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New insights into the noncanonical inflammasome point to caspase-4 as a druggable target

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Abstract

Recent studies indicate that the human lipopolysaccharide sensor caspase-4, unlike its mouse homologue caspase-11, is constitutively expressed and activates pro-IL-18 as well as gasdermin D-mediated pyroptosis. Activation of human caspase-4 causes vascular leakage systemically and at the blood–brain barrier in mice and is implicated in the pathogenesis of a range of inflammatory diseases for which there are currently no effective therapies. These results suggest the therapeutic potential of modulating caspase-4 activity, and structural studies indicate that the caspase-4 exosite might be a promising inhibitory target.

Introduction

The inflammatory caspases (caspases 1, 4 and 5 in humans; caspases 1 and 11 in mice) respond to pathogenic infection, tissue damage and other threats to homeostasis by inducing the release of inflammatory factors that recruit immune cells to sites of 'danger' and promote their activation[1,2]. The inflammatory caspases are expressed in cell types that are most likely to encounter infectious agents, particularly immune sentinel cells (monocytes, macrophages and dendritic cells), mucosal epithelial cells and endothelial cells[3,4]. They are highly homologous and have the same overall structure. Unlike the apoptotic effector caspases (caspase-3, caspase-6 and caspase-7), the inflammatory caspases have an amino-terminal homotypic interaction domain, known as a caspase activation and recruitment domain (CARD), that has a crucial role in their assembly into large, micrometre-sized, multicomponent complexes known as inflammasomes[3–5]. Within inflammasomes, the inflammatory caspases dimerise in an anti-parallel configuration that causes them to

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Author contributions

All authors contributed to all aspects of the article.

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activate each other. Dimerisation causes auto-cleavage of the flexible interdomain linker (IDL) between their two catalytic domains — a larger, 17–20 kDa domain that contains the active site and a smaller 9–12 kDa domain[6–9] (Fig. 1).

There are two classes of inflammasome — canonical and noncanonical. Cytosolic NOD-like receptors (NLRs), AIM2-like receptors (ALRs) and pyrin, which sense pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[5], assemble into canonical inflammasomes that recruit and activate caspase-1. The other inflammatory caspases (caspases 4, 5 and 11) directly bind to lipopolysaccharide (LPS) from Gram-negative bacteria and to some oxidized host lipids to form noncanonical inflammasomes[10,11]. The optimal peptide cleavage sites for the inflammatory caspases are WEHD (for caspase-1) and W/LEHD (for caspase-4 and caspase-5)[12]. Upon activation, the inflammatory caspases all cleave and activate the pore-forming protein gasdermin D (GSDMD) after the aspartic acid (D) residue of the tetrapeptide sequence, LESD, in the linker connecting the N-terminal and carboxy-terminal domains of GSDMD[13]. The N-terminal fragment of GSDMD then assembles onto mitochondrial and plasma membranes to form pores that can rapidly kill cells via pyroptosis[14,15]. In addition, IL-1 family cytokines — which are also activated by some of the canonical inflammatory caspases (see below) — and other host molecules that cause inflammation (known as alarmins), including other cytokines, chemokines, ATP and HMGB1, are released from the cell through these GSDMD pores. In some situations, the membrane damage caused by cleaved GSDMD is repaired and the cell does not die[16]. This has been termed ‘hyperactivation’ because inflammatory cytokines are still released, often in much higher amounts than from dying cells.

Until recently, most research has focused on the canonical inflammasomes, and strategies to interfere with cytokine-related and pyroptosis-related inflammation have mostly involved inhibiting canonical inflammasomes. The canonical inflammasome sensors recognize a diverse set of danger signals to recruit and activate caspase-1 (refs. 5,17). Their expression is inducible but under basal conditions is mainly limited to the myeloid sentinel cells and mucosal epithelial cells that most frequently encounter infection. Caspase-1 not only cleaves and activates GSDMD but also processes the IL-1 family pro-cytokines pro-IL-1β and pro-IL-18 into their highly inflammatory, mature states, which enables their release from cells through GSDMD-induced plasma membrane pores. Because the IL-1 family pro-cytokines do not have a secretion signal and are electrostatically repelled by the intracellular acidic rim of the GSDMD pore, they are only released from cells after cleavage to their mature forms, which have a more basic outer surface[18]. The canonical inflammasomes generally contain adaptor molecules, such as ASC, that have homotypic-interacting CARDs that recruit caspase-1 to the cytosolic sensor (NLR, ALR or pyrin). The ASC-containing canonical inflammasomes are readily visualized by confocal microscopy as ‘ASC specks’ that colocalize with activated caspase-1 (refs. 19,20).

By contrast, the noncanonical inflammasomes are simpler structures, as human caspase-4 and caspase-5 and mouse caspase-11 directly sense and are activated by LPS. Our current understanding is that the noncanonical inflammasomes consist of only the lipid danger signal and the inflammatory caspase. Here, we discuss recent insights into the structure, expression and biological functions of the noncanonical inflammasomes. These include

differences between human caspase-4 and mouse caspase-11 that may have resulted in the in vivo effects of noncanonical inflammasome activation in humans to be underestimated. In particular, pro-IL-18 is now known to be processed by caspase-4 and caspase-5 in addition to caspase-1, but not by caspase-11. These insights point to the inhibition of caspase-4 as a new therapeutic strategy in various diseases where this noncanonical inflammasome has been shown to have an important pathological role. So far, not much is known about caspase-5, which is expressed under basal conditions in a more limited number of tissues and cell types than caspase-4, including intestinal epithelial cells, monocytes and macrophages (Supplementary Fig. 1). The role of caspase-5 in human physiology and pathology requires further study.

Noncanonical inflammasomes

The noncanonical inflammasome caspases contain CARDs that directly recognize and bind to the lipid A domain of LPS in the cell wall of most Gram-negative bacteria[21,22]. Thus, they do not require separate sensor and adaptor molecules. Binding to LPS triggers the proximity-induced dimerisation and activation of caspase-4, caspase-5 and caspase-11, which then cleave GSDMD to trigger pore formation and pyroptosis (Fig. 2).

