Resurrection of an ancient inflammatory locus reveals switch to caspase-1 specificity on a caspase-4 scaffold


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Pyroptosis is a mechanism of inflammatory cell death mediated by the activation of the prolytic protein gasdermin D by caspase-1, caspase-4, and caspase-5 in human, and caspase-1 and caspase-11 in mouse. In addition, caspase-1 amplifies inflammation by proteolytic activation of cytokine interleukin-1β (IL-1β). Modern mammals of the order Carnivora lack the caspase-1 catalytic domain but express an unusual version of caspase-4 that can activate both gasdermin D and IL-1β. Seeking to understand the evolutionary origin of this caspase, we utilized the large amount of data available in public databases to perform ancestral sequence reconstruction of an inflammatory caspase of a Carnivora ancestor. We expressed the catalytic domain of this putative ancestor in Escherichia coli, purified it, and compared its substrate specificity on synthetic and protein substrates to extant caspases. We demonstrated that it activates gasdermin D but has reduced ability to activate IL-1β. Our reconstruction suggests that caspase-1 was lost in a Carnivora ancestor, perhaps upon a selective pressure for which the generation of biologically active IL-1β by caspase-1 was detrimental. We speculate that later, a Carnivora encountered selective pressures that required the production of IL-1β and caspase-4 subsequently gained this activity. This hypothesis would explain why extant Carnivora possess an inflammatory caspase with caspase-1 catalytic function placed on a caspase-4 scaffold.

The inactive precursors of IL-1β and IL-18 (pro-IL-1β and pro-IL-18) become proteolytically activated by removal of their propeptide (18–21). Biochemical studies and genetic animal models suggest that conversion of pro-IL-1β is a function performed mainly by caspase-1 in human and mouse (7, 9, 22). Mouse caspase-11, and human caspase-4 and caspase-5, have inadequate capacity to cleave pro-IL-1β (9, 22). Cleavage of pro-IL-18 is less restricted as it can be cleaved by human caspase-1 and caspase-4 with similar efficiency (9). Caspase-1 is activated downstream of caspase-11 in an NLRP3 (NOD-, LRR-, and pyrin domain-containing 3) inflammasome–dependent manner, providing an alternative mechanism to produce IL-1β during noncanonical pyroptosis (7, 23). Hence, pyroptosis is an essential first line of defense against pathogens by eliminating their replicating niche and infl uences downstream immune responses by producing inflammatory IL-1β and IL-18 (24, 25). The proteolytic efficiency differences among caspases of the canonical and noncanonical pathways on pro-IL-1β highlight a pivotal difference in their contribution to innate immunity.

Inflammatory caspases consist of a caspase activation and recruitment domain (CARD) followed by a catalytic domain with proteolytic function (26). Typically, CASP1 and CASP4 are contiguous genes, except in primates where CASP5 lies between these two genes (27). The dog genome contains a

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single caspase-1 and caspase-4-like gene (Fig. 1A). This gene encodes a protein called “hybrid caspase-1/caspase-4” or “hybrid inflammatory caspase,” (28) which contains a caspase-1 CARD followed by a caspase-4 CARD and a catalytic domain (Fig. 1A). Early studies suggest that this gene originated via the deletion of a chromosomal segment encoding the caspase-1 catalytic domain and exon 1 of CASP4 (28).

In our previous report of inflammatory cell death in mammals of the order Carnivora, we observed that the catalytic domain of the dog inflammatory caspase behaved like caspase-1 catalytically, despite its sequence identity to caspase-4 (29). Moreover, the caspase-1 and caspase-4-like gene seems to be common in mammals of this order (29, 30). Given the importance of caspase-1 in inflammation through pyroptosis signaling and pro-IL-1β processing, we hypothesized that the loss of caspase-1 catalytic domain in Carnivora became compensated by reshaping the caspase-4 catalytic domain and function. We looked for caspases with similar domain architecture in mammals to test this hypothesis and approximate how the caspase-1 and caspase-4-like gene originated. Previous studies suggest that the inflammatory caspase locus is under extreme selective pressure, leading for instance toward a new inflammatory caspase in higher primates (27, 31). To understand the evolution of this locus in Carnivora, we set out to reconstruct the most probable protein sequence that represents the ancestor from whom the inflammatory caspase catalytic domain diverged in these mammals. Finally, we evaluated the substrate cleavage and specificity of this hypothetical ancestral inflammatory caspase.

