Pyroptosis is the caspase-dependent inflammatory cell death mechanism that underpins the innate immune response against pathogens and is dysregulated in inflammatory disorders. Pyroptosis occurs via two pathways: the canonical pathway, signaled by caspase-1, and the noncanonical pathway, regulated by mouse caspase-11 and human caspase-4/5. All inflammatory caspases activate the pyroptosis effector protein gasdermin D, but caspase-1 mostly activates the inflammatory cytokine precursors pro-interleukin-18 and pro-interleukin-1β (pro-IL18/pro-IL1β). Here, in vitro cleavage assays with recombinant proteins confirmed that caspase-11 prefers cleaving gasdermin D over the pro-ILs. However, we found that caspase-11 recognizes protein substrates through a mechanism that is different from that of most caspases. Results of kinetics analysis with synthetic fluorogenic peptides indicated that P1’–P4’, the C-terminal gasdermin D region adjacent to the cleavage site, influences gasdermin D recognition by caspase-11. Furthermore, introducing the gasdermin D P1’–P4’ region into pro-IL18 enhanced catalysis by caspase-11 to levels comparable with that of gasdermin D cleavage. Pro-IL1β cleavage was only moderately enhanced by similar substitutions. We conclude that caspase-11 specificity is mediated by the P1’–P4’ region in its substrate gasdermin D, and similar experiments confirmed that the substrate specificities of the human orthologs of caspase-11, i.e. caspase-4 and caspase-5, are ruled by the same mechanism. We propose that P1’–P4’-based inhibitors could be exploited to specifically target inflammatory caspases.

Caspases constitute a family of cysteine proteases that are obligate dimers in their active conformations (1, 2). They have a stringent requirement for aspartic acid at the primary specificity position in their substrates (P1) (Fig. 1) (3). A great number of studies have aimed to understand the specificity mechanisms of these central proteases of regulated cell death (3, 4). Early works pointed out the requirement for interaction with four amino acid residues to the N-terminal side of a cleavage site (5); thus, caspases can be distinguished by their favored P1–P4 sequences (6).

The main objective of learning the specificity requirements of a caspase is to use this information to develop specific and selective chemical biology tools for their study in cellular biology and disease models. However, success is limited by the overlap in the preferred peptide sequences, which prevents the generation of selective reagents for individual caspases (7). Cleavage sequences have also been explored, aiming to predict natural protein substrates; however, many endogenous substrates are cleaved at suboptimal sites (8). Alternative substrate specificity mechanisms, like exosites, enzyme regions distant from the catalytic site that participate in substrate binding, are emerging as mechanisms explaining the discrepancies between consensus sequences and actual cleaved proteins by caspases (9–11). Investigating these mechanisms is important, because they may reveal novel targeting sites for caspase inhibition.

Inflammatory caspases regulate pyroptosis, a form of regulated cell death central to the innate immune response and clearance of pathogens (12). Pyroptosis is divided into canonical and noncanonical pathways. Caspase-1 is involved in signaling canonical pyroptosis, whereas mouse caspase-11 and human caspase-4 and -5 regulate the noncanonical pathway. Caspase-1 is activated in protein complexes known as inflammasomes, which are assembled upon recognition of pathogen or damage-associated molecular patterns (13, 14). In contrast, lipopolysaccharide from Gram-negative bacteria is the only known activator of the noncanonical pathway and is thought to directly induce the activation of caspases (15–17).

Inflammatory caspases cleave the pyroptosis effector protein gasdermin D to release its N-terminal domain (18, 19). The N-terminal gasdermin D domain oligomerizes in the plasma membrane, causing the distinguishing lytic event of pyroptotic cell death (20, 21). A recognized function of gasdermin D is to allow the release of the proinflammatory cytokines interleukin-1β and -18 (pro-IL1β and pro-IL18) (22, 23). Genetic and biochemical evidence indicates that the precursors of these cytokines (pro-IL1β and pro-IL18) are proteolytically activated by caspase-1 (15, 24). Caspase-11 does not activate the interleukins directly, but caspase-1 is activated downstream of caspase-11 in the noncanonical pathway (15). The detrimental effects of autoinflammatory diseases can be halted in part by anti-IL1 therapy, signifying the potent inflammatory nature of this cytokine (25–27), making pyroptosis an attractive target for therapeutics of inflammation disorders. The development of inhibitors and probes to selectively target each inflammatory caspase would be beneficial to investigate their mechanisms in cell death signaling and disease and provide a path toward anti-inflammatory therapeutics.
Inflammatory caspase specificity