Speck-like structures

The noncanonical inflammasomes, which consist of caspase-4, caspase-5 or caspase-11 bound directly to LPS, were assumed to assemble into large inflammasome structures similar to the ASC specks observed for the canonical inflammasomes. However, owing to a lack of probes, such noncanonical inflammasome ‘specks’ have only recently been visualized in LPS-transfected and *Legionella pneumophila*-infected mouse macrophages, and in LPS-transfected human HEK293T cells that ectopically express a fluorescently labelled caspase-11 fusion protein[23]. Noncanonical inflammasome speck formation in mouse cells requires the autoprocessing of caspase-11 to a catalytically active form as it does not occur in cells expressing catalytically dead caspase-11 or a mutated form that prevents caspase activation by autoproteolysis, or in cells treated with a pan-caspase inhibitor. It will be important to confirm these findings with endogenously expressed caspases. Unlike for some of the canonical inflammasomes[24–29], the high-resolution structure of a noncanonical inflammasome has not yet been solved but is thought to be formed by LPS micelles. Solving the structure of the noncanonical inflammasome may be challenging using available methods as these micelles are likely to be heterogeneous in size.

Activation

Bacterial LPS in the cell wall of cytosol-invasive bacteria or in outer membrane vesicles that are released from bacteria and endocytosed by cells is not recognized by the noncanonical inflammasome caspases until the lipid A component of LPS is exposed by binding to cellular guanylate-binding proteins (GBPs)[30–33] (Fig. 2). Two recent elegant structural studies have helped to understand how GBPs expose lipid A for caspase-4 binding and activation[34,35]. When pathogenic bacteria invade the cytosol, binding of GBP1 to the outer membrane of Gram-negative bacteria recruits GBP2, GBP3 and GBP4 to coat the entire bacterial surface with tens of thousands of GBP complexes within minutes. The GBP

complexes intercalate with LPS, release LPS into the cytosol and make lipid A accessible for the activation of caspase-4. Although previous studies have suggested that caspase-4 assembles and is activated on GBP-coated bacteria[31–33], it is not completely clear whether caspase-4 activation occurs on the LPS micelles that are released by GBPs or on the surface of coated bacteria. In either case, GSDMD cleavage, cytokine release and pyroptosis in response to Gram-negative bacterial invasion do not occur in cells knocked out for *GBP1*.

There is some evidence that the noncanonical inflammasome caspases can also be activated by other lipids, such as lipoteichoic acid in Gram-positive bacterial cell walls[36], unknown lipids from bacteria or invasive fungi or parasites[37–39] or oxidized host phospholipids[11]. These other lipids could activate the noncanonical inflammasome directly by binding to noncanonical caspases or their mechanism of activation could be indirect. For example, some oxidized host phospholipids — produced by reactive oxygen species that are generated during pyroptosis or other forms of programmed cell death, or in cells with damaged mitochondria or undergoing other forms of oxidative stress — bind to caspase-11 (refs. 11,40–42). However, this binding seems to be to the caspase catalytic region rather than the CARD and can lead to the inhibition or activation of GSDMD depending on the context[43,44]. Furthermore, certain oxidized host phospholipids that activate caspase-11 seem to be only weak agonists. In myeloid cells, where this phenomenon has been studied, GSDMD is activated and inflammatory cytokines are released in response to oxidized phospholipids, but the cell is able to repair GSDMD-mediated plasma membrane damage and survive[11,16,45].

It is important to note that caspase-4-mediated activation of pyroptosis and plasma membrane damage can also be sensed by NLRP3 and lead to secondary activation of the canonical inflammasome pathway, including the processing of IL-1 family cytokines, in cells expressing NLRP3 and caspase-1 (ref. 46). Careful studies to compare the kinetics of caspase-4 and GSDMD activation with formation of ASC specks and caspase-1 activation, and knockout experiments or use of GSDMD or NLRP3 inhibitors, could be used to distinguish primary from secondary NLRP3 activation.

Expression

Caspase-4 is unique among the noncanonical inflammasome caspases in that it both is constitutively expressed and can be directly activated by LPS, whereas the activation of mouse caspase-11 and human caspase-5 needs to be ‘primed’ by the expression and/or activation of other plasma membrane or endosomal receptors by extracellular PAMPs or DAMPs[47–51] (Supplementary Fig. 1). Caspase-4 is constitutively expressed, together with GSDMD and pro-IL-18, in a wide variety of cell types, including myeloid sentinel cells (in particular, at high levels in monocytes and tissue macrophages), epithelial cells and endothelial cells. The expression level of caspase-4 can be increased further by exposure to interferon-γ (IFNγ) and DAMPs and PAMPs that activate Toll-like receptors (TLRs), and by specific cellular stresses such as endoplasmic reticulum stress or hypoxia[39,40]. By contrast, most cells need to be primed to express caspase-5, which is encoded by a highly homologous gene duplication of *CASP4* (77% identity). Under basal conditions, caspase-5 expression is highest in intestinal epithelial cells but it is also expressed in the lung, bladder, spleen and lymph nodes, all sites exposed to microorganisms (Supplementary Fig. 1). Both

caspase-5 and caspase-11 are induced and regulated by stimulation with IFNγ and LPS[52,53], although caspase-5 has not been as well studied as caspase-4 and caspase-11. It is also of note that both *CASP4* and *CASP5* can be alternatively spliced to produce proteins that lack parts of their N-terminal LPS-binding domains. We propose that these alternatively spliced protein products might function as dominant-negative variants that inhibit the inflammatory response to LPS. However, the biological relevance of these alternatively spliced proteins and whether they can dimerise with full-length protein requires further study.

