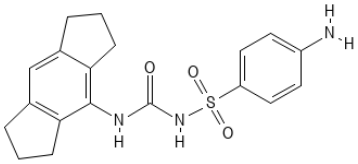
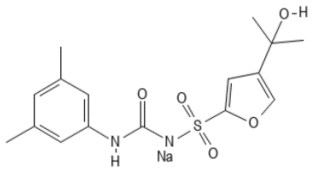
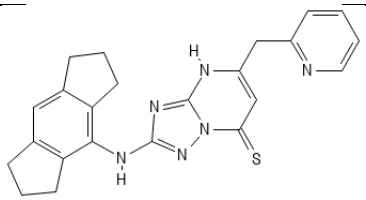
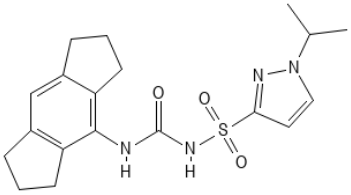
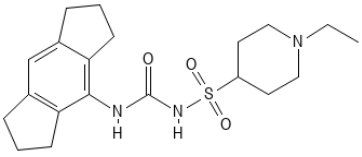
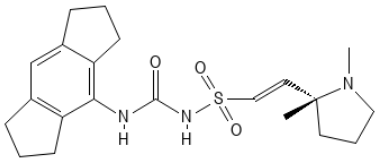
Table 1. NLRP3 inhibitors in clinical and preclinical development. Abbreviations**:** SAD, single ascending dose study. To the best of our knowledge, all listed compounds are part of the current development pipelines of the listed companies.

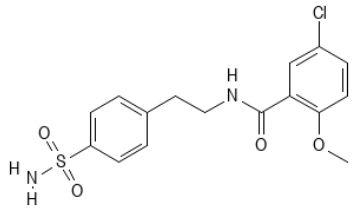
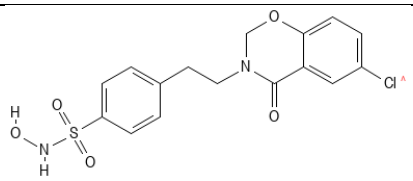
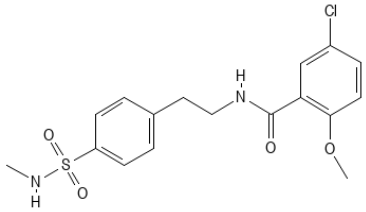
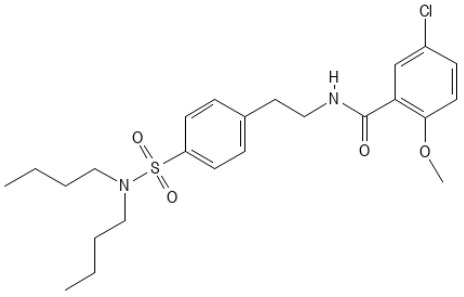
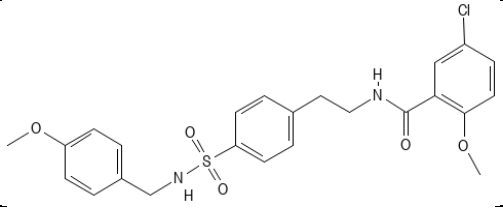
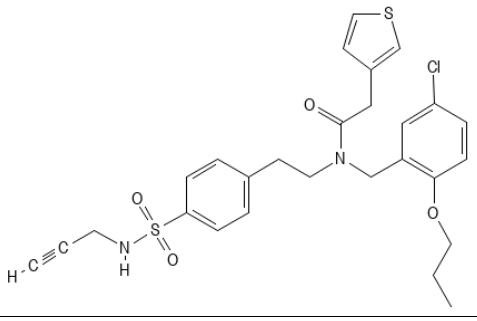
Project	Company	trial
Phase III		
RRx-001, ABDNAZ	Epicentrx	NCT03699956 (REPLATINUM), small cell lung cancer
Phase II		
DFV890, IFM-2427	Novartis (IFM tre)	NCT04868968, FCAS (completes Mar 2023) NCT04886258, knee osteoarthritis (completes Mar 2024)
ZYIL1	Zydus Cadila	NCT05186051, CAPS (completed Jul 2022)
Dapansutrile (OLT1177)	Olatec Therapeutics	Phase I (topical gel, completed, August 2012) Phase I (capsules, completed, December 2014) NCT01768975, osteoarthritis (completed August 2013) EudraCT number 2016-000943-14, gout (completed, Feb 2019)NCT03595371, Schnitzler's syndrome (completes Dec 2022) NCT04540120, COVID-19 Symptoms and Evidence of Early Cytokine Release Syndrome (CRS) (completes Dec 2022),
Phase I		
Selnoflast, Somalix, RG6418, IZD334	Roche (Inflazome)	Phase Ib, Ulcerative colitis (recruitment completed Q2 2022) Phase Ib, Chronic obstructive pulmonary disease (COPD) (first patient enrolled, Q2 2022)

VTX2735	Ventyx Biosciences	Phase I (completed Jun 2022), Phase II planned in CAPS
NT-0796	NodThera	Phase I (completed Sep 2022)
NT-0249	NodThera	Phase I SAD (completed Sep 2022)
HT-6184	Halia Therapeutics	NCT05447546 (completed Nov 2022)
Preclinical		
VTX3232	Ventyx Biosciences	IND-enabling studies for neuroinflammatory diseases. Phase I planned for Q1 2023
VENT-01	Novo Nordisk (Ventus)	IND-enabling studies for NASH, chronic kidney disease and other cardiometabolic conditions
VENT-02	Ventus	Phase I planned in 2023
NT-0527	NodThera	IND-enabling studies for neuroinflammatory diseases.