To control for folding of the substrates, we employed the apoptotic caspase-3, which has been shown to generate loss-of-function cleavages alternative to those of the inflammatory caspases in gasdermin D and the prointerleukins (Fig. 2B) (31, 35, 36). As previously reported, we observed cleavage by caspase-3 (Fig. 2, A and C). These results suggest that caspase-3 inactivates the interleukins as part of a mechanism that blunts pyroptosis (36).

Human caspase-4 and caspase-5 are often considered synonymous enzymes and orthologues of murine caspase-11. However, these enzymes differ in their tissue expression and are up-regulated differentially (37–41). We hypothesized that these enzymes are not interchangeable. Biochemical analysis showed that caspase-4 and caspase-5 have very similar specificity in P1–P4 (6). However, analysis of the human protein substrates revealed a very different picture. Overall, gasdermin D was preferred to the prointerleukins by caspase-4 and caspase-5. However, whereas caspase-4 was more like caspase-1 in its cleavage of gasdermin D and pro-IL18, caspase-5 was more like caspase-11 (Fig. 3). However, caspase-4 does not cleave pro-IL1β, and caspase-5 cleaves it very poorly (Fig. 3).

These data revealed that caspases signaling noncanonical pyroptosis prefer gasdermin D as a substrate. Based on the cleavage site sequence of the protein substrates, previous peptidyl substrate library screens have shown that P1–P4 specificity does not determine specificity for protein substrates in these caspases (caspase-4/5/11) (6, 24).

Extended synthetic substrates confirm the importance of the prime side of the gasdermin D sequence in enhancing activity of caspase-11

Extended interactions at the active-site level beyond the P1–P4 sites are a possible mechanism for enhancing activity. An example of this is caspase-2, which has been demonstrated to cleave pentapeptides at rates 10–40 times higher than those of tetrapeptides (42). Given our previous observations that such interactions occur outside the P1–P4 area and the faster processing of gasdermin D by caspase-11 compared with that of the pro-ILs, we hypothesized that interactions occur at positions outside but proximal to the P1–P4 sites. To investigate such potential interactions, we designed and synthesized extended internally quenched fluorescent (IQF) substrates containing different portions of the mouse gasdermin D sequence around the cleavage site within positions P7–P5′ (Table 1). Caspase-1 was far more efficient at cleaving these substrates than caspase-11 (Fig. 4). Like most caspases, caspase-1 is most influenced by P1–P4. In contrast, caspase-11 is more highly influenced by the prime-side amino acids P1′–P4′ (Fig. 4). These observations confirm that the prime side contains specificity determinants in substrate recognition by inflammatory caspases.

Mutational analysis reveals that cleavage of protein substrates by caspase-11 is strongly influenced by the prime-side residues

Given the influence of the prime-side region of peptidyl substrates on the activity of caspase-11, we asked if these constitute specificity determinants for interaction with gasdermin D. Consequently, we predicted that introducing gasdermin D

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**Figure 1. The convention for naming substrate residues interacting with a protease active site**

The residue to the N-terminal side of the cleaved peptide bond is named P1 and the one to the C-terminal side is P1′; residues are numbered consecutively from this origin. Many substrate residues interact with specificity pockets in the protease and are named with an S and numbered by following the logic used for the substrate. The specificity pockets are defined by several enzyme residues.

One of the main conundrums in the field is the discrepancy of the number of inflammatory caspases in different mammals and the substrates they cleave. Extensive substrate specificity analysis using peptide combinatorial libraries revealed that using traditional active-site-directed strategies is insufficient to selectively target individual inflammatory caspases (6, 24, 28). In contrast, protein substrates can be readily distinguished by caspase-11, leading to the prediction that its specificity is mediated by exosite interactions (24). Indeed, an interaction between gasdermin D and a region of caspase-11 may contribute to catalysis (11) but cannot explain the specificity differences between inflammatory caspases. Therefore, we hypothesize that there are other mechanisms besides the exosite that determine substrate specificity. By using recombinant proteins and peptide substrates, we describe the role of alternative substrate recognition in driving the specificity of inflammatory caspases.