Because of its constitutive, widespread expression — and ability to directly process pro-IL-18 (see next section) — caspase-4 activation of the human noncanonical inflammasome triggers a much more potent response to LPS than its mouse homologue caspase-11. *Casp11*$^{-/-}$ mice knocked in with a human *CASP4* gene that contains the regulatory regions (including the promoter) are much more responsive to LPS than wild-type mice and have increased inflammatory cytokine secretion[54–56]. Indeed, *CASP4* knock-in mice are about 2 logs more sensitive to LPS than wild-type mice; low concentrations of LPS that do not kill wild-type mice are rapidly lethal in *CASP4* knock-in mice. Some bacteria, including *Francisella* and *Neisseria* strains, produce heterogeneous forms of LPS that have fewer lipid A acyl side chains than the hexa-acylated LPS produced by most bacteria. Human caspase-4, but not mouse caspase-11, recognizes and is activated by LPS that contains under-acylated lipid A, although it binds to under-acylated lipid A with lower affinity than to hexa-acylated lipid A[56]. This finding suggests that caspase-4 can detect LPS from a broader range of bacteria than caspase-11. Humans are much more sensitive to LPS than wild-type mice (about 1,000–10,000-fold more sensitive)[57], which may explain why many therapeutic interventions that protect mice from bacterial sepsis have failed in human trials[58]. However, as there are many aspects of innate immunity that are not conserved between mice and humans (or indeed between different mouse strains), differences other than that between caspase-4 and caspase-11, such as in the TLR4 response to LPS and downstream signalling[59,60] and the primate-specific expression of NLRP11 (which enhances caspase-4 responses to LPS[61]), could also contribute to the increased LPS responsiveness in humans.

A new substrate for caspase-4

Until recently, the noncanonical inflammasome caspases were not thought to process IL-1 family pro-cytokines directly. In cells undergoing noncanonical inflammasome-mediated pyroptosis that also express NLRP3 and caspase-1, caspase-1 is secondarily activated when the NLRP3 canonical inflammasome senses pyroptotic plasma membrane damage. Caspase-1 then processes pro-IL-1β and pro-IL-18, and the mature cytokines are secreted through GSDMD pores.

Although some previous studies had suggested that caspase-4 might process pro-IL-18 to its mature cytokine form, these studies were sometimes carried out under non-physiological conditions and did not clearly define whether cleavage of pro-IL-18 was direct or where it was cleaved[32,62–65]. However, two studies now provide more direct and conclusive evidence that human caspase-4 and caspase-5 recognize and cleave pro-IL-18 after Asp36 to its 18 kDa mature form[51,66]. These studies have shown that caspase-4 binds pro-IL-18

with nanomolar affinity (dissociation constant (K_d) = 290 nM)[51], and that caspase-4 and caspase-5 cleave pro-IL-18 similarly and as efficiently as human caspase-1, whereas rodent caspase-11 neither binds nor cleaves pro-IL-18 (refs. 51,66). Rabbit, pig, sheep, dog and lemur caspase-4 also process pro-IL-18 of the same species[66]. Human caspase-4 also cleaves two other IL-1 family members, pro-IL-1β and pro-IL-36γ, in vitro but with 30–100-fold less efficiency than cleavage of pro-IL-18 (ref. 66). Strong cleavage of pro-IL-18 and weak cleavage of pro-IL-1β by caspase-4 were also reported in two independent studies[9,67]. One study suggested that the ability of caspase-4 to cleave pro-IL-1β might be increased if the caspase-4 interdomain linker is cleaved after Asp289 rather than the usual site Asp270 (ref. 9) (Fig. 1). However, the enzymatic activity of the alternate cleavage variant of caspase-4 was not measured. Furthermore, it is unclear whether caspase-4-mediated maturation of IL-1β or IL-36γ has any physiologically relevant role. On the basis of these results, we caution that any future preclinical evaluation of potential treatments for bacterial sepsis should use animals that constitutively express a caspase-4 homologue that activates their pro-IL-18, such as rabbits, sheep or non-human primates, and thus that are as sensitive to LPS as humans. Human caspase-4 only weakly cleaves mouse pro-IL-18 in vitro, which suggests that the *CASP4* knock-in mouse, despite being more sensitive to LPS than wild-type mice, may still underestimate the in vivo effects of noncanonical inflammasome activation in humans, particularly in in vivo situations that depend on IL-18 activity.

Both of the published studies showing that caspase-4 cleaves proIL-18 reported the structure of caspase-4 bound to pro-IL-18 at similar resolution — using X-ray crystallography[51] or cryo-electron microscopy[66] — and revealed that the complex forms a ‘wrapped candy’-shaped dimer, composed of two anti-parallel caspase-4 molecules bound at both ends to pro-IL-18 (Fig. 3a). In both studies, these interactions needed to be stabilized to solve the structure, which suggests that the caspase-4–pro-IL-18 interaction is likely to be flexible. One study[51] took advantage of previous work from the same group[68] that had identified a *Shigella* effector protein, OspC3, as an inhibitor of caspase-4 that binds to its catalytic domain by an ankyrin repeat domain (ARD); this study used the OspC3 ARD to co-crystallize catalytically inactive caspase-4 and pro-IL-18. The other study[66] used chemical cross-linking of catalytically inactive caspase-4 to pro-IL-18 to obtain a stable complex. Both structures show that caspase-4 and pro-IL-18 interact at two sites — the caspase-4 catalytic site and a secondary binding site of caspase-4 termed an exosite (a site outside of the catalytic cleavage site; also known as an allosteric site) that was previously shown to be required for binding of caspase-1 and caspase-4 to GSDMD[69] (Fig. 3b). The catalytic site of caspase-4 binds to a U-shaped cleavage site of the pro domain of pro-IL-18 via electrostatic interactions, whereas the caspase-4 exosite engages the mature domain and part of the pro domain of pro-IL-18 via hydrophobic and electrostatic interactions (Fig. 3c,d).