Table 2. NLRP3 inhibitors with disclosed chemical structures. Abbreviations: BMDM, bone marrow derived macrophages; Nig, nigericin; N/A, not available.

Compound name	Structure	IC_{50} (IL-1β assay)	References
glyburide		20 µM, BMDM, LPS + ATP	[67,93]
CRID3		7.5 nM, BMDM, LPS + ATP 30 nM, PBMC, LPS + ATP	[68]
Second-generation CRID3-based inhibitors			
N-cyano sulfoximineurea Cpd 15 ()		7 nM, THP1	[97]

Ester-substituted urea Cpd 44		36 nM, PBMC, LPS + ATP	98
Ester-substituted urea Cpd 45		30 nM, PBMC, LPS + ATP	98
		35 nM, THP1	101
Aniline sulfonylurea Cpd 8b		15 nM, BMDM, LPS + ATP	93
Cpd 4b		~30 nM, BMDM, LPS + ATP	100
NDT-30805		13 nM, PBMC, LPS + ATP	99
Emlenoflast, Inzomelid, MCC7840		N/A	81 Roche/Inflazome
Selnoflast, RG6418, Somalix, IZD334		N/A	Roche/Inflazome
ZYIL1		N/A	77 Zydus Lifesciences
Second generation glyburide-based inhibitors			

JC121, 16673-34-0		3.25 µM, J774A.1, LPS + ATP	103,105
JC171		8.45 µM, J774A.1, LPS + ATP	105
JC124		3.25 µM, J774A.1, LPS + ATP	102
Cpd 14, JC124 analogue		0.12 µM, BMDM, LPS + ATP	102
Cpd 17 JC124 analogue		0.36 µM, BMDM, LPS + ATP	102
YQ128		0.3 µM, J774A.1, LPS + ATP	106

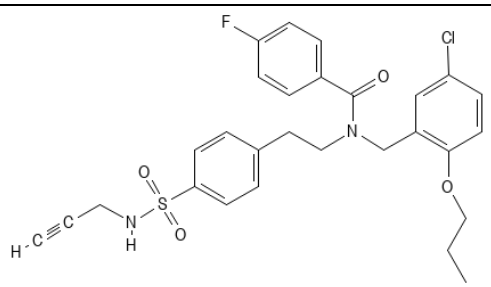
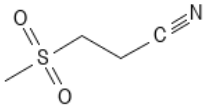
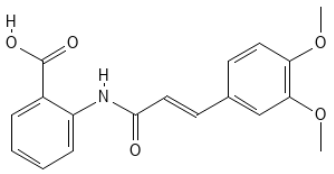
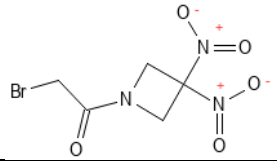
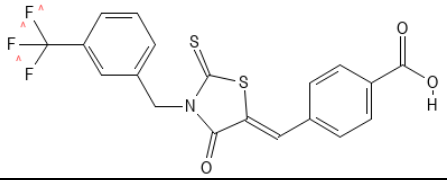
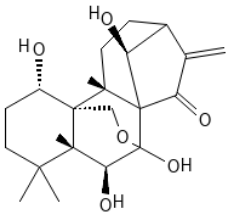
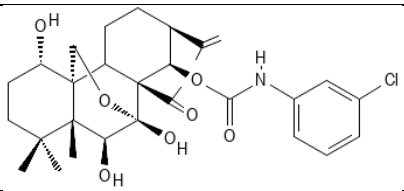
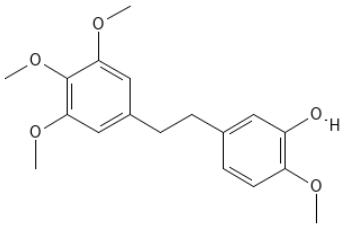
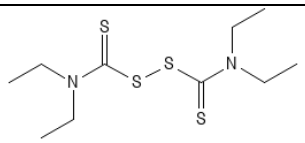
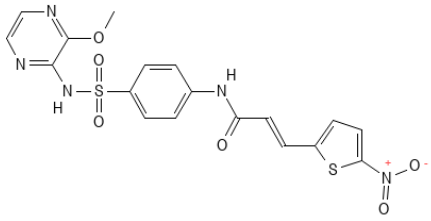
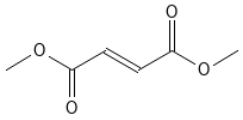
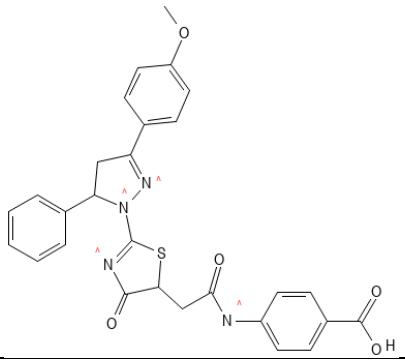
Cpd 19		0.12 µM, J774A.1, LPS + ATP	107
Synthetic NLRP3 inhibitors with distinct pharmacophores			
Dapansutrile, OLT1177, 3-methylsulfonylpropionitrile		N/A	87
Tranilast		~25 µM, BMDM, LPS + Nig	90
RRx-001		117 nM, BMDM, LPS + Nig	91
CY-09		~5 µM, BMDM, LPS + Nig	108
xNatural compounds and derived analogs			
Oridonin		~5.2 µM, THP-1, LPS + ATP	111
E6		~0.45 µM, THP-1, LPS + ATP	111
Erianin		~1 nM, BMDM, LPS + Nig	109

