Extensive peptide and natural protein substrate screens reveal that mouse caspase-11 has much narrower substrate specificity than caspase-1

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Inflammatory cell death, or pyroptosis, is triggered by pathogenic infections or events. It is executed by caspase-1 (in the canonical pyroptosis pathway) or caspase-11 (noncanonical pathway), each via production of a cell-lytic domain from the pyroptosis effector protein gasdermin D through specific and limited proteolysis. Pyroptosis is accompanied by the release of inflammatory mediators, including the proteolytically processed forms of interleukin-1β (IL-1β) and IL-18. Given the similar inflammatory outcomes of the canonical and noncanonical pyroptosis pathways, we hypothesized that caspase-1 and -11 should have very similar activities and substrate specificities. To test this hypothesis, we purified recombinant murine caspases and analyzed their primary specificities by massive hybrid combinatorial substrate library (HyCoSuL) screens. We correlated the substrate preferences of each caspase with their activities on the recombinant natural substrates IL-1β, IL-18, and gasdermin D. Although we identified highly selective and robust peptidyl substrates for caspase-1, we were unable to do so for caspase-11, because caspase-1 cleaved even the best caspase-11 substrates equally well. Caspase-1 rapidly processed pro-IL-1β and -18, but caspase-11 processed these two pro-ILs extremely poorly. However, both caspase-1 and -11 efficiently produced the cell-lytic domain from the gasdermin D precursor. We hypothesize that caspase-11 may have evolved a specific exosite to selectively engage pyroptosis without directly activating pro-IL-1β or -18. In summary, comparing the activities of caspase-1 and -11 in HyCoSuL screens and with three endogenous protein substrates, we conclude that caspase-11 has highly restricted substrate specificity, preferring gasdermin D over all other substrates examined.

The two cellular pathways that transduce recognition of pathogens by innate immune effector cells to induce cell death and accompanying cytokine release are the canonical pathway (which integrates extracellular pathogen recognition) and the non-canonical pathway (which integrates intracellular cytoplasmic pathogen recognition) (1–4). The canonical pathway operates by recognizing pathogen-associated molecular patterns through membrane-associated Toll-like receptors to signal transduction with up-regulation of components of inflammasomes. These macromolecular platforms assemble in the cytoplasm and are essential for recruitment and activation of inflammatory caspases (5, 6), and sensitization of cells to a second signal that activates caspase-1 in an inflammasome-dependent manner. The non-canonical pathway is driven by intracellular cytosolic sensing of pathogens by caspase-11, the mouse ortholog of human caspase-4 and -5, and does not require inflammasome components (1, 7, 8). Although the activation mechanism of caspase-1 and -11 is not defined, it most likely mirrors the proximity-induced dimerization model of initiator caspases in apoptosis (9, 10).

Caspase-1 and caspase-11 cleave gasdermin D (GSDMD),3 forming pores in liposomes by aggregation of N-terminal GSDMD fragments. Although not yet recorded in vivo, these pores are thought to also form in the cell membrane (11–14). The subsequent release of pro-inflammatory mediators via cellular membrane pores defines pyroptosis, a form of regulated inflammatory cell death (15). The requirement of two inflammatory caspases for the different inflammatory pathways is not well-understood.

Caspase-1, formerly known as ICE (interleukin-converting enzyme), was originally identified as the protease responsible for cleaving and activating the 33-kDa pro-IL-1β to its mature 17-kDa form in human monocytes (16–18), a role mirrored in mice (18, 19). Caspase-1 also processes pro-IL-18 into its mature form (20–22). Although these inflammatory messengers lack a signal export sequence, after maturation they are nevertheless released from the cell (23). Akin to caspase-11,

3 The abbreviations used are: GSDMD, gasdermin D; HyCoSuL, hybrid combinatorial substrate library; CARD, caspase activation and recruitment domain; Z, carbobenzoxy; fmk, fluoromethylketone; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ACN, acetonitrile; Fmoc, N-(9-fluorenyl)methoxycarbonyl; Bzl, benzyl.
caspase-1 produces the same cell lytic N-terminal domain of GSDMD (11–14, 24, 25). Together these caspase-1-mediated limited proteolytic events provide a rational explanation of how leaderless cytokines are activated and released from bacterially-infected macrophages, no export mechanism is required because the release is from N-GSDMD-permeabilized cells.