Modelling the caspase-11–pro-IL-18 interaction based on the caspase-4–pro-IL-18 structures provides an explanation as to why pro-IL-18 is not cleaved by caspase-11 (ref. 51). Whereas the sequences of caspase-4 and caspase-5 are almost identical to that of caspase-1 in the catalytic binding site and the exosite, caspase-11 has multiple nonconservative substitutions in both interaction interfaces. A loop in the modelled caspase-11 exosite sterically clashes with pro-IL-18 and mutating the residues in that exosite loop to those present in the caspase-4 exosite enables the mutant caspase-11 to process

pro-IL-18 as efficiently as caspase-4 (ref. 51). Together with previous studies, these new insights highlight the importance of the exosite for inflammatory caspase-mediated cleavage of both IL-1 family pro-cytokines and GSDMD. They suggest that compounds that bind to the exosite of inflammatory caspases could be effective caspase-4 (and caspase-1) inhibitors for therapeutic development.

Emerging roles of caspase-4 in disease

The importance of the noncanonical inflammasome in the pathology of sepsis is well established based on mouse *Casp11*-knockout studies and the effectiveness of GSDMD inhibitors. However, recent studies (as described above) suggest that mouse models may have underestimated the importance of the noncanonical inflammasome in humans. In particular, the induction of pyroptosis and IL-18 activation by caspase-4 in endothelial cells seems to have a crucial role in vascular leakage systemically and in maintaining the integrity of the blood–brain barrier (BBB)[55,70]. Here, we discuss the potential role of caspase-4 in diseases in which vascular leakage is central to life-threatening pathology, such as sepsis, pneumonia, cytokine storm and ischaemia–reperfusion injury. There is some suggestion that caspase-4 may also contribute to the inflammatory pathology of other diseases in which infection or IL-18 exacerbate pathology, such as inflammatory bowel disease (IBD).

Sepsis

Despite hundreds of targeted clinical trials testing treatments for sepsis — which is the leading cause of death of children worldwide and contributes to the death of a large proportion of hospitalized adults — including some therapies that have sought to inhibit individual inflammatory cytokines or LPS, there is still no effective treatment for sepsis other than supportive care[58]. Previous studies have clearly shown a central role of caspase-11 in protection from bacterial pathogens but also in causing the lethal effects of excessive inflammation in mouse models of sepsis[21,71] (Table 1). mRNA and protein expression of the noncanonical inflammasome caspases, human caspase-4 and caspase-5 and mouse caspase-11, are induced by LPS priming and during sepsis[72]. Most mice knocked out for *Casp11* or *Gsdmd* survive a lethal challenge with LPS or polymicrobial sepsis caused by caecal ligation and puncture (CLP), whereas wild-type mice similarly challenged all die within a few days[13,70,73,74]. Caspase-11 activation drives sepsis and its accompanying complications, particularly cytokine release syndrome and the loss of vascular integrity, resulting in hypotension, tissue oedema and multi-organ failure. Disruption of vascular integrity is the main driver of death in sepsis[75,76]. A previous pioneering study[70] showed that the noncanonical inflammasome is activated in mouse and human endothelial cells after LPS is taken up into these cells via TLR4. All the features of acute lung injury in a mouse model of LPS-induced sepsis — disruption of the pulmonary vasculature, pulmonary oedema, neutrophil infiltration and inflammatory cytokine release — were nearly completely abolished in mice knocked out for caspase-11 only in endothelial cells[70]. As caspase-4 is constitutively expressed in human vascular endothelial cells and surrounding pericytes, unlike mouse caspase-11 which is expressed after cell priming, the LPS-induced activation of caspase-4 in human sepsis may have a greater effect on endothelial cell pyroptosis than in mouse models and therefore more rapidly disrupt vascular integrity.

A recent study has shown that lethal doses of LPS in mice also activate caspase-11 and GSDMD in brain endothelial cells to disrupt the BBB[55]. Brain endothelial cell-specific knockout of *Gsdmd* protected BBB integrity after LPS challenge. The BBB was disrupted by much lower doses of LPS in *CASP4* knock-in mice than in wild-type mice, which confirms the increased in vivo sensitivity to LPS of caspase-4 compared with caspase-11. Importantly, wild-type mice were relatively resistant to *Klebsiella pneumoniae* infection compared with *CASP4* knock-in mice, which developed lethal BBB disruption that was dependent on brain endothelial cell expression of GSDMD.

An important next step will be to confirm the activation of caspase-4 in models of sepsis and infection in animal species that naturally express caspase-4 (such as rabbit, pig[77,78] or non-human primates) to better understand the role of noncanonical inflammasome activation in vascular leakage and sepsis. The recent mouse study of the BBB[55] described above also did not evaluate the extent to which pro-IL-18 activation by caspase-4 contributes to vascular leakage or other sepsis pathologies, including disruption of the systemic vasculature. As caspase-4 does not efficiently cleave mouse pro-IL-18 in *CASP4* knock-in mice, additional experiments in animal species that have caspase-4 homologues that also cleave pro-IL-18, such as pigs, may also allow for a better understanding of the contribution of IL-18 to vascular leak and sepsis pathology. Of note, a clinical case report of severe and persistent *Burkholderia pseudomallei* lung infection that did not respond to standard antibiotic treatment was associated with a homozygous mutation in the p10 domain of activated caspase-4 (R344W) that markedly reduced its ability to cleave both GSDMD and pro-IL-18 (ref. 79). This study, combined with the others mentioned above, suggests that caspase-4 activation in response to bacterial infection and LPS can have overall beneficial or pathological effects depending on the setting — GSDMD can directly kill bacteria[80] and intracellular bacteria cannot efficiently survive and replicate in pyroptotic cells[81], but unrestrained pyroptosis can lead to systemic inflammation, cytokine release syndrome and vascular leakage to cause hypotension, acute organ failure, disseminated intravascular coagulation and death.

Currently, most cases of sepsis in the USA are polymicrobial or caused by pathogens that do not produce LPS, such as Gram-positive bacteria or fungi[82]. Wild-type or *CASP4* knock-in mice challenged with CLP resulting in polymicrobial sepsis or with Gram-positive bacterial infection have much less severe disease if they are genetically deficient in caspase-11 or GSDMD[13,36,74,83] or are treated with a caspase-4 inhibitor (E.E., F.G.G. and M.A.C., unpublished observations), which suggests that the noncanonical inflammasome can be directly or indirectly activated by other pathogens in addition to LPS-producing Gram-negative bacteria to induce pathology. However, this needs to be more thoroughly investigated in other models of sepsis.