Table 3. Reported GSDMD inhibitors. *In the presence of copper gluconate Cu(II), which significantly reduces IC_{50} values by stabilizing diethyldithiocarbamate,, the active metabolite of DSF. Abbreviations: Nig, nigericin; PI, propidium iodide.

Compound name	Structure	IC50	References
Disulfiram, Antabuse, C-23		0.2 µM, Liposome leakage assay* 0.41 µM, THP1, LPS + Nig, CytoTox 96® LDH assay*	134
Necrosulfonamide		~2.5 µM, THP1, LPS + Nig, PI assay	135
Dimethyl fumarate, Tecfidera		<25 µM, BMDM, LPS + Nig, CytoTox 96® LDH assay	136
C202-2729		32 µM, Liposome leakage assay	140

[a]In the presence of copper gluconate Cu(II), which significantly reduces IC_{50} values by stabilizing diethyldithiocarbamate, the active metabolite of DSF. LPS, lipopolysaccharide; Nig, nigericin; PI, propidium iodide.

Figure legends

Figure 1. Overview of human inflammasomes with their respective stimuli and expression profiles.

Various stimuli activate the different inflammasome sensors. Activation of the PYD-containing inflammasomes (NLRP3, AIM2 and Pyrin) and the CARD-containing inflammasomes (NLRC4 and NLRP1) promotes recruitment of ASC and activation of caspase-1 through homotypic PYD-PYD and CARD-CARD interactions. The CARD8 inflammasome bypasses ASC and activates caspase-1 directly through CARD-CARD interactions. DPP8/9, dipeptidyl peptidases 8 and 9.

Figure 2. Molecular mechanisms driving NLRP3 inflammasome activation.

NLRP3 activation occurs through three main pathways. **a,** The canonical NLRP3 pathway involves a two-step process of priming and activation. In the priming step, stimulation of cytokine receptors such as IL-1R and PRRs such as TLR4 results in NF-κB-mediated transcriptional upregulation of NLRP3, pro-IL-1β and pro-IL-18. During NLRP3 activation, PAMPs such as bacterial ionophores and RNA viruses, DAMPs such as ATP, medically relevant crystals such as MSU and CPPD, as well as cholesterol and protein fibrils like β-amyloid (Aβ) and α-synuclein, are thought to promote translocation of inactive NLRP3 to subcellular organelles such as the dispersed trans-Golgi network. K^+ efflux has been proposed as a common upstream mechanism sensed by NLRP3 at these subcellular locations, although NLRP3 can also be activated independently of K^+ efflux. In response to the activation signal, NLRP3 forms a complex with NEK7 and recruits ATP, ASC and pro-caspase-1 to assemble the NLRP3 inflammasome, which activates caspase-1. Active caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms and also cleaves GSDMD, allowing GSDMD NT to form pores in the plasma membrane through which IL-1β, IL-18 and ion fluxes are released. Subsequently, GSDMD NT pore formation induces NINJ1-driven plasma membrane rupture, resulting in the release of numerous DAMPs into the extracellular space, including HMGB1, LDH and ASC specks. **b**, In the non-canonical pathway, membrane-bound vacuoles of Gram-negative bacteria that invade the cell are detected by guanylate-binding proteins (GBPs), which coordinate the exposure of cytosolic LPS to caspase-4/5/11, which induces caspase auto-activation. Active caspase-4/5/11 cleaves GSDMD, which induces pyroptosis and activates the NLRP3 inflammasome by lowering intracellular K^+ levels. **c**, In the alternative NLRP3 pathway, TLR4 stimulation activates NLRP3 downstream of the TRIF adaptor in human monocytes through an incompletely understood mechanism involving RIPK1, FADD and caspase-8. NLRP3 activation through this pathway subsequently engages caspase-1-mediated IL-1ß secretion through an unknown mechanism, independent of ASC speck assembly and without inducing pyroptosis.