Thus there exist three well-recognized substrates for caspase-1, and at least one substrate in common with caspase-11 (GSDMD). Genetic evidence based on induction of non-canonical pyroptosis indicates that caspase-1 and the canonical inflammasome components Nlrp3 and ASC are required for processing of pro-IL-1β and pro-IL-18 with the implication that caspase-11 does not, or cannot, process these cytokines (1). Given the 53% sequence identity in their catalytic domains of caspase-1 and -11 and the regulatory role caspase-11 has been implicated to play, a question that has remained unanswered is whether efficient catalysis of caspase-11 on pro-IL-1β and pro-IL-18, the only two members of the interleukin family that are processed by caspase-1, differs from caspase-1 hydrolysis. The biochemical characterization of caspase-1 and -11 presented here addresses this dichotomy.

Screening of proteolytic enzymes with short peptide substrate libraries is a common approach to define their inherent substrate preferences in the active site, and thus distinguish closely related proteases. Several chemical- and biological-based approaches have been developed to dissect protease preferences (26). The most commonly used technology utilized Positional Scanning Synthetic Combinatorial Libraries (PS-SCL), introduced by Thornberry and co-workers (27, 28) to screen human caspases. This seminal research provided a robust screening platform and a framework defining the major differences in specificity between inflammatory and apoptotic caspases. These tetrapeptides have served for many years as gold standards for developing substrates, inhibitors, and activity-based probes to study caspases (29) and many other proteases (30). A substantial extension of PS-SCL termed hybrid combinatorial substrate library (HyCoSuL) (31) employs a wide set of unnatural amino acids in peptide mixtures, allowing for detailed exploration of protease-active sites, as we have employed HyCoSuL to discriminate between human apoptotic caspases (32). Accordingly, we employed HyCoSuL here to explore specificity differences between mouse caspase-1 and -11, and deployed the complementary technique of natural protein profiling and kinetics to address the individual roles of caspase-1 and -11 in pyroptosis and inflammatory cytokine activation.

**Results**

**Expression and purification of mouse inflammatory caspases**

To explore and differentiate the substrate specificity of mouse caspase-1 and caspase-11, we expressed full-length C-terminally His-tagged versions of each enzyme as well as caspase activation and recruitment domain (CARD)-deleted (∆CARD) versions (Fig. 1). When expressed in *Escherichia coli*, the purified soluble material did not render a full-length enzyme (Fig. 1B). The purified material from ∆CARD constructs of caspase-1 and -11 revealed two species: an unprocessed catalytic domain comprised of large and small subunits, and processed large and small subunits (Fig. 1B). The identity of these ∆CARD caspase-11 derivatives was confirmed by MS/MALDI (Fig. 1B). MALDI analysis of caspase-11 recovered from full-length construct expression revealed removal of the CARD consistent with cleavage at Phe77/Ser78, which is likely due to cleavage by an endogenous *E. coli* protease.

CARD-containing proteins belong to the death domain superfamily, which consists of 35 family members, all varying in solubility degree when expressed in *E. coli* (33). The disappearance of CARDs from the full-length expression constructs raised three possibilities: 1) the overexpression resulted in autoprocessing and removal of the CARDs, 2) CARD is insoluble and was trapped in inclusion bodies; and/or 3) CARD is cleaved by an *E. coli* protease. To investigate these possibilities we dissolved cell pellets in 8 M urea and purified all His-tagged proteins under denaturing conditions. Immunoblotting revealed full-length versions of both enzymes (Fig. 1C), suggesting that the CARD rendered the full-length caspases insoluble upon expression in *E. coli* and we are thus only able to purify CARD-removed proteins.

Although CARD-containing proteins, such as human ASC and ICEBERG, have been refolded from denatured preparations (33), our efforts to capture activity following denaturation and renaturation of caspase-1 or -11 were futile after following previously reported protocols (34). Accordingly, because of higher yields, we decided to continue our work with the ∆CARD-purified derivatives of these enzymes.

**Caspase-1 and caspase-11 substrate specificity**

The enzymes were scanned using a HyCoSuL, which employs 19 natural amino acids and 110 unnatural amino acids (32). This tactic allows us to explore in depth the chemical space represented by caspase-active sites. The general formula of the HyCoSuL library is Ac-P_4–P_3–P_2–Asp-ACC where the primary specificity determinant (P_1) is fixed as Asp, and the upstream P_2–P_4 positions are randomized. Caspase-1 and caspase-11 are not synonymous on this library and each displays characteristic differences, as visualized by comparison of preferences for natural amino acids (Fig. 2A). The analysis reveals caspase-1 and -11 both have a strong preference for histidine in the P_2 position, and broad tolerance in the P_3 position. Caspase-11 accepts a wide variety of residues at P_3, but this position is more restrictive for caspase-1. At the P_4 position caspase-1 prefers tryptophan, whereas caspase-11 favors valine. The region of endogenous substrates spanning the cleavage sites for caspase-1 and -11 (Fig. 2B) reveals that P_4 is a somewhat match the optimal substrate sequence motifs, but that P_3 does not, with the optimal histidine not utilized in endogenous protein substrates.

