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The tetrapeptide sequence of IL-18 and IL-1β regulates their recruitment and activation by inflammatory caspases

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SUMMARY

Inflammasomes are multiprotein signaling complexes that activate the innate immune system. Canonical inflammasomes recruit and activate caspase-1, which then cleaves and activates IL-1β and IL-18, as well as gasdermin D (GSDMD) to induce pyroptosis. In contrast, non-canonical inflammasomes, caspases-4/-5 (CASP4/5) in humans and caspase-11 (CASP11) in mice, are known to cleave GSDMD, but their role in direct processing of other substrates besides GSDMD has remained unknown. Here, we show that CASP4/5 but not CASP11 can directly cleave and activate IL-18. However, CASP4/5/11 can all cleave IL-1β to generate a 27-kDa fragment that deactivates IL-1β signaling. Mechanistically, we demonstrate that the sequence identity of the tetrapeptide sequence adjacent to the caspase cleavage site regulates IL-18 and IL-1β recruitment and activation. Altogether, we have identified new substrates of the non-canonical inflammasomes and reveal key mechanistic details regulating inflammation that may aid in developing new therapeutics for immune-related disorders.

Graphical abstract

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AUTHOR CONTRIBUTIONS

C.Y.T. conceived and directed the project, performed experiments, analyzed data, and wrote the manuscript. P.E., C.H.-C., B.D., R.M.R., C.M.B., and M.B.B. performed experiments and analyzed data. M.S.E. and J.Z. performed bacterial infections in Caco-2 cells with supervision from S.S. J.L., T.S., and W.Y. performed cloning and generated plasmids. All authors read and approved the manuscript.

SUPPLEMENTAL INFORMATION

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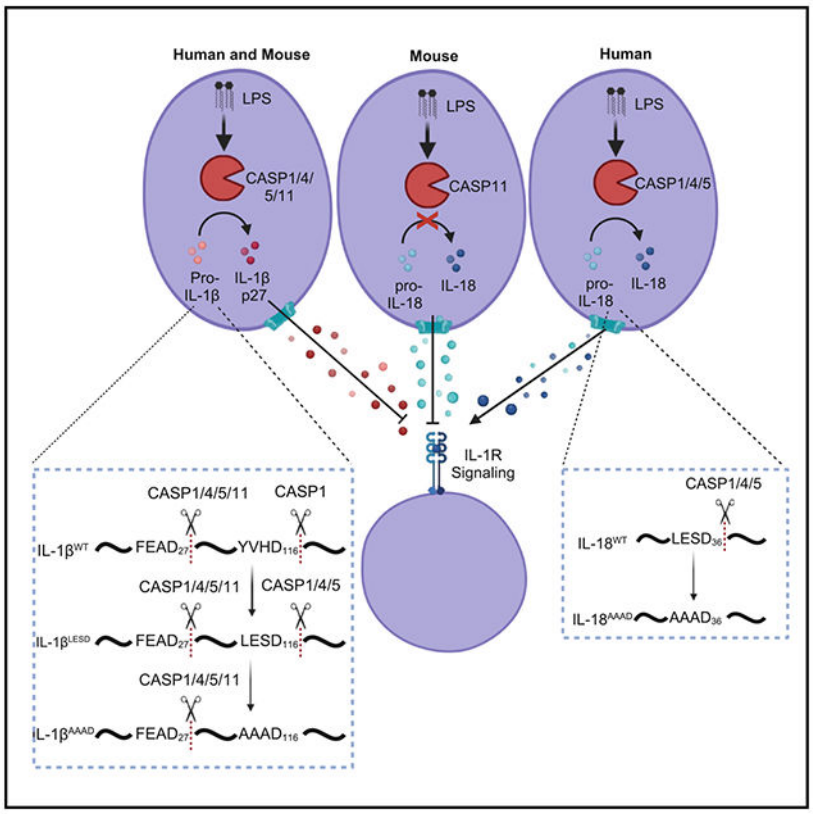
DECLARATION OF INTERESTS

The authors declare no competing interests.



In brief

Exconde et al. report that the non-canonical inflammasomes directly process IL-18 and IL-1β. The tetrapeptide sequence regulates IL-18 and IL-1β processing to generate an active or inactive cytokine respectively. These results suggest that the non-canonical inflammasomes directly modulate inflammation and may have a broader substrate repertoire than previously known.