Figure 3. Binding pocket of CRID3 and model for NLRP3 inflammasome activation.

a, The transition from the inactive ADP-bound state of NLRP3 to the active ATP-bound state requires binding to NEK7 as well as large conformational changes in the NACHT domain of NLRP3 that open the NBD and allow nucleotide exchange. CRID3 (yellow) and ADP (red) stabilize the inactive NLRP3 confirmation. ATP in the active NLRP3 complex is also depicted in red. Subdomains are color-coded as shown in the schematic diagram depicting the NLRP3 domain organization. The structures are based on the reported NLRP3 protein structures with PDB accession codes 7PZC (inactive NLRP3, ADP-bound), 6NPY (NLRP3–NEK7 complex, ADP-bound) and 8EJ4 and 8ERT (active NLRP3 complex, ATP-bound). The relative position of the PYD is arbitrary because of the disordered linker. **b,** Scheme depicting the transition from the closed, inactive NLRP3 cage structure (PDB: 7PZC) to the open and inflammasome-competent flower-shaped NLRP3 complex (PDB: 8EJ4). **c,** Atomic interactions between CRID3 and NLRP3 residues flanking the CRID3-binding pocket in the NACHT formed at the interface of the four NACHT subdomains and flanked by the extended LRR domain (PDB: 7PZC). Colours of the NLRP3 domains match those of panel (a).

Figure 4. Therapeutic targets and inhibitors of the NLRP3 inflammasome pathway.

The clinically licensed biologics canakinumab, rilonacept and anakinra suppress inflammation by preventing IL-1β from engaging IL-1R on effector cells, acting downstream of inflammasome activation. Compounds such as disulfiram, oridonin and tranilast impede NLRP3 inflammasome signaling at various stages, including the initial priming step. The discovery that sulfonylurea-containing inhibitors CRID3 and glyburide selectively inhibit the activation step of NLRP3 paved the way for the development of second-generation clinical-grade NLRP3 inhibitors with improved potency and pharmacological profiles such as ZYIL1, DFV890 and Selnoflast. VTX3232, VTX2735, NT-0796 and NT-0249 are additional NLRP3 inhibitors with undisclosed structures that are in clinical development. HT-6184 modulates NEK7 in addition to NLRP3. The anti-allergic drug tranilast and the candidate anti-tumor drug RRx-001 might not solely target NLRP3 as other immunomodulatory activities have been attributed to them. Other NLRP3 inhibitors in preclinical development include VENT-01, VENT-02 and NT-0527. CRID3 binds reversibly to the NACHT domain of NLRP3. Other compounds suggested to inhibit NLRP3 by binding to its NACHT domain include CY-09, tranilast and the covalent NLRP3 inhibitors erianin and oridonin. Compound E6 is a novel oridonin derivative with increased potency and selectivity. ASC, GSDMD and NINJ1 have emerged as additional inflammasome targets that act downstream of NLRP3 and other inflammasome sensors. The small molecule ASC inhibitor MM01 inhibits ASC oligomerization, and the ASC-targeted biologics IC-100 and VHH_{ASC} nanobody target extracellular ASC specks. C202-2729 is thought to non-covalently bind and inhibit

GSDMD NT translocation into the plasma membrane. The FDA-approved drugs disulfiram and dimethyl fumarate and the necroptosis inhibitor necrosulfonamide covalently modify and inhibit GSDMD. However, these molecules have a poly-pharmacological profile. GSDMD-targeted nanobodies $VHH_{GSDMD\text{-}1}$ and $VHH_{GSDMD\text{-}2}$ inhibit GSDMD oligomerization in the plasma membrane without interfering with GSDMD NT membrane insertion. The antagonistic NINJ1 monoclonal antibody (mAb) clone D1 inhibits NINJ1 polymerization and pyroptotic cell lysis downstream of GSDMD NT pore formation.

Boxes

Box 1. Auto-inflammatory diseases

Auto-inflammatory diseases (AIDs) are thought to primarily arise from antigen-independent overactivation of inflammatory pathways. The clinical presentation of AIDs varies significantly, but can involve recurrent episodes of spiking inflammation as well as a wide variety of clinical symptoms that affect multiple systemic tissues and organs such as the intestinal tract, skin, lungs, synovial tissues, skeleton and central nervous system. Serum amyloid A amyloidosis is a severe long-term complication of uncontrolled systemic inflammatory disease with poor prognosis in AID patients[169]. Prototypic examples of systemic AIDs are the hereditary monogenic AIDs that are caused by gain-of-function mutations in genes involved in inflammasome signaling (inflammasomopathies) and those primarily driven by excessive type I IFN signaling (interferonopathies). AIDs caused by loss-of-function mutations in genes that suppress activity of inflammasomes or type I IFN pathways have also been discovered. Examples of inflammasomopathies are familial Mediterranean fever (FMF, with mutations in the MEFV gene), cryopyrin-associated periodic syndromes (CAPS, with NLRP3 mutations), autoinflammation with infantile enterocolitis (AIFEC, with NLRC4 mutations) and deficiency of the IL-1 receptor antagonist (DIRA, with IL1RN mutations) (reviewed by [169]). Inflammasomes are thought to have a prominent role in the etiology of other monogenic AIDs because of the clinical efficacy of IL-1-blocking biologics. Such diseases include mevalonate kinase deficiency (or hyperimmunoglobulinemia D syndrome, MDK/HIDS; with MVK mutations) and tumor necrosis factor receptor associated periodic syndrome (TRAPS, with TNF-R1 mutations), as well as complex, multi-factorial inflammatory diseases such as gout, systemic juvenile idiopathic arthritis and Still's disease.