Expanding the screen with unnatural amino acids reveals that in the P_2 position caspase-11 can also recognize His(Bzl), whereas caspase-1 has much broader specificity recognizing multiple amino acids, including His(Bzl). Both enzymes present broad specificity in P_3 with caspase-11 even tolerating D-amino acids to a small extent, however, several amino acids (Bip, Tyr(Bzl), Bpa) are significantly better recognized by caspase-11 than by caspase-1. The P_4 position shows broad specificity for aliphatic and aromatic unnatural amino acids for both caspases with
Trp(Me) being the best for caspase-1 and Tle (tert-leucine) being the most active toward caspase-11. Detailed amino acid screening profiles for caspase-1 and -11 are found in Figs. S1–S3. These characteristics suggested that we may be able to generate individual caspase-selective substrates, and to this end we synthesized the top hits for P2, P3, and P4 using natural amino acids and determined \( k_{cat}/K_m \) values (Table 1 and Table S1). WEHD and LEHD-based substrates had already been used to measure inflammatory caspase activity (35), and thus served as prior indicators. Indeed, WEHD is the best substrate for caspase-1 with a \( k_{cat}/K_m \) of 52,200 M\(^{-1}\) s\(^{-1}\). We found the WQPD substrate sequence to be highly selective for caspase-1 over caspase-11 (selectivity factor \( 5,140 \)), and over a panel of other caspases (Table S3). The best substrates for caspase-11 included PYHD and PMHD tetrapeptides, however, caspase-1 cleaved these substrates even more efficiently with 2–3-fold higher \( k_{cat}/K_m \) values. Based on these results, caspase-1 is a more active enzyme and outpaces caspase-11 on all substrates containing natural amino acids.

Taking advantage of the unique side chains that unnatural amino acids present to explore the active sites of caspase-1 and -11, we synthesized distinctive substrates based on the preferred unnatural amino acid library screening results (Table 2, Table S2). To account for potential subsite cooperativity issues (36, 37) we covered a large amount of sequence combinations, synthesizing 57 individual substrates, 20 with natural amino acids and 37 with natural and unnatural amino acids (Tables S1 and S2). We found several substrates that display significant selectivity for caspase-1 over caspase-11. All these substrates share some structural similarities: cyclic, aliphatic (Cha), or aromatic (Phe and Trp derivatives) amino acids at the P4 position, Glu or Met(O2) at P3, and Tic or His(Bzl) at P2. Despite the large diversity of unnatural amino acids used in HyCoSuL none of these substrates was more selective than the substrate containing the natural amino acids WQPD, and the Cha-Glu-Tic-Asp unnatural substrate was only slightly more active than the reference natural amino acid WEHD sequence.

In contrast to caspase-1, more success in obtaining better substrates for caspase-11 was achieved. We found that the use of branched, aliphatic amino acids (Tle) at P4, and large, aromatic amino acids (Bip, Bpa) at P3 generated substantially improved substrates, as the Ac-Tle-Bpa-His(Bzl)-Asp-ACC was hydrolyzed 15.6- and 27-fold faster than the best substrate containing natural amino acids (Ac-PMHD-ACC) and reference (Ac-WEHD-ACC) substrates, respectively (Fig. 3). The extended nature of the cyclic side chains that comprise these unnatural amino acids may indicate that they could reach surface hydrophobic pockets that natural amino acids cannot.

![Figure 1. Expression and purification of mouse caspase-1 and -11. A, two constructs, full-length (FL) and CARD-deleted (Δ), were designed for each enzyme, see "Materials and methods" for details of the constructs. B, purified ΔCARD caspase-1 and -11 resulted in a mixture of soluble, unprocessed catalytic subunit with processed large and small subunits. MALDI-MS analysis revealed molecular mass derivatives of ΔCARD caspase-11 that coincides with the expected engineered protein molecular weights. The CARΔ of FL caspase-11 was removed during expression to generate derivatives whose mass is consistent with processing at Glu97/Ser98. C, cell pellets expressing full-length enzymes were resuspended in 8 M urea, 50 mM Tris-Cl, 100 mM NaCl buffer, pH 8.0. Purified unfolded full-length enzymes were detected on 4–12% BisTris SDS-PAGE with Instant Blue and anti-His or caspase-11 antisera.](image)
explore, much as seen in the case of binding of peptides based on unnatural amino acids to the serine protease neutrophil elastase (38). Unfortunately, even the best caspase-11 substrates were also efficiently hydrolyzed by caspase-1 so we could not obtain a substrate reasonably selective for caspase-11. Nevertheless, we demonstrated that the application of unnatural amino acids is a good strategy to enhance catalysis for caspase-11.