Results

Sequence-based classification of the Carnivora inflammatory caspase

To investigate the occurrence of inflammatory caspases with the CARD–CARD–catalytic domain architecture in mammals, we performed a BLAST analysis search using the dog inflammatory caspase sequence as a query (UniProt: A9YEF4). We found a total of 24 proteins (Table S1), 23 of which were found in mammals of the order Carnivora (of the more than 270 reported species of Carnivora), and only one belonging to the order Artiodactyla, namely sheep. The high prevalence of the hybrid inflammatory caspase in Carnivora and the lack of a caspase-1 ortholog in this clade suggest that CASP1 deletion occurred early in the evolution of these mammals.

Cladograms revealed that the first CARD in the hybrid inflammatory caspase grouped with caspase-1, whereas the subsequent CARD grouped with caspase-4 CARDs (Fig. S1). In addition, the catalytic domain grouped with caspase-4 (Figs. 1B and S1). Consequently, we hypothesize that, regardless of the CARD-dependent activation mechanism, the catalytic activity and specificity of the catalytic domain in the Carnivora inflammatory caspases would be caspase-4 like.

Dog inflammatory caspase shows caspase-1-like specificity

Human caspase-4 and mouse caspase-11 cleave gasdermin D more efficiently than they cleave pro-IL-1β (9, 22). Accordingly, our hypothesis predicts that the catalytic domain of the Carnivora inflammatory caspase would cleave gasdermin D better than pro-IL-1β. We recombinantly expressed and purified the CARD-depleted version of the dog inflammatory caspase. We obtained an active and processed protein consisting of a large (p20) and a small (p10) subunit (Fig. S2).

Utilizing in vitro cleavage assays, we calculated the catalytic parameter $k_{cat}/K_m$ of the dog inflammatory caspase toward recombinant protein substrates gasdermin D, pro-IL-18, and pro-IL-1β. Strikingly, the dog inflammatory caspase cleaved gasdermin D, pro-IL-18, and pro-IL-1β with similar efficiency to mouse and human caspase-1 (9, 22) (Fig. 2). We conclude that the dog inflammatory caspase is endowed with the biochemical properties that allow it to perform caspase-1 functions.

Caspase specificity is dictated by interactions with amino acids in a bipartite sequence motif surrounding the cleaved peptide bond. Most caspases, including caspase-1, recognize the N-terminal portion of this motif. In contrast, caspase-4 recognizes the C-terminal side (9). Because the usage of substrate recognition subsites is an elemental distinction between caspase-1 and caspase-4, we tested the specificity of the dog inflammatory caspase on peptides corresponding to the recognition motif by using a set of internally quenched fluorogenic substrates. Like caspase-1 (9), the dog inflammatory caspase was more influenced by the N-terminal region than by the C-terminal region (Table 1 and Fig. S3).

We employed positional scanning tetrapeptide libraries to further explore this concept and reveal subtle differences between closely related enzymes (32). The dog inflammatory caspase was tolerant of a broad range of amino acids, and it preferred His in position $P_2$ and Val in position $P_3$. It also demonstrated the typical preference of inflammatory caspases for bulky and hydrophobic amino acids in the $P_4$ position favoring Trp and Tyr (Fig. 3B).

Exploring of the specificity determinants in more depth revealed that the dog inflammatory caspase showed the highest...
correlation with human and mouse caspase-1 within the recognition motifs (Fig. 3C). Consequently, our prediction that the catalytic domain of Carnivora inflammatory caspase would have a caspase-4-like specificity is incorrect, and it has a caspase-1-like specificity.