Box 2. NLRP3 and neuroinflammation

With mounting evidence implicating the NLRP3 inflammasome as a central disease mechanism in neuroinflammation, there has been a surge of interest to explore inflammasome targeting in neurodegenerative diseases such as multiple sclerosis, Alzheimer disease, Parkinson's disease and stroke[170]. Multiple sclerosis is a progressive inflammatory neurological disorder with a highly variable clinical presentation, ranging from minimal symptoms such as fatigue, numbness and blurred vision to loss of neurological function and ascending paralysis. Myelin-specific autoreactive $CD4^+$ T lymphocytes that cross the blood-brain barrier and blood-spinal cord barrier have a key role in multiple sclerosis pathogenesis by attacking the protective myelin coating that surrounds neurons in the brain and spinal cord, leading to neurodegeneration[171]. An important role for inflammasome signaling has been

demonstrated in multiple sclerosis pathogenesis. Patients exhibit elevated serum levels of IL-1β and increased caspase-1 activation, ASC speck formation and GSDMD processing in peripheral blood mononuclear cells (PBMCs)[136,172]. Studies in the experimental autoimmune encephalomyelitis (EAE) and cuprizone-induced models of multiple sclerosis showed that deficiency in NLRP3, ASC or GSDMD suppresses priming of peripheral immune cells and their subsequent infiltration into the CNS, resulting in diminished T cell-mediated myelin damage and disease pathology[59,173-175]. Furthermore, pharmacological inhibition of NLRP3 and GSDMD with small molecule inhibitors and IC-100 antibody-based neutralization of ASC suppressed inflammatory cell infiltration in the CNS and demyelination, significantly delaying disease progression[68,105,118,136,140,175,176]. Interestingly, the NLRP3 inhibitors JC-171 and CRID3 had potent *in vivo* therapeutic activity, suggesting that NLRP3 and downstream inflammasome mediators may be promising therapeutic targets in multiple sclerosis[105].

Alzheimer's disease is characterized by extracellular accumulation of β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein. These protein deposits in the brain currently represent the two most promising therapeutic targets. However, strategies designed to treat Alzheimer's disease-associated dementia by targeting the pathogenic build-up of Aβ and tau have had little success in late-stage clinical trials, and strategies targeting additional disease mechanisms are urgently needed. Several reports have implicated inflammasomes as disease targets. The brains of Alzheimer's disease patients display increased caspase-1 cleavage and elevated levels of mature IL-1β[57,177]. Moreover, phagocytosis of fibrillar Aβ activates NLRP3 in microglia[178]. NLRP3, ASC, and caspase-1 deficiency enhanced microglial clearance of Aβ, resulting in reduced Aβ deposits and improved cognition in the transgenic APP/PS1 mouse model of Alzheimer's disease[57,179]. Additionally, ASC specks released by microglia promoted Aβ aggregation by binding to Aβ, spreading Aβ pathology in the brain of APP/PS1 animals[179]. Interestingly, oral administration of NLRP3 inhibitor CRID3 reduced Aβ deposition in the brain of APP/PS1 transgenic animals and improved cognitive function[180].

Parkinson's disease is characterized by accumulation of α-synuclein–rich Lewy bodies (LBs), accompanied by chronic microglial neuroinflammation and loss of dopaminergic neurons in the substantia nigra. This results in lower dopamine concentrations and motor function impairment. Experimental Parkinson's disease models have implicated microglial NLRP3 inflammasome activation as a central event in dopaminergic neurodegeneration. Extensive inflammasome activation was noted in the brain of Parkinson's disease patients and various preclinical Parkinson's disease models[58]. α-Synuclein aggregates in microglia activate the NLRP3 inflammasome[181], which drives striatal dopaminergic neurodegeneration and behavioral defects in several experimental Parkinson's disease models[58,182-184]. Finally, CRID3-

mediated NLRP3 inhibition markedly reduced pathologic α-synuclein aggregates in the α-synuclein preformed fibril (PFF) mouse model of Parkinson's disease[58]. However, the precise mechanisms by which NLRP3 activation drive α-synuclein pathology has yet to be elucidated.