In vitro cleavage of pro-IL-1β, pro-IL-18, and GSDMD by mouse inflammatory caspases

To compare the catalytic efficiency of mouse inflammatory caspases for endogenous substrates, we incubated the active site-titrated enzymes with 4 μM recombinant mouse pro-IL-1β, pro-IL-18, and GSDMD (Fig. 4). To determine the enzyme concentration needed to cleave half of the substrate, and thus derive $k_{cat}/K_m$ values according to Equation 2, the incubated samples were run on SDS-PAGE gels, and the stained gels were quantified by densitometry scanning. Because the apoptotic executioner caspase-3 has also been implicated in cleavage of the proteins we included this enzyme in our analyses. The respective $k_{cat}/K_m$ are presented in Fig. 4, from which it is apparent that caspase-1 efficiently processes pro-IL-1β, pro-IL-18, and GSDMD, while caspase-1-11 cleaves GSDMD with somewhat comparable efficiency, but is unable to cleave the pro-interleukins, and the

Table 1
Unique preferred substrates with natural amino acids

Based on screening of a natural amino acid HyCoSuL library, single substrates were synthesized for caspase-1 and caspase-11. Each substrate was assayed with either caspase-1 or caspase-11. All synthesized natural amino acid substrates were preferred by caspase-1, even those that had the lowest catalytic numbers for caspase-11. The selectivity factor represents the catalytic parameter $k_{cat}/K_m$ for caspase-1 divided by that for caspase-11. Each experiment was repeated at least three times, and the $k_{cat}/K_m$ values are presented as an average. Standard deviations were below 15%.

| Substrate sequence: | $k_{cat}/K_m$ | Selectivity factor |
|---------------------|---------------|--------------------|
| Ac-P$_4$P$_3$P$_2$P$_1$-ACC | Caspase-1 | Caspase-11 |
| Reference substrates | | |
| WEHD | 522,000 | 4,100 | 127 |
| LEHD | 179,000 | 3,850 | 47 |
| Caspase-1 preferred | | |
| WQPD | 257,000 | <50 | >5,140 |
| FEAD | 171,000 | <50 | >3,420 |
| WQVD | 328,000 | 244 | 1,340 |
| Caspase-11 preferred | | |
| PYHD | 22,400 | 7,070 | 3.2 |
| PMHD | 16,100 | 7,200 | 2.2 |

Table 2
Unique preferred substrates with unnatural amino acids

Based on screening of an unnatural amino acid HyCoSuL library, optimal single substrates were synthesized for caspase-1 and caspase-11. Each substrate was assayed with either caspase-1 or caspase-11. Most synthesized unnatural amino acid substrates were preferred by caspase-1, with only the best caspase-11 substrates demonstrating about equal catalytic rates with caspase-1. The selectivity factor represents the catalytic parameter $k_{cat}/K_m$ for caspase-1 divided by that for caspase-11. Each experiment was repeated at least three times, and the $k_{cat}/K_m$ values are presented as an average. Standard deviations were below 15%.

| Substrate sequence: | $k_{cat}/K_m$ | Selectivity factor |
|---------------------|---------------|--------------------|
| Ac-P$_4$P$_3$P$_2$P$_1$-ACC | Caspase-1 | Caspase-11 |
| Caspase-1 preferred | | |
| Cha-Glu-Tic-Asp | 544,000 | 609 | 893 |
| Phe(2Cl)-Glu-Tic-Asp | 525,000 | 1,010 | 519 |
| Trp(Me)-Glu-His(Bzl)-Asp | 524,000 | 1,700 | 106 |
| Trp-Met(O2)-His-Asp | 512,000 | 7,100 | 72 |
| Caspase-11 preferred | | |
| Tle-Bpa-His(Bzl)-Asp | 89,000 | 112,000 | 0.80 |
| Tle-Bip-His-Asp | 80,200 | 109,000 | 0.74 |
| Pro-Bip-His(Bzl)-Asp | 25,900 | 69,000 | 0.38 |
Inflammatory caspases

Figure 3. Structures of the best natural and unnatural fluorogenic substrates for caspase-11. A, one of the most active natural substrate contains the Pro-Tyr-His-Asp sequence. B, two of the most active caspase-11 substrates containing unnatural amino acids. Extended cyclic and hydrophobic side chains enhance catalysis compared with substrates with natural amino acids.