INTRODUCTION

The mammalian innate immune system uses multiprotein complexes termed inflammasomes to defend against invading pathogens.[1,2] Canonical inflammasome assembly typically involves recruitment of the adapter protein ASC and pro-caspase-1 (pro-CASP1) to the inflammasome, which leads to autoproteolytic activation of pro-CASP1. Active CASP1 then processes the inflammatory cytokines IL-1β and IL-18 into their bioactive forms as well as the pore-forming protein gasdermin D (GSDMD) to induce pyroptosis.[3–8]

In contrast to the canonical pathway, non-canonical inflammasome activation involves direct detection of cytosolic lipopolysaccharide (LPS) from gram-negative bacteria by caspases-4/5 (CASP4/5) in humans and caspase-11 (CASP11) in mice.[9–12] Binding of LPS induces oligomerization and autoproteolytic maturation of CASP4/11, which then cleave GSDMD to induce pyroptosis.[13–15] Formation of GSDMD pores during non-canonical inflammasome activation results in K^+ efflux, which activates CASP1 to induce IL-1β and IL-18 processing via the canonical NLRP3 inflammasome.[16,17] Interestingly, both CASP4 and CASP11 have

been implicated in IL-18 processing, but as the canonical pathway is activated downstream, whether this is a direct processing event in cells or if CASP5, which is thought to be functionally similar to CASP4, can analogously process IL-18 remains to be defined.[18–21]

During inflammasome activation, caspases undergo autoproteolysis at multiple sites to generate distinct species (Figure 1).[22–25] The inflammatory caspases are composed of a CARD domain, followed by a 20-kDa large catalytic subunit (LS) and a 10-kDa small subunit (SS) (Figure 1A). Generally, autoproteolysis first occurs in the interdomain linker (IDL) to generate a p35/10 species, followed by further processing in the CARD domain linker (CDL) to generate a p20/10 species (Figure 1). It is thought that IDL processing confers full protease activity to the inflammatory caspases.[14,15,22,23] In support of this idea, recent studies suggest that the CASP1 p33/10 species is the active species in cells.[23] However, current structural studies suggest that CASP1/4/11 p20/10 is the species that binds GSDMD[26,27] via exosite-mediated interactions. However, if and how the non-canonical inflammasomes interact with other putative substrates such as IL-18 and IL-1β are unclear. The mechanisms employed by inflammatory caspases for substrate recognition and processing need to be clearly defined as this has important ramifications for innate immune regulation.

Here, we report that distinct inflammatory caspase species interact with specific substrates with varying affinities, which likely regulates substrate processing. We also discovered that the non-canonical inflammasomes directly process IL-18 and IL-1β in a tetrapeptide sequence-dependent and species-specific manner, revealing key insights into the molecular regulation of inflammation.

RESULTS

Autoproteolyzed inflammatory caspase species differentially bind to inflammatory substrates

Because the distinct caspase species (Figure 1A) may have different substrate specificities, we first wanted to determine which species interact with specific substrates. We generated plasmids to express the different subunits of CASP1/4/5 as separate polypeptides, which can form the active species in the absence of any stimulating ligand, for functional interrogation.[27] Since IDL cleavage generates a CARD-LS that is ~35 kDa, and CDL processing generates the LS that is ~20 kDa when averaged, we will henceforth refer to the p35/10 and p20/10 when discussing the inflammatory species collectively (Figure 1A).[22,23] We transiently expressed the catalytically inactive (cysteine to alanine mutant) versions of the different CASP1/4/5 species harboring a 2xFLAG N-terminal tag in HEK293T cells stably expressing GSDMD-V5 and IL-1β-Myc, or IL-18-V5, and then we subjected them to anti-FLAG immunoprecipitation (Figures 1B–1G). Consistent with recent structural data[26,27], the p20/10 species bound strongly to the inflammatory substrates (GSDMD, IL-18, IL-1β) (Figures 1B–1G). We could not detect binding of IL-1β to any of the CASP4/5 species tested (Figures 1D and 1F), but IL-18 was bound to all the p20/10 species (Figures 1C, 1E, and 1G). Notably, no processing of GSDMD, IL-1β, or IL-18 was observed, and the p10 subunits were pulled down by the large subunits, indicating a functional catalytically inactive enzyme complex was formed. We noticed that the CASP1

p12/10 antibody had significant cross-reactivity with the CASP4 p10 subunit, so we used that to probe CASP4 p10 expression, but none of the antibodies tested could detect CASP5 p10. Altogether, these data suggest that the autoproteolyzed p20/10 species of the inflammatory caspases are the species that bind strongly to the inflammatory substrates.