Vascular leakage and lung pathology

A role for caspase-4 activation in other diseases that are driven by vascular leakage should also be evaluated (Table 1). For example, infection with SARS-CoV-2 increases caspase-4 expression in human cells in vitro and drives pyroptosis in lung vascular endothelial cells in vivo[84]. Moreover, caspase-11-deficient mice are protected from lung

pathology caused by infection with mouse-adapted SARS-CoV-2, without any effect on viral load[84]. A potential contribution of caspase-4 activation to lung vascular leakage in acute respiratory distress syndrome triggered by various pathogens, including influenza A virus and *Streptococcus pneumoniae*, should be investigated in in vivo models. Models of non-infectious pulmonary artery hypertension also implicate caspase-4 in the tumour necrosis factor-induced pyroptosis of human vascular cells and suggest a role for caspase-11 in pulmonary vascular pathology in mice[85] (Table 1). A role for caspase-4 and caspase-11 in asthma has been suggested by a study showing that *Casp11*-knockout mice are protected from disease in the ovalbumin-induced model of allergic asthma[86]. Caspase-4 and caspase-11 expression are down-regulated by prostaglandin E_2, which is a known allergy-protective agent, and aspirin (which inhibits prostaglandin synthesis) can induce asthma[86]. The increased levels of IFNγ in the blood during checkpoint inhibitor-induced cytokine release syndrome and capillary leak, combined with the known capacity of IL-18 to induce IFNγ production and of IFNγ to induce caspase-4 expression in vascular endothelial cells and pericytes, suggest that caspase-4 might also promote vascular leakage in this condition[49].

Inflammatory bowel disease

IBD is another disease in which caspase-4 activation might contribute to inflammation and tissue damage, leading to loss of barrier integrity of the gut epithelium, in response to changes in the microbiota or the presence of LPS-containing outer membrane vesicles. Increased levels of caspase-4 and caspase-5, as well as of IL-18 and GBPs, have been observed in individuals with IBD[87–89]. Caspase-4 was also shown to regulate IL-18 secretion in human intestinal epithelial cells in response to infection with *Salmonella enterica* serovar Typhimurium[90]. Unlike in mouse intestinal epithelial cells, pathogenic infection of a human intestinal epithelial cell line with *Salmonella* was independent of the canonical inflammasome — knockout or knockdown of *NAIP–NLRC4*, *NLRP3*, *ASC* or *CASP1* did not reduce pyroptosis of these cells. Further studies in primary human epithelial cells or organoids, and involving other bacterial pathogens, are necessary to confirm this finding and to determine which inflammasome pathways are triggered in intestinal epithelial cells by other gastrointestinal pathogens.

Ischaemia–reperfusion injury

Several recent papers suggest that the noncanonical inflammasome might also be involved in ischaemia–reperfusion injury (Table 1). Caspase-4 expression is upregulated by cellular hypoxia followed by reoxygenation in human cardiac microvascular endothelial cells[91,92]. Caspase-11, but not caspase-1, is similarly upregulated in mouse cardiomyocytes and is responsible for GSDMD cleavage and pyroptosis under hypoxia–reoxygenation conditions[91]. *Gsdmd* deficiency reduces the size of myocardial infarcts in a model of ischaemia–reperfusion injury in mice[91]. There is also evidence of pyroptosis following cardiac ischaemia–reperfusion injury in humans. For example, the level of serum GSDMD is increased in patients with signs of cardiac ischaemia within an hour of angiographic stent placement to resume perfusion of the tissue[91]. It will be important to examine more carefully whether caspase-11 or *CASP4* knock-in in mice, and caspase-4 in other species, drive pyroptosis, inflammation and increased infarct size not only in the heart but also in

the brain, kidney and other organs during ischaemia–reperfusion injury. Although genetic manipulation of caspases can provide strong evidence in mouse models, caspase-4 inhibitors (as recently identified, see next section) could potentially be used to study the role of caspase-4 in other species.

Neurodegenerative disease

There are also hints that caspase-11 is involved in the pathogenesis of neurodegenerative diseases in mice — specifically, MOG peptide-induced experimental autoimmune encephalomyelitis (a model of multiple sclerosis)[93]. However, in the superoxide dismutase 1-mutant model of amyotrophic lateral sclerosis, caspase-11-deficient mice did not show any marked difference in pathology[94]. Nevertheless, caspase-4-mediated cleavage has been shown to remove a nuclear localization sequence in TDP43 — which regulates RNA processing and has been implicated in the pathogenesis of amyotrophic lateral sclerosis — and cause TDP43 cytoplasmic accumulation[95]. Further study of these diseases in *CASP4* knock-in mice or in caspase-4-expressing pigs or non-human primates would help to determine whether caspase-4 inhibitors might be useful therapeutics for these indications.

IL-18-dependent disease

Disease conditions in which IL-18 levels are increased, including Still's disease (a systemic inflammatory disorder) and some monogenic inflammatory diseases, are currently being investigated using biologics that directly target IL-18 (ref. 96). In view of the recent discovery that pro-IL-18 is a substrate of caspase-4, these diseases might also be sensitive to caspase-4 inhibitors. However, it should be noted that in some of these diseases, activation of the canonical inflammasome and of caspase-1 might be more (or equally) important than activation of the noncanonical inflammasome. Which inflammasome pathway is most important for IL-18 production will need to be determined for each specific disease. Another disease to consider is hidradenitis suppurativa, a severe form of acne where skin bacteria cause inflammation and severe local tissue damage. Skin biopsies from individuals with this disease show increased levels of IL-18 that are not reduced by an inhibitor of the canonical NLRP3 inflammasome and might instead be driven by bacterial activation of the noncanonical inflammasome pathway[97]. Although there are no animal models of hidradenitis suppurativa available to study, the effects of a caspase-4 inhibitor on patient skin biopsies could be investigated.