$k_{\text{cat}}/K_m$ of 9.0 M$^{-1}$ s$^{-1}$ is a maximal estimate, and orders of magnitude slower than caspase-1 cleavage of the pro-interleukins. Caspase-3 efficiently cleaves pro-interleukins, but displays a low $k_{\text{cat}}/K_m$ on GSDMD (Fig. 4). Importantly, the caspase-3 cleavage sites are different from the inflammatory caspase cleavage sites, and the consequence of this is discussed later.

Discussion

Genetic evidence indicates that caspase-1 processes pro-IL-1β and pro-IL-18 but caspase-11 does not (1, 39). Both of these caspases are nevertheless able to induce pyroptosis by cleaving GSDMD (24, 25). Thus there must be biochemical characteristics that distinguish these two otherwise closely related caspases. Working with recombinantly expressed enzymes, we found these enzymes to be soluble and active after purification. The lack of CARD domain does not inhibit their activity as seen in the library profiles and in vitro cleavages of endogenous substrates. A question of whether CARDs influence specificity cannot yet be determined. However, substrate specificity mapping of the CARD-containing caspase-9 reveals no role in specificity or catalysis for its CARD, and from this perspective we predict that the specificity and activity of caspase-1 and -11 would not be influenced by their CARDS.

Since the discovery of human caspase-1 more than two decades ago, various studies have focused on understanding its kinetic parameters by designing multiple length substrates using different approaches (18, 28, 40–42). The optimal length of peptide substrates is four residues (41). Using this peptide length, initial substrate specificity studies conducted on caspase-11, and its closest human orthologs caspase-4 and -5, reported similar specificity to caspase-1 (28, 43), which we confirm in Tables S3 and S4.

To better understand the distinctions between caspase-1 and -11 we employed a screening strategy utilizing HyCoSuL, followed by synthesis of individual substrates containing unnatural amino acids. Analysis of these substrates allowed us to draw several conclusions. First, the use of unnatural amino acids had no impact on the activity or the selectivity of caspase-1 substrates, as the sequence WEHD was still among the best recognized substrates, and WQPD was the most selective substrate over caspase-11 with a selectivity factor over 5,140. Thus, the WQPD substrate can serve as an excellent distinguishing marker for caspase-1 detection in cells undergoing pyroptosis. On the other hand, unnatural amino acids were very useful in caspase-11 profiling. We found that two substrate containing unnatural amino acids (Tle-Bip-His-Asp and Tle-Bpa-His(Bzl)-Asp) displayed a 15-fold higher $k_{\text{cat}}/K_m$ toward caspase-11 than the best substrate containing natural amino acids (PMHD). The high increase in enzyme activity was due to Tle at P4 and Bip/Bpa at P3. We reasoned that these amino acids produce new binding interactions with the enzyme that could not be obtained using natural analogs. For instance, Ac-Pro-Bip-His(Bzl)-Asp-ACC displayed almost 3-fold higher $k_{\text{cat}}/K_m$ for caspase-11 over caspase-1, whereas all alleged caspase-11 selective substrates containing only natural amino acids were hydrolyzed substantially faster by caspase-1. However, the very best selective substrates for caspase-11, suboptimal for caspase-1, are still cleaved by caspase-1 with equal or greater efficiency. Even the very best peptide substrates for caspase-11 have $k_{\text{cat}}/K_m$ values of less than $10^4$ M$^{-1}$ s$^{-1}$, which is generally very poor for caspase peptidyl substrates (32).