Resurrection of a Carnivora inflammatory caspase ancestor

Interested in the evolutionary process that generated the caspase-1 function on the caspase-4 catalytic domain scaffold, we utilized ancestral sequence reconstruction (ASR) to examine the characteristics of the protein from which the Carnivora inflammatory caspases descended. ASR calculates a phylogenetic tree based on statistical analysis of sequence conservation and substitutions of existing proteins within a family (33). These relationships allow for calculation of sequences that represent the diverging nodes within the phylogenetic tree and thus the ancestor of each branch. The resurrected protein represents the node from which the Carnivora inflammatory caspases diverged. We termed this caspase “node 22” based on the position on the phylogenetic tree (Figs. 4A and 5A).

The results of ASR analysis are site-specific probabilities for each position in the protein sequence (33, 34). Only 5% of the node 22 caspase sequence was identified as ambiguous, defined as sites with <70% probability (Fig. 5A). All ambiguous residues in the node 22 sequence are indicated in Fig. S5. The node 22 sequence shares more than 80% identity with the dog inflammatory caspase and human caspase-4 but only 60% identity with human caspase-1 (Fig. S5). Because node 22 is the predicted ancestor of the catalytic domain of the Carnivora inflammatory caspases, we expected that these proteins would have the same specificity. Accordingly, we hypothesized that the Carnivora inflammatory caspase evolved from a protein that should have been able to convert pro-IL-1β.

To test the specificity of node 22, we examined the cleavage efficiency on protein and peptide substrates. Node 22 appeared to have similar cleavage efficiencies of inflammatory substrates to the dog inflammatory caspase (Figs. 2 and 4, B and C). However, close observation highlights a significant distinction in the cleavage pattern of pro-IL-1β (Fig. 4B). While pro-IL-1β is preferably converted into the 17 kDa active form by caspase-1 (9, 22), the major observed proteolytic product of caspase node 22 was 27 kDa, an alternative product of unknown function and that can also be generated by caspase-1 (35).

To investigate whether caspase node 22 can produce a bioactive IL-1β, we used a cellular reporter assay. When we cleaved pro-IL-1β with equal amounts of caspases (Fig. 6A), we observed similar levels of IL-1β signaling generated by mouse caspase-1 and the dog inflammatory caspase (Fig. 6B). Conversely, we observed 2.5-fold lower IL-1β signaling by caspase node 22 compared with caspase-1 (Fig. 6B). Mouse caspase-11 served as a negative control. These results signify

<table>
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<tr>
<th>Substrate sequence</th>
<th>kcat (s⁻¹)</th>
<th>Km (μM)</th>
<th>10⁵ × kcat/Km (s⁻¹ M⁻¹)</th>
<th>kcat/Km (fold change)</th>
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<tr>
<td>SLLSDG</td>
<td>1.06 ± 0.3</td>
<td>87 ± 16</td>
<td>12.0 ± 1.3</td>
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<tr>
<td>SLLSGLI</td>
<td>3.75 ± 0.7</td>
<td>52 ± 16</td>
<td>7.44 ± 11</td>
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<td>SLLSGID</td>
<td>2.23 ± 1</td>
<td>49 ± 6.8</td>
<td>44.5 ± 14</td>
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<tr>
<td>SLLSGIDG</td>
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<td>38 ± 8</td>
<td>79.6 ± 4.9</td>
<td>7</td>
</tr>
<tr>
<td>SLLSGIDGE</td>
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<td>47 ± 4</td>
<td>58.6 ± 5.1</td>
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</tr>
<tr>
<td>SLLSGIDDEG</td>
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<td>38 ± 0</td>
<td>0.77 ± 0</td>
<td>1</td>
</tr>
<tr>
<td>SLLSGIDE</td>
<td>0.30 ± 0.05</td>
<td>115 ± 36</td>
<td>2.6 ± 0.42</td>
<td>3</td>
</tr>
<tr>
<td>SLLSGXID</td>
<td>4.93 ± 1.5</td>
<td>22 ± 8</td>
<td>222.6 ± 13</td>
<td>288</td>
</tr>
</tbody>
</table>