CASP4/5 cleave IL-1β and IL-18

We next sought to investigate the functional impact of the distinct caspase species on inflammatory substrate processing. We hypothesized that when present at similar levels, the p20/10 species would be the most active given that they exhibited stronger binding to the inflammatory substrates. To prevent processing of the p35 constructs, we made the corresponding CDL mutations for CASP1 (D103A, D119A), CASP4 (D59A, D80A, D104A), and CASP5 (D137A) to generate constitutive p35/10 enzymes. As hypothesized, transient transfection of the catalytically competent p35/10 or p20/10 species in HEK293T cells expressing GSDMD and IL-1β or IL-18 resulted in the most significant LDH (lactate dehydrogenase) release for the p20/10 species (Figures 2A and 2B), except for CASP5, which was expressed at lower levels (Figure S1A). Interestingly, all the CASP4/5 species tested appeared to cleave GSDMD less efficiently than CASP1 (Figures 2C and S1A). Both CASP1 p33/10 and p20/10 were able to process IL-1β, and unexpectedly, we observed alternative processing of IL-1β by all the CASP4/5 species (Figures 2C and S1A). CASP4 also cleaved IL-1β to generate the active 17-kDa fragment, but this processing event was minimal compared to CASP1 (Figures 2C and S1A). Remarkably, unlike GSDMD or IL-1β, all caspase species seemed to process IL-18 equivalently (Figures 2D and S1B). It is worth noting that we combined the lysates and supernatants for immunoblot analysis to rule out loss of processed substrates due to secretion through GSDMD pores or cell death (Figures 2C and 2D).

We noticed that there was PARP cleavage in caspase-transfected cells, implying that the apoptotic pathway may be engaged. As both caspase-3 (CASP3) and caspase-8 (CASP8) have been implicated in cleaving inflammatory substrates,[28,29] we wanted to confirm direct processing of IL-18 and IL-1β by CASP4/5. We transfected CASP1/4/5 p20/10 species and activated CASP3 using etoposide and staurosporine and CASP8 using TRAIL (TNF-related apoptosis-inducing ligand)[30] in wild-type or *CASP3* knockout (KO) HEK293T cells (Figure 2E). IL-18 and IL-1β were processed in CASP1/4/5-transfected parent and *CASP3 KO* cells but not in CASP3/8-activated cells, suggesting that CASP1/4/5 directly cleave IL-18 and IL-1β (Figure 2E).

To gain more insight into the molecular basis of why CASP4/5 were deficient in IL-1β and GSDMD, but not IL-18 processing compared to CASP1, we tested the binding of these substrates to the p20/10 species. In agreement with our previous data, neither CASP4 p19/10 nor CASP5 p22/10 bound GSDMD or IL-1β as strongly as CASP1 p20/10 (Figure 2F). Conversely, more IL-18 was bound to both CASP4 and CASP5 compared to CASP1 (Figure 2G), suggesting that this increased binding affinity permits processing to the same degree as CASP1. Notably, a functional p20/10 complex was required for binding and processing, as expression of just the catalytically competent p20 subunit alone failed to bind or process IL-18 in the absence of the p10 subunit (Figure S1C).

The P4–P1 tetrapeptide sequence of IL-18 and IL-1β regulates their recruitment and processing by inflammatory caspases