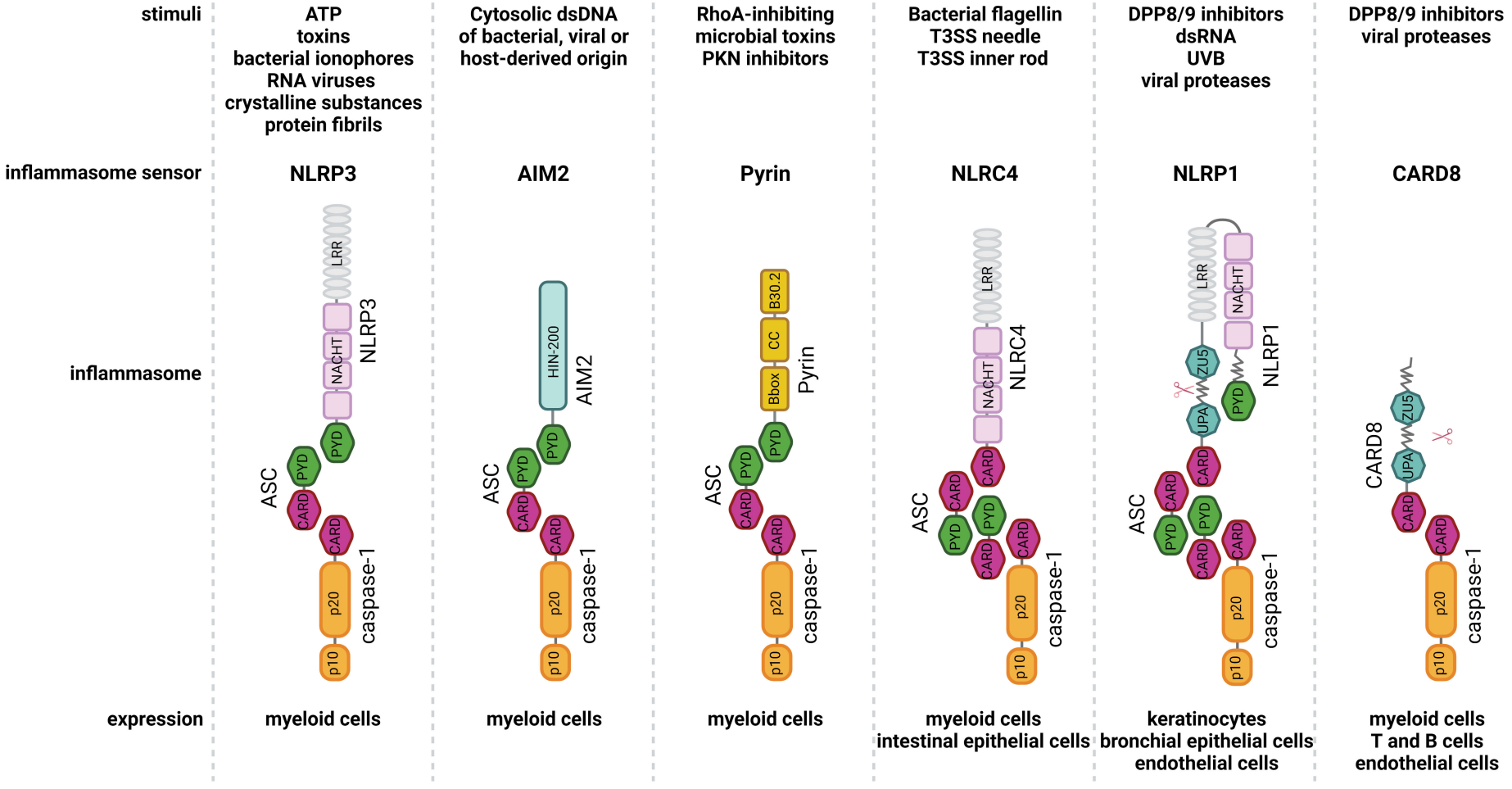
Box 3. Lessons from clinical studies on the role of inflammasomes in diseases

Three IL-1-neutralizing biologics have been licensed for clinical use in a subset of autoinflammatory diseases: canakinumab (Ilaris), rilonacept (Arcalyst) and anakinra (Kineret). Clinical experience with these biologics has provided strong proof-of-concept that inflammasome activation is detrimental in a subset of autoinflammatory diseases (see Box 1). Anakinra has also been approved for treatment of rheumatoid arthritis. As in gouty arthritis, patients suffering from FMF are primarily treated with the microtubule polymerization inhibitor colchicine, which suppresses periodic inflammatory attacks in the majority of patients and prevents amyloidosis, a major long-term complication that can result in renal failure and death. A small subset of gout and FMF patients are colchicine-resistant or –intolerant, and are effectively treated with IL-1-targeted biologics as second-line therapies[169]. Although colchicine has been a gold standard therapy in gout and FMF for many decades, its clinical efficacy is still not well understood nor why it is ineffective in treating other IL-1-mediated autoinflammatory diseases.

Exciting results from the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) phase III trial with the IL-1ß-targeted monoclonal antibody canakinumab have significantly expanded the potential clinical scope of inflammasome-targeted drugs. The primary objective of CANTOS was to assess whether reducing vascular inflammation rather than lowering plasma levels of cholesterol reduces rates of cardiovascular events in atherosclerosis patients[185]. CANTOS recruited 10,061 high-risk patients with prior myocardial infarction and a persistent systemic inflammatory response, defined by CRP levels ≥ 2 mg/L. The patients were treated with different doses of canakinumab and the incidence rate of recurrent cardiovascular events was assessed for a median of 3.7 years. Canakinumab was found to dose-dependently reduce the rate of major adverse cardiovascular events (MACE), independent of cholesterol control[185]. Remarkably, the CANTOS trial also registered a 77% reduction in lung cancer mortality and 67% reduction in lung cancer incidence in patients treated with the highest dose of canakinumab (300 mg)[186]. Secondary exploratory analyses of the CANTOS cohort further revealed significant reductions in risk for gout attacks independently of serum uric acid levels[187], a substantially reduced risk for osteoarthritis[188], and a reduction in the incidence of anemia by 16%[189]. These findings provide strong support for the premise that inflammasome-targeted therapies might be beneficial in multiple diseases beyond the rheumatic and hereditary autoinflammatory syndromes for which IL-1 blocking therapies are currently

licensed for clinical use. However, CANTOS also showed that IL-1ß inhibition confers an increased risk for upper airway infections, consistent with results from clinical trials with several IL-1-targeted biologics in inflammasomopathies and rheumatoid arthritis patients.