The peptides are flanked by an N-terminal fluorophore and a C-terminal quencher as described in the Experimental procedures section. Cleavage occurs after the underlined Asp residue. Fold change in kcat/Km was calculated relative to the lowest value within each group. Data show average and SD of two independent experiments.
that caspase node 22 has decreased ability to produce bioactive IL-1β compared with caspase-1 and the dog inflammatory caspase, implying that it has caspase-4-like specificity. If this is the case, the specificity of caspase node 22 on synthetic peptides should match caspase-4. Indeed, the catalytic efficiency of caspase node 22 toward the inflammatory caspase reference substrate WEHD-7-amino-4-trifluoromethylcoumarin (AFC) was highly efficient. However, cleavage of caspase-1-optimized substrates by node 22 was substantially less efficient than cleavage by the dog inflammatory caspase (Table 2 and Fig. S6). We conclude that a caspase-4-like protease that lacked the ability to convert pro-IL-1β gave rise to the inflammatory caspase of extant Carnivora.

Discussion

The innate immune response is important in the control of infection; however, the very mechanisms that lead to its activation can also result in damaging responses to the host, such
as cytokine storm, also known as hypercytokinemia (36). Host responses such as this can be lethal and thereby lead to loss of inflammatory genes through selective pressure. For example, some mammals have suffered from dampened immune responses against pathogens by downmodulating their IL-1β-converting capacity (29, 37, 38) or other innate immune–sensing mechanisms (29, 39–41). Given the importance of inflammatory caspases in programing innate immunity, we sought to understand the evolution of this response. The loss of caspase-1 in the Carnivora ancestor may represent an adaptation of this kind.

The catalytic domain of caspase-1 is absent in Carnivora and thus was lost in an early ancestor of this clade via the deletion of a chromosomal segment encompassing this domain, leaving caspase-4 as the single catalytically competent inflammatory caspase at this locus (28). The event that caused the loss of caspase-1 likely occurred in an early Carnivora ancestor 62 to 34 million years ago, while the clade was radiating from its origins (42). Seeking to construct the most likely sequence of the last Carnivora inflammatory caspase ancestor (node 22), we utilized every existing Carnivora inflammatory caspase catalytic domain sequence and performed ASR. Our data suggest that caspase node 22 had the catalytic profile of current day caspase-4. This resurrected caspase would have been able to induce pyroptosis through cleavage of gasdermin D but could not produce biologically active IL-1β. This finding indicates that the early Carnivora could likely mount the first step of innate response via pyroptosis and release DAMPs but could not amplify the response by producing active IL-1β. However, during the evolution of the Carnivora, this caspase became more caspase-1 like, such that the current day Carnivora possess a caspase-1-like activity on a caspase-4 scaffold. Consequently, Carnivora may activate pyroptosis and amplify the response with cytokine production by using a repurposed caspase-4.

Seeking to understand the features that may drive the specificity of caspase-1 on a caspase-4 scaffold, we compared protein sequences between inflammatory caspases in the Carnivora. A, snapshot covering the ancestral node from the phylogenetic tree (Fig. S4) used to resurrect the antecedent of the inflammatory caspase catalytic domain of the order Carnivora (caspase node 22). B, cleavage of protein substrates by caspase node 22. Recombinant protein substrates—mouse 4 μM gasdermin D, pro-IL-18, and pro-IL-1β—were cleaved in vitro with a dilution series of recombinant caspase node 22 and analyzed by Coomassie blue–stained SDS-PAGE. S and E represent the substrate- and enzyme-only controls. C, the catalytic parameter, kcat/Km, of caspase node 22 against protein substrates was calculated based on this SDS-PAGE analysis. The figure shows average and SD of three experiments. Pro-IL-1β, inactive precursor of interleukin-1β; pro-IL-18, inactive precursor of interleukin-18.
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Figure 6. Caspase node 22 has decreased IL-1-convertase activity. A, cleavage of pro-IL-β by 40 nM mouse caspase-1, dog inflammatory caspase, and caspase node 22, or 4.5 μM mouse caspase-11.8, the cleavage products of pro-IL-β from (A) were analyzed for biological activity using an IL-1 receptor cellular reporter assay. Data are presented as mean and SD of triplicate assays aligned under the respective cleavage reactions shown in (A). IL-1, interleukin 1; pro-IL-β, inactive precursor of interleukin-1β.

Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat (s⁻¹)</th>
<th>Km (μM)</th>
<th>10⁴ × kcat/Km (s⁻¹ M⁻¹)</th>
<th>Substrate</th>
<th>kcat (s⁻¹)</th>
<th>Km (μM)</th>
<th>10⁴ × kcat/Km (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-WEHD-AFC</td>
<td>8.87 ± 0.18</td>
<td>38 ± 1.7</td>
<td>233 ± 5.4</td>
<td>Ac-WEHD-AFC</td>
<td>9.2 ± 0.17</td>
<td>17 ± 0.12</td>
<td>533 ± 5.8</td>
</tr>
<tr>
<td>Ac-WQPD-ACC</td>
<td>2.96 ± 0.26</td>
<td>16 ± 1.5</td>
<td>180 ± 0.26</td>
<td>Ac-WQPD-ACC</td>
<td>1.53 ± 0.55</td>
<td>73 ± 37</td>
<td>21.7 ± 3.5</td>
</tr>
<tr>
<td>Ac-FEAD-ACC</td>
<td>1.22 ± 0.02</td>
<td>21 ± 0.81</td>
<td>57 ± 3.4</td>
<td>Ac-FEAD-ACC</td>
<td>0.58 ± 0.03</td>
<td>19.2 ± 0.03</td>
<td>30 ± 1.8</td>
</tr>
</tbody>
</table>

Data show average and SD of two independent experiments.
benzylxoycarbonyl-Val-Ala-Asp–fluoromethyl ketone. The remaining activity was measured with Ac-WEHD-AFC (100 μM) substrate in caspase assay buffer 20 mM Pipes (pH 7.2), 10% sucrose, 100 mM NaCl, 1 mM EDTA, and 10 mM DTT supplemented with 0.75 M sodium citrate (48).

**Fluorescent substrates**

Internally quenched fluorescent substrates flanked by the N-terminal fluorophore 7-amino-4-carbamoylmethylcoumarin (ACC) and a C-terminal quencher 2,4-dinitrophenyl-lysine were synthesized by solid-phase peptide synthesis as previously described (9, 49). The ACC fluorophore–containing substrates Ac-WQPD-ACC and Ac-FEAD-ACC were synthesized as described (22). All in-house synthesized substrates were purified using reversed-phase HPLC, dissolved in dimethyl sulfoxide to the concentration of 10 mM, and stored at −20 °C until use. The AFC fluorophore–containing substrate Ac-WEHD-AFC was obtained from Enzo Life Sciences.

**Activity assays**

Enzymatic assays of recombinant caspases consisted of 100 μl final volume and were performed in 96-well opaque plates (Costar, Corning). Caspases were incubated in caspase assay buffer for 10 min at 37 °C before measuring activity, which was initiated upon addition of substrate. Activity assays consisted of kinetic fluorescence measurement in a CLARIOstar plate reader (BMG LabTech). The ACC fluorophore was detected at excitation/emission 355/460 nm and AFC at 400/505 nm. Reaction velocity was defined as the linear portion of the kinetic curve (relative fluorescence unit/second). Relative fluorescence unit values were converted to concentration by comparison with known standards.

Specificity parameters of caspases for the fluorogenic substrates were determined from enzyme kinetic data of 30 min reactions. The reaction velocity was plotted against substrate concentration, and $V_{\text{max}}$ and $K_m$ were calculated with Prism 7 (GraphPad Software, Inc) using the Michaelis–Menten equation. $k_{\text{cat}}$ was obtained by using Equation 1, where [E] is the enzyme concentration measured by active-site titration.