**Results**

*In vitro cleavage assays of protein substrates suggest that inflammatory caspases have specificity determinants outside the P1–P4 region*

Caspase specificity is generally dictated by the S1–S4 pockets that interact with the P1–P4 region of substrates (Fig. 1) (3, 4). We postulated that extended substrate recognition elements account for the restricted specificity of caspase-11 and its human orthologues.

The expression of full-length caspase-3 in *Escherichia coli* results in a fully functional enzyme (29). We found that the expression of the CARD-deleted version of caspase-1, caspase-4, and caspase-5 resulted in fully functional enzymes, as previously demonstrated for caspase-11 (24).

Recombinant inflammatory caspases and apoptotic caspase-3 were used for *in vitro* cleavage assays of gasdermin D, pro-IL18, and pro-IL1β. Cleavage products were visualized on SDS-PAGE (Fig. 2A). The inflammatory caspases cleaved the protein substrates at the expected positions previously reported (Fig. 2B) (18, 19, 30–34) to generate characteristic products that were quantitated by densitometry. Band quantitation allowed the calculation of the cleavage efficiency (kcat/Km) (Fig. 2C). Caspase-1 cleaved all three substrates at high efficiency. Gasdermin D was the preferred substrate of caspase-11. Pro-IL18 was also cleaved by caspase-11 but 50 times slower than gasdermin D. Only minimal cleavage of pro-IL1β was observed with caspase-11.
sequences from this region into pro-IL18 would make this a better substrate for caspase-11. The hypothesis was supported for caspase-11, where we observed a 40-fold increase in cleavage of the mutant containing P1⁻⁹–P4⁻⁹ substitution (Fig. 5). In contrast, we observed minimal enhancement in caspase-1 catalysis.

We sought to pinpoint which particular subsite has the most influence on caspase-11, and we individually substituted the amino acids in the P1⁻⁹–P4⁻⁹ region of pro-IL18 for those of gasdermin D. P1⁻⁹ and P4⁻⁹ mutations in pro-IL18 demonstrated the largest effect on the activity of caspase-11, both leading to a 5-fold efficiency increase (Fig. 5). In contrast, similar mutations moderately enhanced the cleavage of pro-IL1β by caspase-11 (Fig. 6). We conclude that pro-IL18 is a better protein substrate scaffold than pro-IL1β for caspase-11.

Caspase-3 was used as a control for cleavage of the mutant proteins. It cleaved all pro-IL18 mutant proteins at alternative sites, producing the same product sizes as those for WT pro-IL18. Caspase-3 had the same activity in each of the introduced mutations, suggesting that these mutations did not result in generally more accessible or better sites for any caspase.

**Human caspase-4 and -5 are similarly influenced by prime-side residues from gasdermin D**

To investigate if caspase-4 and caspase-5 specificity is determined by the prime side, we used the mouse protein substrates and their mutants. Gasdermin D was a good substrate for both caspase-4 and caspase-5. Pro-IL1β was not cleaved by caspase-4 and was a poor substrate for caspase-5, being cleaved at an alternative upstream site. Cleavage of pro-IL1β by caspase-5 was enhanced by introduction of the gasdermin prime region, which also resulted in a shift of the cleavage site to generate the active cytokine. Pro-IL18 was not cleaved by caspase-4 but was cleaved at a moderate rate by caspase-5. Introducing the gasdermin D prime-side region in pro-IL18 enhanced catalysis by both caspases to levels comparable with those of gasdermin D (Fig. 7). The prime-side residues of gasdermin D differ between human and mouse (Fig. 7C). Nevertheless, both substrates are cleaved by caspase-4 and caspase-5. These data revealed that caspase-4 and caspase-5 share with caspase-11 a requirement for prime-side residues to enhance specificity and catalysis.