At this point we reasoned that the active form of caspase-11 produced in E. coli might lack activity due to folding or conformational issues, and so we tested both caspases on natural substrates obtained from recombinant expression. We were struck by the very poor cleavage of pro-IL-1β and pro-IL-18 by caspase-11. The $k_{\text{cat}}/K_m$ values are so poor (>10,000-fold lower than caspase-1) that we estimate, through applying Equation 3 and an estimated enzyme concentration of 10 nM, that it would take over 3 months for caspase-11 to cleave 50% of pro-IL-1β and pro-IL-18. In contrast, caspase-1 would take about 5–10 min to accomplish cleavage under the same conditions. However, the cleavage rate of GSDMD by caspase-1 and -11 is more comparable with only a 13-fold difference in $k_{\text{cat}}/K_m$ values (half-times for cleavage of 10 min to 2 h).
Figure 4. Cleavage rates of substrate proteins by caspases. To test catalytic efficiency of caspases on A, pro-IL-1β, B, pro-IL-18, and C, GSDMD, the indicated enzymes were active site titrated using Z-VAD-fmk, incubated with 4 μM substrates for 30 min at 37 °C, and run in SDS-PAGE. Gel densitometry of the remaining uncleaved substrate yielded IC50 values from which catalytic rates $k_{cat}/K_m$ were determined according to Equation 1 ("Materials and methods"). The precursor proteins are shown by a solid black arrowheads, derivatives of each substrate are indicated by the gray or white arrowheads. Importantly, although the apoptotic caspase-3 cleaved the proteins, each site was distinct from those generated by inflammatory caspase-1 and -11 (white arrowheads). The gels above are representative of two biological replicates employing different caspase preparations. D, comparative $k_{cat}/K_m$ values are described by the colored symbols, as indicated in the key. Caspase-3 cleaved all three proteins, but at sites distinct from those generated by caspase-1 and -11, as discussed in the text. Data are averages from two biological replicates, and the error bars indicate standard deviations.
**Inflammatory caspases**

Taking these results into account we conclude that caspase-11 has a very restricted substrate specificity, preferring GSDMD over all other substrates examined. The P$_4$–P$_1$ residues in GSDMD are LLSD and those in pro-IL-18 are LSED, and so the only difference in this region that occupies the active site cleft is at P$_9$, where GSDMD has Leu, and pro-IL-18 has Glu (Fig. 2B). Examination of Fig. 2 reveals that Glu is preferred to Leu at P$_9$, so from this perspective pro-IL-18 should be a better substrate than GSDMD for caspase-11. Clearly this is not the case, so another explanation must be considered to explain the rapid and preferred cleavage of GSDMD by caspase-11.

Mechanistically, this is likely to represent a case where exquisite substrate specificity is created by an exosite on the scaffold of an otherwise poor enzyme. Originally identified in thrombin as a mechanism to enhance cleavage of its key substrate fibrinogen (44), exosites are emerging as a critical substrate recognition function in several distinct protease families including matrix metalloproteases, small ubiquitin-like modifier (SUMO), and ubiquitin-deconjugating enzymes, and cathepsins (45–48). Exosites are generally distant from the enzyme active site and tend to direct proteases toward specific substrates that are normally not well-cleaved. Caspase-11 may represent such a case, cleaving peptide substrates and pro-interleukins poorly, but cleaving GSDMD well, and has thus developed a highly selective substrate specificity via a yet to be identified exosite. Notably, both pro-IL-1β and pro-IL-18 possess negatively charged residues in the P$_9$–P$_{10}$ region upstream of the cleavage site (Fig. 2B). This may preclude efficient binding by caspase-11 and constitute part of an exosite.

Despite the finding that caspase-11 is an unlikely candidate for activation of pro-interleukins there is nevertheless evidence based on in vivo studies and ablation of inflammasome components in mice that non-canonical stimuli lead to pro-interleukin processing (1). One potential pathway is via direct activation of pro-caspase-1 by active caspase-11. We have not been able to produce pro-caspase-1 by recombinant expression, so have been unable to test this straightforwardly. However, mice and human cells ablated in Nlrp3 and ASC, key inflammasome components, are unable to process the pro-interleukins, leading to the conclusion that canonical activation of caspase-1 is required and activation should not be directly via caspase-11 (49–51) (Fig. 5). The identity of the pathway(s) leading to the indirect activation of caspase-1 by caspase-11 remains elusive.

Although we have focused on the pro-inflammatory caspase-1 and -11, it is noteworthy that the apoptotic caspase-3 also cleaves pro-IL-1β, pro-IL-18, and GSDMD, but at different sites. Cleavage of human GSDMD by caspase-3 inactivates lytic potential, preventing caspase-1 and -11 from generating a cell-permeabilizing product (52). Cleavage of human pro-IL-18 by caspase-3 generates biologically inactive products (53). Together these observations give credence to the hypothesis that apoptotic caspases blunt inflammation by inactivating pro-inflammatory cytokines (54) and GSDMD.