Recent *in vitro* work using recombinant CASP4/5 demonstrated that the identity of the residues adjacent to the caspase cleavage site can influence the processing of substrates.[28,31] While some enhancement in the catalytic efficiency of non-canonical inflammasomes for processing IL-18 was achieved by substituting the P1′–P4′ region with the sequence of GSDMD, these substitutions in IL-1β had limited effect on catalysis.[28] This raises three possibilities that are not mutually exclusive: (1) the P4–P1 (rather than the P1′–P4′) region is the major determinant of IL-1β catalysis, (2) the GSDMD sequence is not the optimal sequence for enhancing catalysis, or (3) some other structural features modulate IL-1β recognition and catalysis, such as an exosite. Since the processing of GSDMD by inflammatory caspases is sequence independent,[27] we wondered if processing of IL-18 and IL-1β was similarly sequence independent. We reasoned that processing of IL-18 and IL-1β would be sequence dependent as CASP4/5 engaged and processed IL-1β at an alternative site other than the canonical site (D116) to generate a 27-kDa (IL-1β p27) fragment but not the bioactive p17 fragment (Figure 2C). To test this, we mutated the P4–P1 tetrapeptide sequence of IL-18 and IL-1β to match the tetrapeptide sequence of known caspase substrates (Figure 3A). For example, CASP1 cleaves wild-type IL-18 (IL-18^{WT}) at D36, which harbors an LESD$_{36}$ sequence, and IL-1β (IL-1β^{WT}) at D116, which harbors a YVHD$_{116}$ sequence. We mutated the LESD sequence of IL-18 to FLTD or YVHD to match the tetrapeptide sequence of GSDMD (IL-18^{FLTD}) or IL-1β (IL-18^{YVHD}), respectively, and the YVHD sequence of IL-1β to LESD to match the tetrapeptide sequence of IL-18 (IL-1β^{LESD}) (Figure 3A). We also mutated the native YVHD sequence in IL-1β to IAND (IL-1β^{IAND}) to match the tetrapeptide sequence of IL-1α, which was reported to be processed by CASP5,[32] and we generated IL-18^{AAAD} and IL-1β^{AAAD} mutants, which abolished all specificity.

We transiently co-expressed the IL-18 and IL-1β tetrapeptide mutants with the active caspase p20/10 constructs in HEK293T cells (Figures 3B and 3C). As expected, all the caspases cleaved IL-18^{WT}, but only CASP1 was able to process IL-18^{FLTD} and IL-18^{YVHD}, consistent with the notion that CASP1 cleaves GSDMD and IL-1β more efficiently than CASP4/5 (Figure 3B). None of the caspases were able to process IL-18^{AAAD}, signifying that the tetrapeptide sequence of IL-18 plays an important role in its recognition and processing by inflammatory caspases (Figure 3B). We reasoned that the tetrapeptide sequence of IL-1β would similarly regulate its processing and that changing the YVHD sequence in IL-1β to the LESD sequence found in IL-18 would permit IL-1β processing by CASP4/5. However, only CASP5 would process IL-1β^{IAND}, and none of the caspases would process IL-1β^{AAAD}. As predicted, only CASP1 significantly processed IL-1β^{WT} into the active p17 fragment, while CASP4 and CASP5 processed IL-1β^{WT} to generate the p27 fragment (Figure 3C), but CASP1/4/5 all processed IL-1β^{LESD} to generate the active p17 fragment (Figure 3C). Notably, all caspases processed IL-1β^{AAAD} into the p27 fragment, and only CASP1 was able to generate the p17 fragment, but this was significantly attenuated compared to IL-1β^{WT}. All caspases preferentially processed IL-1β^{IAND} to generate IL-1β p27, and only CASP1 was able to modestly generate IL-1β p17 (Figure 3C). These data indicate that the tetrapeptide sequence at the canonical IL-1β process site regulates its activation by inflammatory caspases (Figure 3C). Intriguingly, despite CASP5 being reported to cleave

IL-1α, CASP5 failed to cleave IL-1β^{IAND} into the p17 product but, rather, generated the p27 product (Figure 3C). These findings hinted that CASP5 may not cleave IL-1α as previously reported, and indeed, we did not observe processing of wild-type IL-1α by any of the caspases when activated by overexpressing the active species, using an inducible system (described below), or by intracellular LPS (Figures S2A–S2C).