**Discussion**

Inflammatory caspases are central regulators of pyroptosis, because they activate the pyroptosis effector protein gasdermin D by limited proteolysis (18, 19). A function of caspase-1, which is not shared with caspase-11, is the processing of IL18 and IL1β precursors to produce the active forms of these inflammatory cytokines (15, 24). This differential substrate recognition is centered on caspase-11 and likely involves sites on the protease that are distinct from the conventional active-site cleft (S1–S4).

Caspase-11 has two orthologues in human, caspase-4 and caspase-5, but their individual functions are not yet defined.
Caspase-4 and caspase-5 may be influenced by different activators (43). There are also differences in their expression profiles that indicate cell type-dependent diversification (40, 44). Consistent with their function in signaling the pyroptotic noncanonical pathway, we observed that, similarly to caspase-11, caspase-4 and caspase-5 favor gasdermin D processing over the pro-ILs. A point of divergence of caspase-4 and caspase-5 is the distinct preference of the former for pro-IL18. The preference of caspase-4 over caspase-5 for pro-IL18 is consistent with the observation that epithelial cell lines respond to Gram-negative bacteria by activating pro-IL18 in a caspase-4-dependent manner (39). These observations suggest that caution should be exercised in assuming equivalence between caspase-4 and caspase-11.

The preference of caspase-11 for gasdermin D is, in part, explained by the recently described exosite interaction between inflammatory caspases and gasdermin D (11). Caspase-11 is intrinsically less efficient than caspase-1 (lower catalytic rates on substrates) (24). The exosite implicates interaction between inflammatory caspases and the C-terminal gasdermin D domain that stabilizes the caspase active dimeric conformation, thereby increasing the activity toward gasdermin D (11). Cleavage of fluorogenic peptidyl substrates based on the gasdermin D cleavage site motif sequence led us to hypothesize that the C-terminal region adjacent to the cleavage site (P1′−P4′) contains amino acids that enhance caspase-11 activity beyond the established P1–P4 specificity-determining region.

A limitation of the peptidyl fluorogenic substrates containing the gasdermin D cleavage site motif sequence is that it does not take into consideration the overall protein substrate scaffold. Therefore, we used protein substrates to test our hypothesis. If the gasdermin D P1′−P4′ region contains specificity determinants for caspase-11, then substitution of these sites in pro-ILs would enhance caspase-11 catalysis. Indeed, introducing the gasdermin D P1′−P4′ region in pro-IL18 enhanced catalysis by caspase-11 to levels comparable with those of gasdermin D. Individual mutations in the P1′−P4′ region in pro-IL18 had moderate effects, signifying that amino acids in this region have additive effects on caspase-11 catalysis. The most influential positions were P1′-Gly and P4′-Glu. Glycine is generally preferred in P1′ by caspases, and this was previously corroborated in inflammatory caspases (42, 45, 46). P1′-Gly is strongly conserved in gasdermin D (Fig. 8); hence, P1′ steric hindrance is a limiting factor for caspase-11 activity. Pro-IL18 contains Lys or Arg in the P4′ position, whereas gasdermin D most often contains a negatively charged or a neutral amino acid residue (Fig. 8). The positive charge in P4′ possibly disfavors interaction with pro-IL18.

In contrast to pro-IL18, we had only limited success on improving IL1β as a substrate for caspase-4, caspase-5, and caspase-11 with P4′ substitutions. This implies that IL1β is a better substrate protein scaffold than pro-IL1β for these caspases and underscores the importance of caspase-1 as the most efficient IL1β-converting enzyme. Our data indicated that the
# Inflammatory caspase specificity