Future studies will seek to delineate the pathway that leads from active caspase-11 to active caspase-1. The pathway may be cell autonomous or it may involve sensing of cellular contents released from pyroptotic cells by adjacent cells, leading to canonical caspase-1 activation. Moreover, evidence presented here implies that an exosite exists on caspase-11 to secure rapid cleavage of GSDMD. Given that we have not been able to capture a substrate selective for caspase-11 over caspase-1, despite massive peptide-based screening, it is unlikely that a simple active site-directed strategy will succeed in developing compounds to interfere with the non-canonical pathway over the canonical one. Accordingly, directing compounds against the putative exosite may lead to highly selective compounds targeting caspase-11, but this will first depend on the characterization of the exosite.

**Materials and methods**

**Protein construct design, expression, and purification**

Constructs encoding full-length mouse caspase-1 and caspase-11 in pcDNA3(+) Zeocin were the kind gifts of Irma Stowe and Nobuhiko Kayagaki (Genentech, San Francisco, CA). Sequences encoding mouse full-length GSDMD, pro-IL-1β, and pro-IL-18 were purchased from Integrated DNA Technologies (San Diego, CA) and cloned into the pET29b(+) (Novagen) containing a C-terminal His tag. CARD deleted (ΔCARD) modified versions of mouse caspase-1 (minus amino acids 2–129) and caspase-11 (minus amino acids 2–99) were also cloned into the pET29b(+) containing a C-terminal His tag. ΔCARD-modified versions of human caspase-1 (minus amino acids 2–118) and caspase-4 (minus amino acids 2–93) were cloned into pET15b(+) containing an N-terminal His tag. ΔCARD Caspase-5 (minus amino acids 2–132) was cloned into the pET21b(+) containing a C-terminal His tag. All other caspases were expressed as previously described (55). All constructs were transformed into BL21 (DE3)-competent E. coli. Expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 25 °C shaking at 250 rpm. Cells were collected and pelleted at 4 °C for 10 min at 3,900 × g. The caspase cell pellets were resuspended in 50 mM Tris–Cl, 100 mM NaCl, pH 8.0, and lysed via sonication. The GSDMD, pro-IL-1β, and pro-IL-18 cell pellets were resuspended in 50 mM HEPES, 100 mM NaCl, pH 8.0.
Cell lysates were centrifuged at 4 °C for 30 min at 28,000 × g, supernatants were filtered through a 0.22-μm filter (Millipore) and the soluble fractions were applied to a 1-ml Ni-chelating-Sepharose resin (GE Healthcare Life Sciences) in a chromatography column. The enzyme-bound resin was washed in 50 mM Tris-Cl, 500 mM NaCl, pH 8.0, and then enzymes were eluted in 25 mM Tris-Cl, 100 mM NaCl, pH 8.0, with stepwise increments of imidazole from 12.5 to 100 mM. Human caspase-3 was prepared as previously described (55). Purified protein concentration was determined by absorbance at 280 nm. Active enzyme concentration was calculated by carboxbenzoxo-varyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VD-fmk, Cayman Chemical, 14467-5) titration, see below. Proteins were analyzed by 4–12% BisTris SD-PAGE gels (Novex Life Technologies) and stained with Instant Blue (Exppeo), or transferred onto a nitrocellulose membrane and detected with antibodies.

Urea protein denaturation and refolding

Full-length caspase-1 and -11 were expressed and purified as described above, except that cell pellets were resuspended in 8 M urea, 50 mM Tris-Cl, 100 mM NaCl, pH 8.0. The enzyme-bound resin was washed in 8 M urea, 50 mM Tris-Cl, 500 mM NaCl, pH 8.0, and then enzymes were eluted in 8 M urea, 25 mM Tris-Cl, 100 mM NaCl, pH 8.0, with stepwise increments of imidazole from 12.5 to 200 mM. Human caspase-3 was prepared as previously described (55). Purified protein concentration was determined by absorbance at 280 nm. Active enzyme concentration was calculated by carboxbenzoxo-varyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VD-fmk, Cayman Chemical, 14467-5) titration, see below. Proteins were analyzed by 4–12% BisTris SD-PAGE gels (Novex Life Technologies) and stained with Instant Blue (Exppeo), or transferred onto a nitrocellulose membrane and detected with antibodies.

Antibodies

Caspase-11 antibody was from Novusbio (Littleton, CO) (NB120–10454). His-tag polyclonal antibody was from Cell Signaling Technology (Danvers, MA) (number 2365).