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

(1)

**Screening of caspase specificity**

A fluorogenic substrate library was utilized to define the amino acid cleavage preference of the dog inflammatory caspase, as described before for other caspases (49). Briefly, three sublibraries that scan positions surrounding the cleavage site (P2, P3, and P4) were screened at a concentration of 100 μM with the dog inflammatory caspase, human caspase-1, caspase-4, and caspase-5, in a final volume of 100 μl in caspase assay buffer. Substrate hydrolysis was monitored to determine reaction velocity. For each sublibrary, percent activity was calculated in relation to the optimal amino acid. We used the relative activity values obtained here for the dog inflammatory caspases and human inflammatory caspases, together with previously reported data for mouse inflammatory caspases (22), to compare their substrate preference by means of the Pearson correlation coefficient ($r$) using Prism 7.

**Cleavage assays of recombinant protein substrates**

We used SDS-PAGE to analyze cleavage of protein substrates by the caspases. Recombinant caspases were serially diluted and incubated with 4 μM of recombinant mouse mouse protein substrates obtained as previously described (9). Controls consisted of substrate or enzyme alone. After incubation, reactions were stopped by the addition of 30 μl of 3x SDS loading buffer and incubated at 95 °C for 5 min. Products were separated in Bolt 4 to 12% Bis–Tris SDS-PAGE (Thermo Fisher Scientific) and stained with InstantBlue (Expedeon). The gels were scanned with an ODYSSEY CLx imager (LI-COR). Images were exported to Image Studio software (LI-COR) for band intensity quantification of protein substrate remaining after cleavage. Band intensity values were normalized relative to those of noncleaved substrate, and values were plotted against log [E] estimated by active-site titration. $E_{1/2}$ values were calculated with Prism 7 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 (48). Where $k_{\text{cat}}/K_m$ is the second-order rate constant for substrate hydrolysis, $E_{1/2}$ is the concentration of caspase for 50% hydrolysis of substrate, and $t$ is the reaction time in seconds.

$$k_{\text{cat}} / K_m = \ln 2 / (E_{1/2} \times t)$$

(2)

**Phylogenetic trees and computation of ancestral sequences**

Seeking proteins with the caspase CARD–CARD–catalytic domain arrangement, we used BLAST on UniProt (50), Ensembl (51), and the National Center for Biotechnology Information to retrieve related proteins employing the dog inflammatory caspase (UniProt: A9YEF4) as a query. The
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origin of caspase-4 is found in early mammals; hence, we focused on Mammalia in our homology search. To resurrect a highly probable sequence of the last common ancestor of the Carnivora clade (node 22), we utilized a database of curated caspase sequences (CaspBase.org) that provided inflammatory caspase protein sequences from the chordate lineage (52) (Table S2). PROMAL53D (prodatabwmed.edu/promals3d) generated structure-based alignments (53), and sequences were pruned on Jalview (jalview.org) (54) to remove the CARDS so that we could focus our analysis on the catalytic domain. Finally, ancestral protein reconstruction proceeded as described by Grishpon et al. (55). Structural model of the ancestral reconstructed caspase was obtained by the PHYRE2 protein fold recognition server (http://www.sbg.bioic.ac.uk/~phyre2/html/page.cgi?id=index) (56).

IL-1 receptor signaling assay

To measure signaling through the IL-1 receptor by IL-1β proteolytic products, we utilized IL-1β reporter human embryonic kidney 293 cells with an NF-κB reporter cell supernatants and mixed with 180 μl of IL-1β, interleukin-1β; IL-18, interleukin-18; pro-IL-1β, inactive precursor of IL-1β; pro-IL-18, inactive precursor of IL-18.

Data availability

All data generated and analyzed during this study are included in this published article and its supporting information file.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ACC, 7-amino-4-carbamoylmethylcoumarin; AFC, 7-amino-4-trifluoromethylcoumarin; ASR, ancestral sequence reconstruction; CARD, caspase activation and recruitment domain; DAMP, damage-associated molecular pattern; IL-1β, interleukin-1β; IL-18, interleukin-18; pro-IL-1β, inactive precursor of IL-1β; pro-IL-18, inactive precursor of IL-18.

References

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