Pharmacological inhibition of caspase-4

These studies of the noncanonical inflammasome suggest that specific inhibitors of caspase-4 would be invaluable for investigating the role of caspase-4 in *CASP4* knock-in mice and in other species that naturally express caspase-4 (provided that the human inhibitors also inhibit caspase-4 in these species), in various disease models involving pathogenic infection, vascular leakage, IBD, ischaemia–reperfusion injury, neurodegenerative disease or increased levels of IL-18. These investigations would set the stage for evaluating caspase-4 inhibition as a new therapeutic strategy.

A caspase-4 inhibitor, the peptide-based Z-LEVD-FMK, was previously developed based on the preferred cleavage sequence of caspase-4 (W/LEHD)[12]. However, peptide-based caspase inhibitors have limited utility for in vivo studies or clinical use owing to their poor bioavailability and pharmacokinetics. Two dual caspase-1 and caspase-4 active-site inhibitors have been reported: the peptide-based Z-YVAD-FMK, which has the same limitations as Z-LEVD-FMK[98] and the small molecule VX-765 (ref. 99). Further development of VX-765 was halted, probably because it required high doses for inhibition and had a poor therapeutic index[100,101]. VX-765 has superb potency in solution but relatively poor cellular potency, similar to other caspase catalytic site inhibitors[99]. Active-site inhibitors of inflammatory caspases also generally lack specificity because their substrate sequence preferences overlap[102]. It has been hypothesized that an allosteric caspase inhibitor (targeting the exosite) could potentially overcome these issues[103]. Allosteric inhibitors of caspase-1 and caspase-7 were previously reported and shown to inhibit substrate access to the catalytic site[104,105]. We have recently identified cell-potent allosteric inhibitors that target both caspase-4 and caspase-5, which inhibit cleavage of the only validated direct substrates of these noncanonical inflammasome caspases, GSDMD and pro-IL-18 (E.E., F.G.G. and M.A.C., unpublished observations). As caspase-4 and caspase-5 are so similar in amino acid sequence (77% identity), it may not be possible to identify inhibitors that are specific for only one of them. These recently identified compounds will be valuable tools to help understand the emerging biological roles of caspase-4 and caspase-5 and potential therapeutic opportunities.

Concluding remarks and future perspectives

Recent insights into the expression of caspase-4, the identification of pro-IL-18 as a substrate of caspase-4, the determination of structural models of how caspase-4 interacts with GSDMD and pro-IL-18, and the demonstration that activation of caspase-4 leads to vascular leak strongly suggest that caspase-4 may be a valuable therapeutic drug target for numerous diseases that lack effective treatment options. Current drug development to interfere with pyroptosis-related inflammation has mostly focused on inhibiting the canonical inflammasome, particularly NLRP3. The discovery of small-molecule tool compounds selective for caspase-4 and caspase-5 will further our understanding of the biochemistry, and cellular and in vivo biology of the noncanonical inflammasome pathway and might pave the way for developing noncanonical inflammasome-targeting therapeutics.

Supplementary Material

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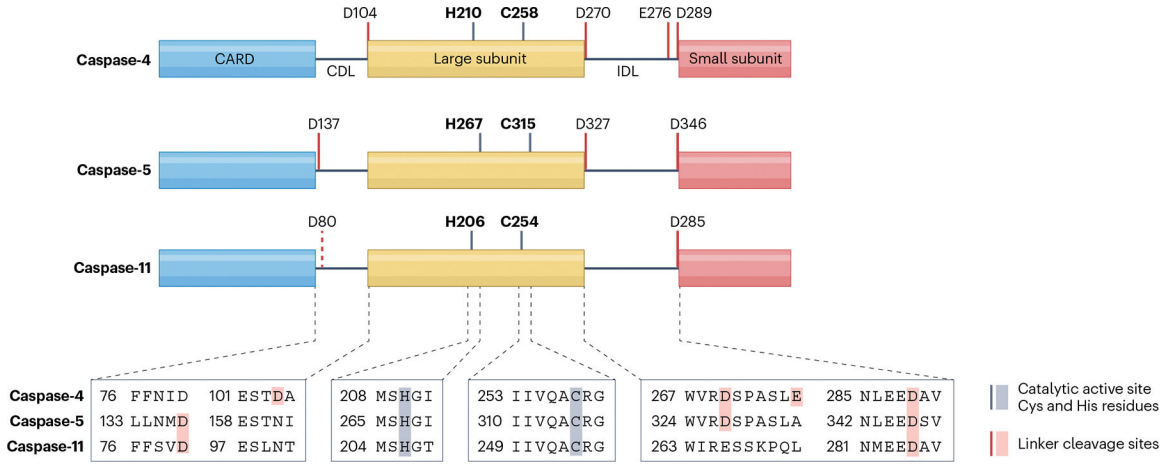

Fig. 1 |. Sequence and domain structure of the noncanonical inflammasome caspases. Caspase-4 and caspase-5 (human) and caspase-11 (mouse) have a similar domain organization that consists of an N-terminal caspase activation and recruitment domain (CARD), and large and small subunits. The large subunit contains the catalytic cysteine and histidine residues of the active site. The three domains are connected by two linkers, the CARD domain linker (CDL) and the interdomain linker (IDL). The auto-cleavage sites in the linkers are indicated with red lines. The IDL must be cleaved to activate the caspase, but cleavage of the CDL may disrupt the noncanonical inflammasome 'speck' and suppress caspase activity[1]. Cleavage of caspase-11 after D80 in the CDL has been observed when caspase-11 is overexpressed but may not occur under physiological conditions[7] (as indicated by the dashed line). Cleavage of caspase-4 after D80 in the homologous sequence has not been reported. Sequence alignment of key regions highlights the similarities of the auto-cleavage sites and the active site cysteine and histidine residues.