## Table 1
Structures and mass spectrometry analysis of IQF substrates

| IQF substrate | [m/z + H]^+ expected (Da) | [m/z + H]^+ observed (Da) |
|---------------|----------------------------|----------------------------|
| ACC-SLSSDGK(dnp)-NH₂ | 1085.4535 | 1085.4523 |
| ACC-SLSSDGK(dnp)-NH₂ | 1198.5375 | 1198.5377 |
| ACC-SLSSDGK(dnp)-NH₂ | 1313.5645 | 1313.5635 |
| ACC-SLSSDGK(dnp)-NH₂ | 1442.6071 | 1442.6058 |
| ACC-SLSSDGK(dnp)-NH₂ | 1571.6497 | 1571.6482 |
| ACC-SLSSDGK(dnp)-NH₂ | 1258.4495 | 1258.4507 |
| ACC-SLSSDGK(dnp)-NH₂ | 1242.4910 | 1242.4929 |
| ACC-SLSSDGK(dnp)-NH₂ | 1226.5325 | 1226.5315 |
| ACC-SLSSDGK(dnp)-NH₂ | 1198.5375 | 1198.5405 |
| ACC-SLSSDGK(dnp)-NH₂ | 1198.5375 | 1198.5394 |
| ACC-SLSSDGK(dnp)-NH₂ | 1326.5961 | 1326.5972 |
dominant specificity sites for caspase-1 are S1–S4, consistent with pro-IL1β recognition (5, 47). In contrast, dominant sites for caspase-11 are S19–S49, consistent with gasdermin D recognition. This is supported by P19–P49 deletion mutations of gasdermin D. Although initially interpreted to shorten the distance between the exosite and the scissile bond (11), we interpret this to reflect sequence alterations of the prime side that interfere with caspase-11 catalysis.

Cleavage of gasdermin D, pro-IL18, and pro-IL1β by inflammatory caspases induces gain of function for these proteins (18–20, 30). Upon cleavage, the gasdermin D N-terminal domain induces pyroptosis, and mature ILs can exert their proinflammatory properties. In contrast, caspase-3, an apoptosis executioner, cleaves these proteins at alternative sites, inducing loss of function (31, 36). Caspase-3 activity on the pyroptotic substrates is thought to be part of the interplay mechanism between apoptosis and pyroptosis to ensure that apoptotic cells remain inflammatory silent (36, 48). Caspase-3 cleavage occurred at the expected sites. Mutations did not alter caspase-3 cleavage. This signifies that P19–P49 recognition is specific to inflammatory caspases.

We have shown that prime-side interactions (the C-terminal region adjacent to the cleavage site) are determinant for substrate recognition by inflammatory caspases. We propose that in addition to the previously described exosite interaction (11), the P19–P49 region also influences caspase-11 specificity for gasdermin D (this also applies to its human orthologues, caspase-4 and caspase-5, with the caveat that in our study the protein scaffolds were mouse, not human). Ours is a mechanistic study that seeks to identify the specificity determinants of substrate recognition. Therefore, it should not matter whether the sequences of our test substrates match the natural species-specific sequences (Fig. 7C). Naturally, these conclusions are independent of any hypothetical regulatory proteins that may exist in cells undergoing pyroptosis.

To date, there are inhibitors preferentially targeting inflammatory caspases over apoptotic caspases (49, 50). In contrast, targeting individual inflammatory caspases has been less successful. Inflammatory caspases differ in their set of protein substrates, and learning their specificity determinants will be conducive for inhibitor design. In fact, prime-side exploring inhibitors have been investigated to target the activity of caspases (51). Future studies will expand the use of inhibitor libraries to explore the prime side in inflammatory caspases.

**Experimental procedures**

**Plasmids and cloning**

Constructs encoding pro-IL18, pro-IL1β, and caspase recruitment and activation domain (CARD)-deleted modified versions of caspase-1 and caspase-11 in pET29b(+) containing...
a C-terminal 6×His tag were previously described (24). Deletion of the N-terminal CARD of inflammatory caspases results in a form that encompasses only the catalytic domain, enabling direct comparisons between caspase activity and specificity. DNA encoding the CARD-deleted versions of caspase-4 and caspase-5 containing a C-terminal 6×His tag were purchased from Integrated DNA Technologies (IDT; San Diego, CA) and cloned into pET29b(+) by using NdeI and XhoI. The expression construct for caspase-3 containing a C-terminal 6×His tag was in pET23b, as previously described (52). Cloning of pro-IL18 and pro-IL1β mutants was performed with DNA gene block, purchased from IDT, or by mutagenesis using PCR-driven overlap extension and cloned into pET29b(+) containing a C-terminal 6×His tag. Gasdermin D sequence was amplified from a pET29b(+) construct using primers to add an N-terminal 8×His tag and cloned into pET15b using NdeI and XhoI restriction enzymes.