We next tested the ability of the inflammatory caspases to directly process IL-18, IL-1β, and the tetrapeptide mutants using *in vitro* assays. CASP1 is known to process IL-1β at D27 to generate a fragment that is ~27 kDa, and CASP1/4 were reported to cleave IL-18 at D36 to generate the active species.[33,34] We reasoned that the non-canonical inflammasomes (CASP4/5/11) cleave IL-18 at D36 and IL-1β at D27. To test this, we mutated the processing sites to alanine to generate IL-18^{D36A} and IL-1β^{D27A} and then expressed and purified the IL-18 and IL-1β variants from HEK293T cells. We then incubated the purified proteins with equivalent activity units of recombinant caspases for 1 h or 24 h and then assessed processing by immunoblotting (Figures 3D and 3E). When incubated for 1 h, CASP1/4 processed IL-18^{WT} to generate the mature species, which was abolished in IL-18^{D36A}, IL-18^{YVHD}, and IL-18^{AAAD} (Figure 3D), supporting our findings that the tetrapeptide sequence regulates IL-18 processing. We could not detect processing of IL-18^{WT} by CASP5 even after 24 h, but we observed CASP11-mediated processing of human IL-18 at D36 (Figure 3D). At equivalent activity units, CASP4 exhibited the most robust processing of IL-18 and was the only caspase that fully processed IL-18 in 24 h. We could also detect CASP4-mediated processing of IL-18^{YVHD} and IL-18^{AAAD} in 24 h, but it was markedly attenuated compared to IL-18^{WT} (Figure 3D). Notably, CASP1/4/5 processed IL-1β^{WT} into the p27 fragment, which was abolished in IL-1β^{D27A}, indicating that this was the site of processing. Only CASP1 was able to cleave IL-1β^{WT} to generate the active p17 species, but CASP1/4/5 were all able to cleave IL-1β^{LESD} to generate the p17 species, consistent with our data in cells (Figure 3E). Importantly, none of the caspases tested processed IL-1β^{AAAD} *in vitro* to generate the p17 species (Figure 3E), indicating the tetrapeptide sequence regulates processing. Intriguingly, although CASP11 was able to cleave human IL-18, it was unable to process human IL-1β to generate the p27 fragment (Figure 3E).

We next wanted to determine the molecular basis governing the processing of IL-1β^{LESD} by CASP1/4/5 to generate the bioactive p17 fragment but not the other tetrapeptide mutants. Immunoprecipitations demonstrated that wild-type IL-1β does not bind to CASP4/5 p20/10, but IL-1β^{LESD} and IL-1β^{AAAD} bind to CASP4/5 (Figures 3F and 3G). We also observed that while CASP1 p20/10 binds IL-18 weakly compared to CASP4/5, CASP1 can bind strongly to IL-1β^{LESD} when expressed in the same cells (Figure 3H). Taken together, our findings suggest that the tetrapeptide motifs of IL-18 and IL-1β play critical roles in regulating their molecular interactions with inflammatory caspases to fine-tune processing.

Dimerization and LPS-activated CASP1/4/5 induce processing of inflammatory substrates

The consensus mechanism for initiator caspase activation involves initial dimerization, which allows the caspase to gain basal activity for itself, leading to IDL autoproteolysis and subsequent maturation.[35] Recently, the DmrB dimerization system has been used to induce the dimerization and activation of caspases.[15,22] Briefly, replacing the CARD domain

of the caspase with the DmrB domain enables precise and controlled dimerization and activation in the presence of the small molecule AP20187. To further probe the processing of inflammatory substrates, we generated HEK293T cells stably expressing either GSDMD-V5 or IL-18-V5 along with either ΔCARD DmrB-CASP1, −4, or −5 (hereafter referred to as DmrB-CASP1/4/5). We treated these cells with AP20187, and in agreement with our previous data, we observed GSDMD (Figure 4A) and IL-18 (Figure 4B) processing. Of note, DmrB-CASP1 exhibited the most GSDMD processing and exhibited the highest LDH release in both cell lines (Figures 4A and 4B). However, DmrB-CASP5 processed IL-18 nearly to the same degree as DmrB-CASP1 within 1 h, and all caspases processed IL-18 after 24 h (Figure 4B).