The CANTOS findings also raise several intriguing questions. What drives IL-1ß production in this patient subset and how is IL-1ß production causally and mechanistically linked to increasing atherosclerosis risk and risk for lung cancer development? Is the clinical effect of canakinumab associated with local neutralization of IL-1ß signaling at the affected tissue or does it require neutralization of IL-1ß at a more systemic level? Is IL-1ß production in atherosclerosis, lung cancer, anemia and osteoarthritis driven by the NLRP3 inflammasome, and what is the role of other inflammasomes? Would upstream targeting with NLRP3-specific inhibitors provide a safety and efficacy advantage over IL-1ß blockade? Undoubtedly, the future availability of clinical-grade NLRP3 inhibitors will help to address some of these questions.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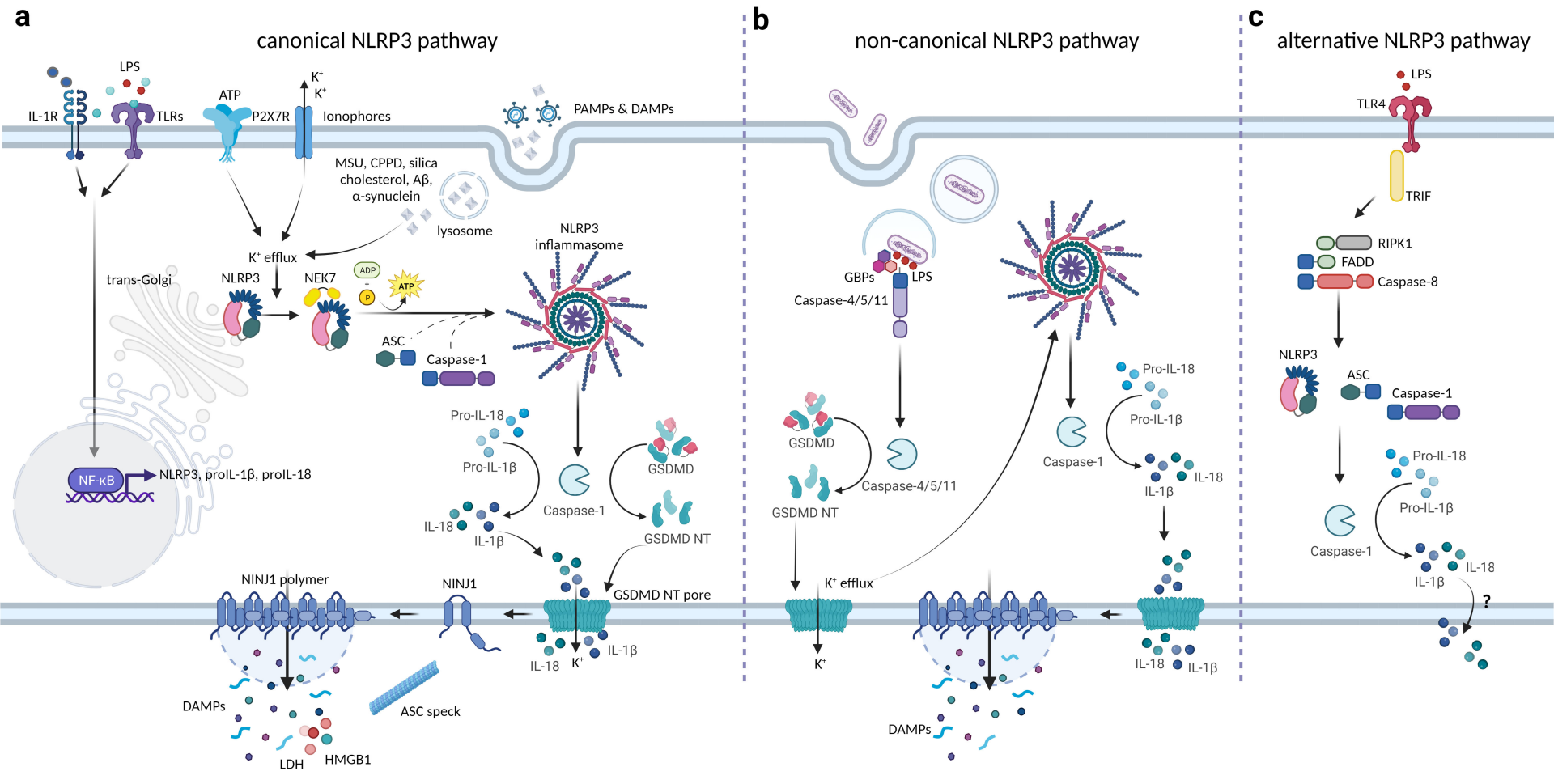
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canonical NLRP3 pathway
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lysosome
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trans-Golgi
NLRP3
NEK7
ADP
P
ATP
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inflammasome
ASC
Caspase-1
NF-κB
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IL-1β
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GSDMD NT
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IL-1β
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K+
Caspase-1
Pro-IL-18
Pro-IL-1β
IL-18
IL-1β
IL-18
IL-1β
DAMPs
c
alternative NLRP3 pathway
LPS
TLR4
TRIF
RIPK1
FADD
Caspase-8
NLRP3
ASC
Caspase-1
Pro-IL-18
Pro-IL-1β
Caspase-1
IL-18
IL-1β
?