MALDI time-of-flight (TOF)-MS

Purified recombinant ΔCARD caspase-11 was analyzed by MALDI TOF/TOF mass spectrometry on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA), in the Sanford Burnham Prebys Proteomics Facility, to accurately determine the molecular weights of the bands observed in SDS-PAGE. Enzyme was desalted and concentrated using C18 ZipTip (EMD Millipore). A ZipTip was prepared as described previously (55).

Z-VD-fmk active site titration

To determine the active concentration of inflammatory enzymes so that substrate $k_{cat}/K_m$ values could be calculated, a serial dilution series of Z-VD-fmk was incubated with purified enzyme for 30 min at 37 °C. The fluorogenic substrate Ac-LEHD-AFC was added to a final concentration of 100 μM in 100 μl of assay buffer and enzymatic activity was measured as above. Velocities were plotted against the inhibitor concentration allowing the calculation of the active enzyme concentration (55).

Screening of caspase-1 and -11 substrate specificities

A HyCoSuL substrate scanning library was utilized to define the substrate specificity cleavage preferences of caspase-1 and -11, essentially as reported before for apoptotic caspases (32). In brief, three fluorogenic substrate sublibraries ($P_4$, $P_3$, and $P_2$) were each screened at 100 μM with mouse recombinant caspase-1 and -11 in a 100-μl final volume. Both enzymes were used immediately after purification because caspase-11 tends to become unstable upon storage. The progress of substrate hydrolysis was monitored for 45 min, but for each substrate only the linear portion of the hydrolysis curve (15–30 min) was analyzed to calculate the reaction velocity (relative fluorescent units/s). Screening of each sublibrary was performed three times, and the averages were used to calculate caspase-1 and -11 specificity matrices. For each sublibrary the amino acid with the highest cleavage rate (relative fluorescent units/s) was set to 100%, and other amino acids were adjusted accordingly.

Synthesis and kinetic analysis of substrates containing natural and unnatural amino acids

Individual ACC-labeled substrates were obtained via solid-phase peptide synthesis in N,N-dimethylformamide and purified as described previously (32). Briefly, ACC-OH was attached to the Rink Amide resin, Fmoc-Asp(tBu)-OH was coupled, and elongated by coupling $P_2$, Fmoc-protected amino acids and acetylation of the N terminus. Side chain protecting groups were removed and the peptide substrate was cleaved from the resin using a TFA procedure. Crude products were precipitated in diethyl ether and purified by HPLC. Purity of each substrate was confirmed via analytical HPLC and the molecular weight was determined by mass spectrometry on a high resolution mass spectrometer WATERS LCT premier XE with electrospray ionization (ESI) and TOF module.

For each fluorogenic substrate we determined the $k_{cat}/K_m$ parameters toward caspase-1 and -11. To do this, serial dilu-
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tions of substrate were placed in the eight wells of 96-well plates followed by addition of caspase. The reaction was monitored for 30 min and the hydrolysis velocity from each well (V) was plotted against substrate concentration (S) and the V/S slope was calculated. The $k_{cat}/K_m$ parameter was then calculated according to the Equation 1.

$$k_{cat}/K_m = \text{slope}/E \quad (\text{Eq. 1})$$

Each experiment was performed at least three times and the average values with S.D. were calculated.

**In vitro cleavage of recombinant protein substrates**

Active site-titrated recombinant ΔCARD caspase-1 or -11 was subjected to 2-fold series dilutions and incubated separately for 30 min at 37 °C with 4 μM pro-IL-1β, pro-IL-18, or GSDMD caspase buffer in a final assay volume of 60 μL. Reactions were terminated by heating at 86 °C with 30 μL of 3× SDS loading buffer to give a final concentration of 1× loading buffer. Samples were analyzed on 4–12% BisTris SDS-PAGE gels by Instant Blue staining. The gels were then scanned and imported to Image Studio (LI-COR Biosciences) for protein band intensity quantification. Band intensity values were plotted against enzyme concentration and IC$_{50}$ values were determined via GraphPad Prism. Those values were then used to measure catalytic efficiencies of mouse caspase-1 and -11 for natural substrates according to the relationship described previously (56).

$$k_{cat}/K_m = \ln2/ \left( t_{1/2} \times E \right) \quad (\text{Eq. 2})$$

Rearranging this equation and applying a pseudo-first order approximation yields the half-life for cleavage of a substrate.

$$t_{1/2} = \ln2/(k_{cat}/K_m \times E) \quad (\text{Eq. 3})$$

Here, $k_{cat}/K_m$ is the second order rate constant for substrate hydrolysis, $E_0$ is the concentration of protease for which half the substrate is consumed, $E$ is the concentration of enzyme, and $t$ is the incubation time (57).

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