We next assessed the processing of IL-18 and IL-1β tetrapeptide mutants using the DmrB caspases. We transiently transfected IL-18 or IL-1β tetrapeptide mutants into HEK293T cells expressing either IL-18-V5 or GSDMD-V5 and DmrB-CASP1, −4, or −5 and then treated with AP20187 (Figures 4C and 4D). As anticipated, DmrB-CASP1/4/5 all processed IL-18^{WT} and IL-18^{FLTD} (Figure 4C). However, processing of IL-18^{FLTD} was less in DmrB-CASP4/5 activated cells compared to DmrB-CASP1, and only DmrB-CASP1 was able to process IL-18 harboring the IL-1β tetrapeptide sequence, IL-18^{YVHD}. Again, none of the caspases were able to process IL-18^{AAAD}, demonstrating that the tetrapeptide sequence controls IL-18 maturation. It's worth noting that these cells also expressed GSDMD, which was cleaved in all AP20187-treated samples despite the lack of tetrapeptide mutant substrate processing (Figure 4C). Unlike IL-18, which was cleaved by DmrB-CASP1/4/5, only DmrB-CASP1 cleaved IL-1β^{WT} at the canonical site to yield the active p17 fragment. However, IL-1β^{LESD} was cleaved by DmrB-CASP1/4/5, and none of the caspases cleaved IL-1β^{AAAD} or IL-1β^{IAND} (Figure 4D), consistent with our prior observations. These cells also expressed IL-18, which was cleaved by all the activated caspases, indicating that the proteases were active but lacked the ability to cleave certain tetrapeptide mutants (Figure 4D).

The non-canonical inflammasomes are activated by intracellular LPS.[9–12] We next sought to determine if activation by the native ligand (LPS) would induce cytokine processing. We first confirmed that LPS-activated CASP4/5 or 2xFLAG-CASP4/5 induced IL-18 processing, and moreover, the maturation of IL-18 was comparable between the tagged and untagged CASP4/5-expressing HEK293T cells (Figure 4E). We then generated cells expressing either CASP4 or CASP5 and the IL-18 tetrapeptide mutants and again observed tetrapeptide sequence-dependent processing of IL-18 upon LPS activation (Figure 4F). IL-18^{WT} was significantly processed by both CASP4 and CASP5, and the processing of the tetrapeptide mutants was considerably less than that of IL-18^{WT} (Figure 4F). Altogether, these data suggest that CASP4/5 do not process GSDMD as efficiently as CASP1 in cells, but IL-18 maturation occurs nearly as efficiently with CASP4/5 as with CASP1. Furthermore, these results reaffirm that the P4-P1 tetrapeptide motif of IL-18 and IL-1β regulates cytokine maturation by inflammatory caspases.

Cytosolic LPS and pathogenic infections induce non-canonical inflammasome-mediated processing of inflammatory substrates in human macrophages and epithelial cells

The non-canonical inflammasomes are expressed in both myeloid and epithelial cells and have a well-documented function in playing an important role in mediating host protection against invading pathogens.[9,10,18,20,21] While epithelial cells express IL-18, there is limited IL-1β expression even with toll-like receptor (TLR) stimulation.[18,36,37] On the other hand, monocytes and macrophages robustly express IL-1β upon TLR stimulation, which can occur during infection with gram-negative bacteria.[37] We thus endeavored to gain more insight into the function of non-canonical inflammasomes in different physiologically relevant cells. Because prior studies have implicated CASP4/11 in IL-18 processing in epithelial cells, we first wanted to examine the role of non-canonical inflammasome activation on IL-18 processing in epithelial cells. We infected Caco-2 cells (Figure 5A) with *Salmonella* Typhimurium, which was previously documented to activate CASP4.[18,20] As expected, *Salmonella* infection induced CASP4-dependent GSDMD and IL-18 maturation and release into the supernatants (Figure 5A). As previously reported, IL-18 processing was CASP1-independent (Figure 5A).[18,20] We next infected HeLa cells with *Salmonella* and observed GSDMD cleavage and IL-18 processing into the active cytokine, which was abrogated by a broad-spectrum pharmacological inhibitor (ZVAD) that targets all caspases and by an inhibitor that is more specific for CASP1 (VX765) (Figure 5B).[38,39] We then generated *CASP4* KO HeLa cells (Figure S3A), and like Caco-2 cells, we observed CASP4-dependent processing of IL-18 (Figure 5C). Intriguingly, we also detected CASP4-dependent activation of CASP3 in *Salmonella*-infected HeLa cells (Figure 5C).