a

Inactive NLRP3
ADP-bound

NLRP3

NACHT

PYD FISNA NBD HD1 WHD HD2 LRR

NEK7

HD2

WHD

LRR

HD1

NBD

FISNA

PYD

NLRP3/NEK7 complex
ADP-bound

LRR

HD2

WHD

HD1

NEK7

FISNA

NBD

ADP + P

ATP

active NLRP3 complex
ATP-bound

NEK7

LRR

HD2

HD1

WHD

FISNA

NBD

PYD

b

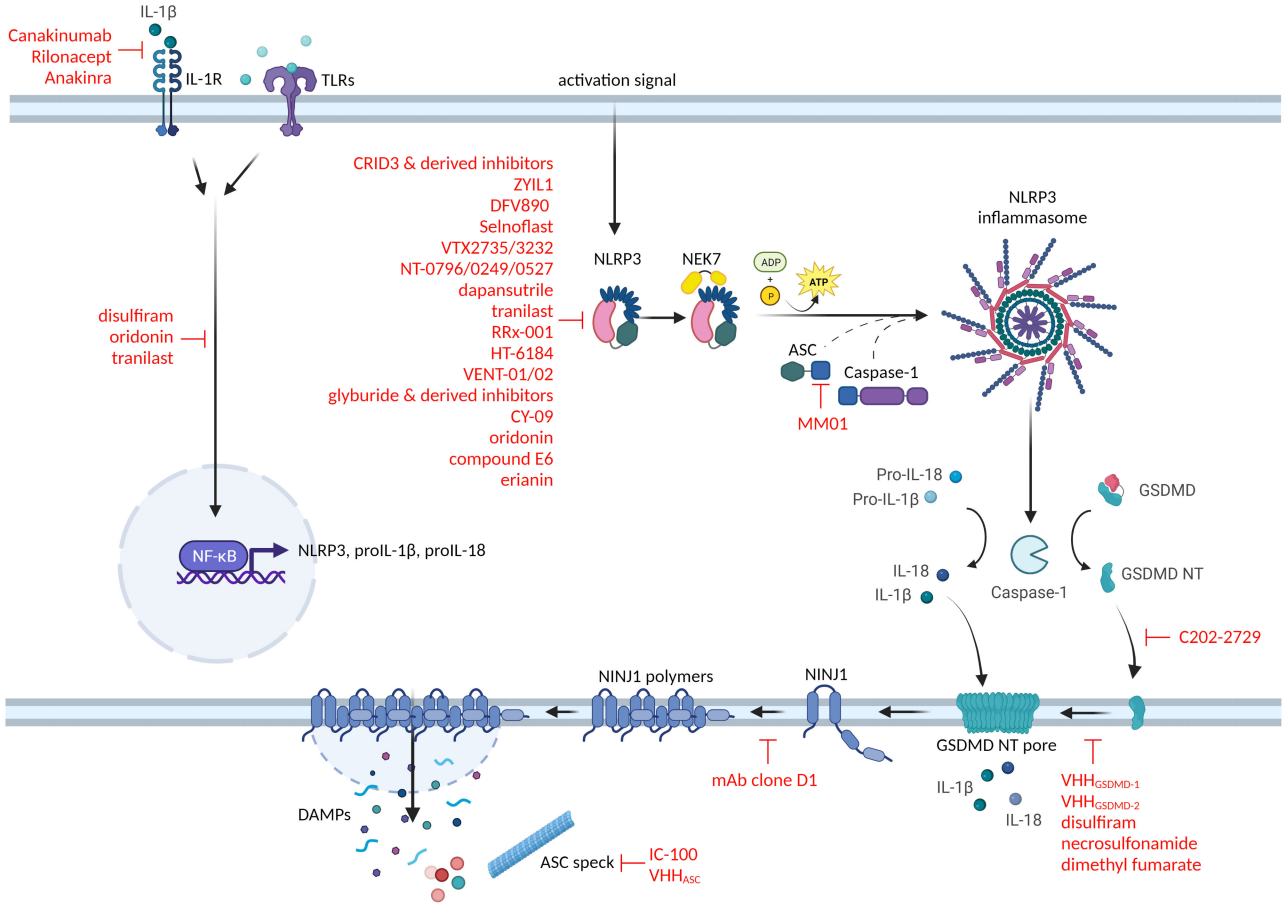
c

R578

A228

A227

R351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oridonin
compound E6
erianin
NLRP3
NEK7
ADP
P
ATP
ASC
Caspase-1
MM01
NLRP3
inflammasome
disulfiram
oridonin
tranilast
NF-κB
NLRP3, proIL-1β, proIL-18
Pro-IL-18
Pro-IL-1β
GSDMD
IL-18
IL-1β
Caspase-1
GSDMD NT
C202-2729
NINJ1 polymers
NINJ1
GSDMD NT pore
mAb clone D1
IL-1β
IL-18
VHHGSDMD-1
VHHGSDMD-2
disulfiram
necrosulfonamide
dimethyl fumarate
DAMPs
ASC speck
IC-100
VHHASC