**Protein expression and purification**

All constructs were transformed into BL21(DE3) competent E. coli and cultured in 2× YT medium containing appropriate antibiotics, expressed, and purified as described.
Protein concentration was calculated by absorbance at 280 nm, and the concentration of active enzyme was calculated by active-site titration against benzoxycarbonyl-VAD-fluoromethyl ketone in 20 mM PIPES, 10% sucrose, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT supplemented with 0.75 M sodium citrate to enhance caspase activity, using Ac-WEHD-7-amino-4-methylcoumarin as the substrate.

Enzymatic assays with fluorogenic substrates

Enzymatic assays of recombinant caspases in a 100-μl final volume were done in 96-well opaque plates (Costar, Corning). Assay buffer was 20 mM PIPES, 10% sucrose, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT supplemented with 0.75 M sodium citrate to enhance caspase activity, using Ac-WEHD-7-amino-4-methylcoumarin as the substrate.

Synthesis of peptidyl fluorogenic substrates

IQF substrates with the ACC-Lys(dnp) donor-acceptor pair were synthesized as previously described. In brief, IQF substrates were synthesized on Rink Amide AM polystyrene resin using Fmoc solid-phase peptide synthesis. Fmoc-protected amino acids were subsequently coupled to the resin using HATU/2,4,6-collidine reagents dissolved in N,N,N,N,N,N,N,N,N-dimethylformamide (DMF). Fmoc-protecting groups were removed after each coupling cycle using 20% piperidine in DMF. Finally, Fmoc-ACC-OH was coupled twice to the N terminus of peptides using hydroxybenzotriazole/N,N,N,N-diisopropyl-O-(7-methoxycoumarin-4-yl)-methylisourea reagents dissolved in DMF (24 h), followed by the removal of the Fmoc-protecting group. The resin then was dried, and the substrates were cleaved from the resin using TFA. All substrates were purified using reverse-phase HPLC, dissolved in DMSO to a concentration of 10 mM, and stored at −20 °C until use.

Enzyme kinetics of peptidyl substrates

We determined \( k_{cat}/K_m \) parameters for fluorogenic substrates toward caspases. For this, serial dilutions of substrates were prepared in eight wells of 96-well plates, and activity assays were initiated by the addition of enzyme. Reactions were monitored for 30 min, and the reaction velocity was plotted against substrate concentration. Kinetic parameters \( V_{max} \) and \( K_m \) were calculated with Prism 7 (GraphPad) using the...
allosteric sigmoidal equation, and \( k_{\text{cat}} \) was calculated by using Equation 1, where \([E]\) is enzyme concentration.

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \tag{1}
\]

In vitro cleavage of recombinant protein substrates

Recombinant inflammatory caspases were subjected to 2-fold dilution series and incubated for 30 min at 37 °C with 4 \( \mu \text{M} \) gasdermin D, pro-IL18, pro-IL1β, or mutant proteins. Reaction controls consisted of incubation of substrate or enzyme-only reactions. Reaction mixtures consisted of a 60-\( \mu \text{l} \) final volume and were performed using assay buffer without sodium citrate supplementation. After incubation, reactions were terminated by the addition of 30 \( \mu \text{l} \) of 3 \( \times \) SDS loading buffer and incubated at 95 °C for 5 min, and reaction tubes were centrifuged for 1 min at 16,000 \( \times \) g. Reaction products were separated on 4–12% Bis-Tris SDS-PAGE and stained with Instant Blue (Expedeon). The gels were scanned by using an ODYSSEY CLx imaging equipment (LI-COR), and images were exported to Image Studio software for band intensity quantification corresponding to protein substrate remaining after assay. Band intensity values were normalized relative to those of noncleaved substrate, and values were plotted against \( \log[E] \), estimated as active-site titration. \( E_{1/2} \) values (the concentration of caspase at which 50% of substrate is converted) were calculated with Prism 7 (GraphPad) using the log(inhibitor) versus response (three parameters) equation. \( E_{1/2} \) values were used to calculate the catalytic efficiency of caspases for protein substrates according to Equation 2 (53), where \( k_{\text{cat}}/K_M \) is the second-order rate constant for substrate hydrolysis and \( t \) is the reaction time in seconds.

\[
k_{\text{cat}}/K_M = \frac{\ln 2}{(E_{1/2} \times t)} \tag{2}
\]

Data availability

All data are contained within the article.

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