We next sought to determine if the non-canonical inflammasomes similarly cleave cytokines in human macrophages. We differentiated THP1 cells into macrophages and transfected them with LPS to induce endogenous CASP4/5 activation. As anticipated, LPS induced significant LDH release in control and *CASP1* KO cells, indicating CASP4/5-mediated pyroptosis (Figure 5D). Indeed, LPS-transfected THP1 macrophages displayed GSDMD, IL-1β, and IL-18 processing in both control and *CASP1* KO cells (Figure 5E). Notably, activation of CASP4/5 in control cells led to robust processing of IL-1β into the p27 fragment, which was nearly completely abrogated by ZVAD and significantly attenuated by VX765 (Figure 5E). NLRP3 is activated downstream of non-canonical inflammasomes, which activates CASP1 to mediate IL-1β processing into the p17 fragment.[16,17] Consistent with this, we also observed IL-1β maturation into the bioactive p17 fragment only in control cells, which was completely abrogated by both inhibitors (Figure 5E).

Importantly, we observed processing of IL-1β into the p27 fragment in *CASP1* KO cells, indicating that this processing event likely results from CASP4/5 activity (Figure 5E). We did not detect the presence of the mature IL-1β p17 fragment in *CASP1* KO cells, suggesting CASP4/5 do not induce processing of IL-1β at the canonical site to generate the active cytokine in human macrophages (Figure 5E). Critically, the appearance of the p27 fragment was abrogated in the presence of caspase inhibitors in *CASP1* KO cells (Figure 5E). It should be noted that pharmacological inhibitors of caspases can cross-react with other caspases, and the CASP1 inhibitor VX765 also inhibits CASP4/5.[39,40] Like IL-1β, we observed CASP1-independent release of mature IL-18, which was abrogated by ZVAD

and VX765, suggesting that CASP4/5 cleave IL-18 to generate the active species in human macrophages (Figure 5E).

To directly test the role of CASP4/5 in cytokine processing in human macrophages, we transfected wild-type and *CASP4/5* double KO THP1 macrophages with LPS and assessed LDH release (Figure 5F), IL-18 secretion by ELISA (Figure 5G), and immunoblotting (Figure 5H). While IL-18 secretion was CASP4/5-dependent, LDH release was only partially dependent on CASP4/5 (Figures 5F and 5G). Immunoblot analysis revealed that IL-1β processing into the p27 fragment, IL-18 release, and CASP3 activation were CASP4/5-dependent. Stunningly, we observed partial activation of CASP1 in *CASP4/5* KO cells treated with LPS, which displayed attenuated GSDMD and IL-1β processing into the p17 fragment (Figure 5H). The partial activation of CASP1 in *CASP4/5* KO cells compared to wild-type cells is consistent with the partial LDH release observed (Figure 5F).

We next asked if CASP4/5 mediate processing of IL-1β and IL-18 in the context of bacterial infections. We infected wild-type and *CASP1* KO THP1 macrophages with *Legionella pneumophila*, and like LPS, *Legionella* infection resulted in increased LDH release in control and *CASP1* KO cells compared to uninfected cells (Figures S3B and S3C). *Legionella* infection induced the processing of IL-1β into both the p27 and p17 fragments in control cells, but only the p27 fragment was produced in *CASP1* KO cells (Figure S3C). It was difficult to ascertain IL-18 processing in *Legionella*-infected cells because the antibody could not detect the processed fragment even in control cells where CASP1 was activated. This was the case whenever lysates were combined with supernatants, suggesting that perhaps only a small fraction of available IL-18 is processed and cannot be sufficiently detected over the background cleavage when all IL-18 is pooled. Regardless, these data collectively demonstrate that CASP4/5 induce IL-18 processing into the active p17 fragment and IL-1β processing into IL-1β p27 in response to intracellular LPS and bacterial infections. Curiously, we observed CASP1 activation in LPS-treated cells independently of CASP4/5, suggesting that the non-canonical pathway may not absolutely be required for downstream canonical inflammasome activation in response to LPS.