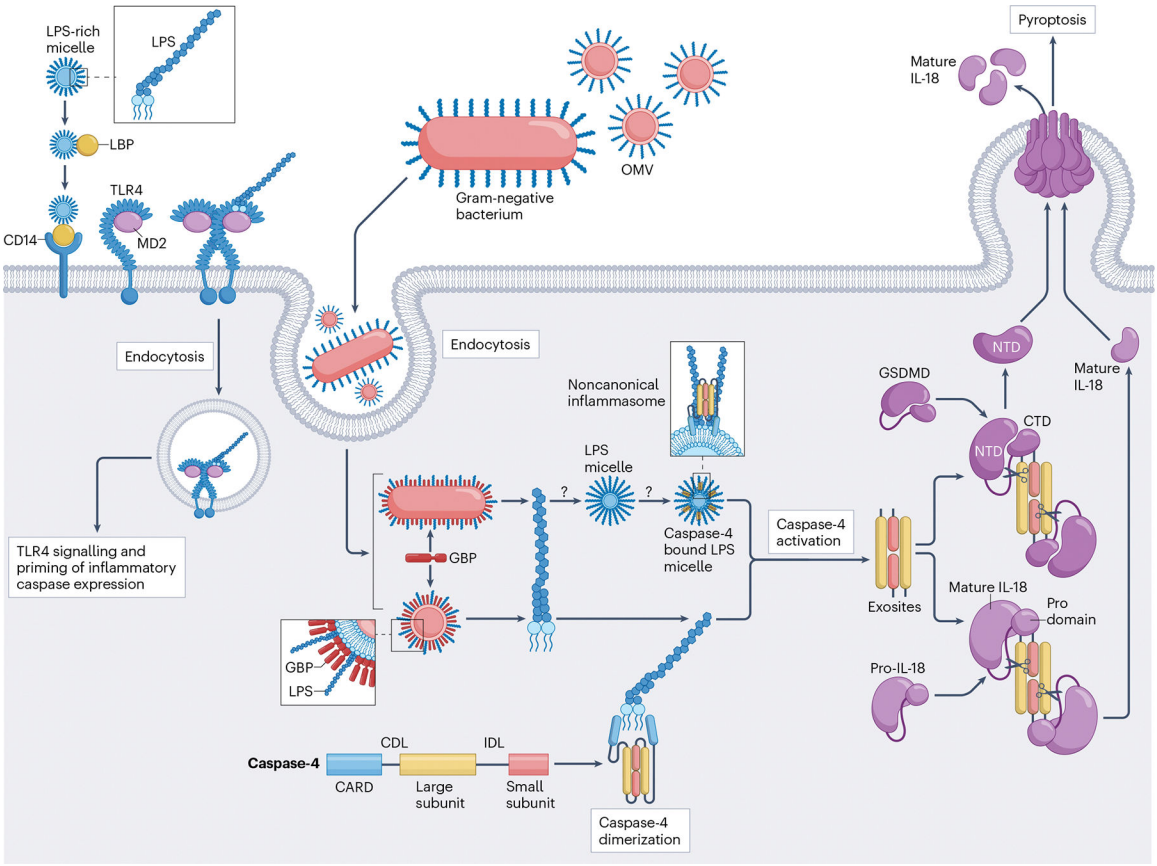

Fig. 2 |. The noncanonical inflammasome pathway.

Lipopolysaccharide (LPS)-rich micelles in the serum bind to LPS-binding protein (LBP). LBP is recognized by cell-surface CD14, which transfers single LPS molecules to Toll-like receptor 4 (TLR4)-associated MD2, resulting in the dimerisation and endocytosis of TLR4 and TLR4 signalling to prime inflammatory caspase expression. Pathogenic Gram-negative bacteria or their outer membrane vesicles (OMVs) are endocytosed and released into the cytosol, where they are recognized by cytosolic guanylate-binding proteins (GBPs), which bind and coat bacteria and OMVs to release LPS. Released LPS, either as free molecules or possibly in micelles, then binds to the caspase recruitment and activation domain (CARD) of the noncanonical inflammasome caspases (caspase-4 (shown), caspase-5 or caspase-11). This interaction forms the noncanonical inflammasome, in which the caspases dimerise and are proteolytically autoactivated by cleavage of their interdomain linker (IDL) between large and small subunits. After the IDL is cleaved, exosites formed by both large and small subunits of the caspase create a platform to recruit its substrates, gasdermin D (GSDMD) and pro-IL-18. All the noncanonical inflammasome caspases cleave GSDMD, but only caspase-4 and caspase-5 recruit and cleave pro-IL-18. The noncanonical inflammasome caspases cleave the linker between the N-terminal domain (NTD) and C-terminal domain (CTD) of GSDMD, leading to binding of the GSDMD-NTD to cell and mitochondrial membranes, its oligomerization and membrane pore formation to trigger pyroptosis. Simultaneously, caspase-4 and caspase-5 cleave and activate pro-IL-18 to generate mature

IL-18, which is secreted from the cell through GSDMD-NTD cell membrane pores. CDL, CARD domain linker.

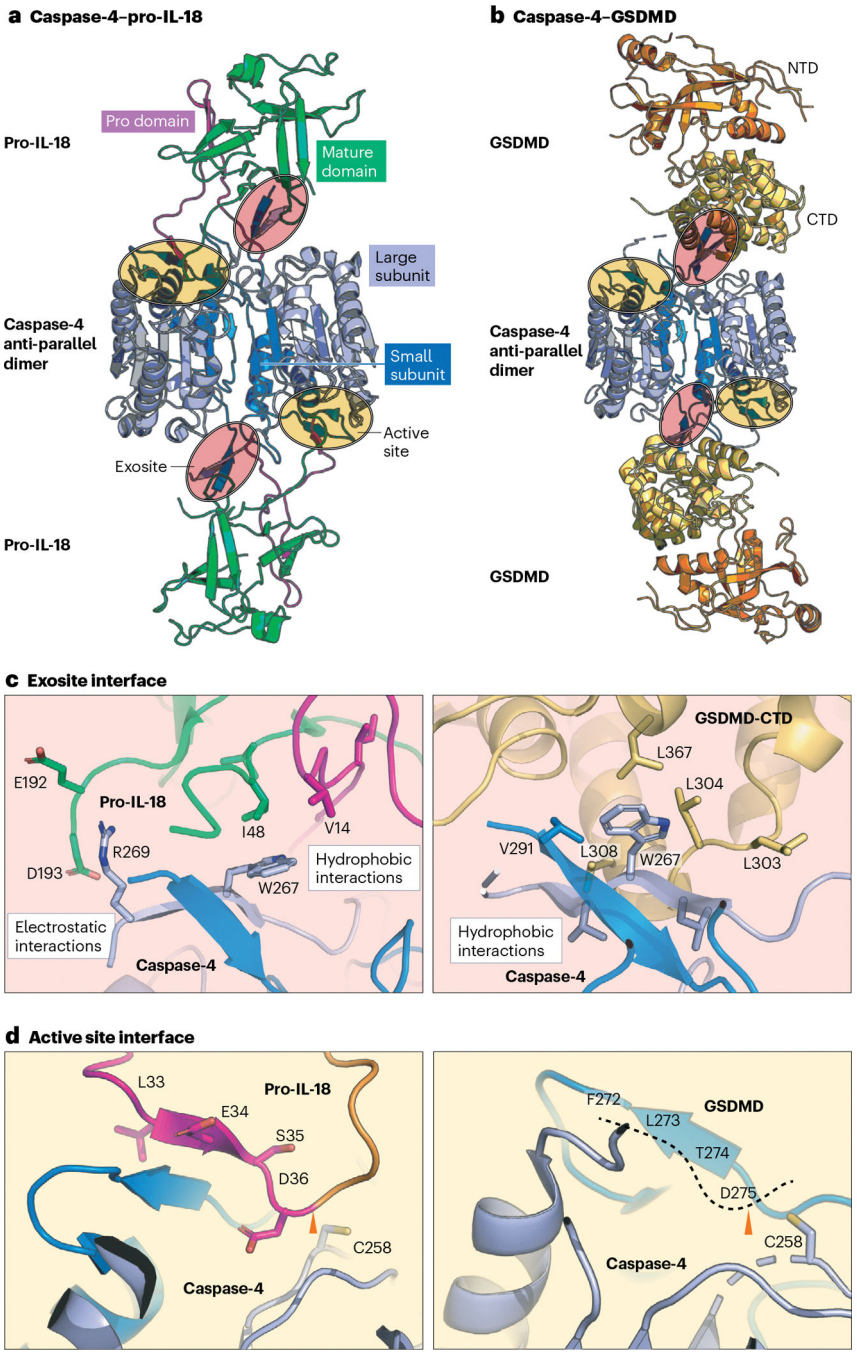

Fig. 3 |. Caspase-4 structure and substrate recognition.

a,**b**, Overall structure of the caspase-4 dimer (each monomer in blue and light blue for small and large subunits, respectively) in complex with: pro-IL-18 (in green and magenta)[66] (**a**) and gasdermin D (GSDMD) (C-terminal domain (CTD) in yellow[69] and N-terminal domain (NTD) in orange[110]) (**b**). Pro-IL-18 structure from Protein Data Bank (PDB) code 8J6K[66]; GSDMD-CTD structure from PDB code 6KMZ[69]; GSDMD-NTD structure modelled using the full-length GSDMD structure PDB code 6N9O[110]. Caspase-4 interacts with its substrates at two sites — the caspase-4 catalytic sites (highlighted in yellow) and a secondary binding

site termed an exosite (highlighted in red). Caspase-4 interacts with pro-IL-18 and GSDMD using two exosites, one at each end of the caspase-4 anti-parallel dimer. **c**, The exosites, which are comprised of residues from both the large and small subunits of caspase-4, form hydrophobic and electrostatic interfaces with both the pro and mature domains of pro-IL-18, whereas the caspase-4 exosites interact with GSDMD-CTD via hydrophobic interactions. **d**, The substrate-binding pocket of caspase-4 recognizes the LESD sequence in the linker connecting the pro and mature domains of pro-IL-18 to align D36 of pro-IL-18 with the caspase-4 active site catalytic residue C258 for cleavage of pro-IL-18 after D36 (shown by orange arrow). Similarly, the FLTD sequence in the linker connecting NTD and CTD of GSDMD aligns with the caspase-4 active site residue C258 for cleavage of GSDMD after D275 (shown by orange arrow). The C258 side chain was modelled from A258 (active site mutant) used for structural studies.

Table 1l

Potential roles of noncanonical inflammasome activation in human disease

Disease	Evidence	Refs.
Sepsis	Increased survival of caspase-11-deficient mice	36,41,55,70,76,106
COVID-19 and ARDS	Increased protection of caspase-11-deficient mice in a model of SARS-CoV-2 infection	84
Pulmonary arterial hypertension	Increased protection of caspase-11-deficient mice	85
Capillary leak syndrome and acute lung injury	IFN-induced expression of caspase-4 in vascular endothelial cells; lack of vascular leakage and acute lung injury in caspase-11-deficient mice	49,70
Ischaemia–reperfusion injury	Caspase-11-deficient cardiomyocytes resist hypoxia–reoxygenation-induced pyroptosis	91,107
Multiple sclerosis	Increased protection of caspase-11-deficient mice in an EAE model	93
Amyotrophic lateral sclerosis	Caspase-4, but not caspase-11, cleaves TDP43 resulting in its cytoplasmic redistribution	95
Diseases with increased levels of IL-18 (such as Still's disease)	Caspase-4 activation may contribute to the observed high levels of IL-18 in various settings	96,108,109
Hidradenitis suppurativa	High levels of IL-18 in patient biopsies are not reduced by an NLRP3 inhibitor	97
Inflammatory bowel disease	Increased levels of caspase-4, caspase-5, IL-18 and GBPs in individuals with disease; pathogenic *Salmonella* infection of human intestinal epithelial cells activates caspase-4-dependent pyroptosis	87–89
Allergic airway inflammation	Increased protection of caspase-11-deficient mice	86

ARDS, acute respiratory distress syndrome; EAE, experimental autoimmune encephalomyelitis; GBPs, guanylate-binding proteins; IFN, interferon.