CASP4/5 preferentially cleave IL-1β at D27 to deactivate IL-1R signaling and IL-18 at D36 to activate cytokine signaling

Although our cell data demonstrated that CASP5 cleaves IL-18, we could not detect CASP5-mediated IL-18 processing *in vitro*. Furthermore, we primarily detected processing of IL-1β into the p27 fragment by CASP1 *in vitro*, but in cells, we generally observed formation of the p17 fragment. Thus, we wanted to confirm the sites of IL-1β and IL-18 processing by CASP4/5 in cells, as well as determine the functional impact. We postulated that in cells, CASP1 prefers to cleave IL-1β at D116 to generate IL-1β p17, but CASP4/5 preferentially cleave IL-1β at D27 to generate IL-1β p27. We co-transfected wild type or IL-1β^{D27A} or IL-18^{D36A} with the catalytically active species of CASP1/4/5 into HEK293T cells, and as anticipated, CASP1 processed IL-1β^{WT} and IL-1β^{D27A} to generate IL-1β p17, whereas CASP4 primarily processed IL-1β^{WT} to generate the p27 and modestly generated the p17 fragment (Figure 6A). Notably, CASP4/5 failed to cleave IL-1β^{D27A} to generate IL-1β p27, and despite D116 being the only processing site available, CASP4/5 failed to significantly

process IL-1β^{D27A} to generate the active p17 fragment, implying a preference for the D27 site (Figure 6A). All caspases processed IL-18^{WT} in cells but failed to process IL-18^{D36A}, indicating that D36 is indeed the site of processing (Figure 6B). A summary of the caspase processing sites on IL-1β and IL-18 is depicted in Figure 6C.

CASP4/5 processing removes the first 27 residues from IL-1β to generate the p27 fragment, which was reported to be inactive and unable to signal to the IL-1 receptor (IL-1R).[41] However, Δ27 IL-1β (IL-1β^{Δ27}) still retains the canonical D116 site that is processed by CASP1, which is activated downstream of CASP4/5. We thus wanted to determine if CASP1 can further process IL-1β^{Δ27} to generate the active cytokine. We observed that activated DmrB-CASP1 processing of IL-1β^{Δ27} to generate the active p17 fragment was reduced compared to processing of IL-β^{WT}, but IL-18 processing remained unaffected (Figure 6D), suggesting CASP4/5 prevent further activation of IL-1β by CASP1.

To investigate the functional impact of CASP1/4/5 processing on IL-1β and IL-18, we subjected the supernatants from LPS-transfected wild-type, *CASP1* KO, and *CASP4/5* KO THP1 macrophages to an IL-1R signaling assay recently described (Figures 6F and S4).[41,42] As expected, CASP1- and CASP4/5-deficient cells had significantly reduced active IL-1β and IL-18 compared to wild-type cells, signifying that both the canonical and non-canonical pathways contribute to maximal IL-18 and IL-1β signaling in response to intracellular LPS (Figures 6F and S4). No active IL-1β was observed in *CASP1 KO* cells, and *CASP4/5 KO* cells had reduced levels of active IL-1β compared to wild-type cells, in agreement with the immunoblot that revealed partial CASP1 activation in *CASP4/5* KO cells (Figures 6E and 6F). Interestingly, we observed CASP4/5- but not CASP1-dependent release of IL-18; however, the levels of released IL-18 in LPS-treated *CASP1 KO* cells were not sufficient to be detected over the untreated samples in the IL-1R assay (Figures 6E and 6F).

To better understand the functional impact of IL-1β p27 production by CASP4/5 in a more physiologically relevant context, we generated THP1 monocytes with doxycycline (dox) inducible plasmids coding for IL-1β^{WT}, IL-1β^{Δ27}, or IL-1β^{D27A}. We then induced expression of these IL-1β variants, treated the cells with nigericin to activate the NLRP3 inflammasome, and then measured LDH release and subjected the supernatants to IL-1R signaling (Figure 6G). The lysates and supernatants were then combined for immunoblot analysis (Figure 6G). As a control, we primed only parent THP1 cells with LPS to induce endogenous IL-1β expression, then treated these cells with nigericin, and compared the amount of released active cytokines to the unprimed (to prevent endogenous IL-1β expression) THP1 cells expressing the dox-inducible IL-1β variants. CASP1 was activated in all nigericin-treated cells and induced GSDMD processing (Figure 6G). Importantly, CASP1 generated IL-1β p17 in all IL-1β variants except for IL-1β^{Δ27}, consistent with our HEK293T data (Figures 6D and 6G). IL-1β p17 could not be detected by immunoblotting or in the IL-1R signaling assay in CASP1-activated THP1 cells expressing IL-1β^{Δ27}, implying that processing of IL-1β into the p27 fragment by CASP4/5 is a deactivating event that prevents downstream IL-1β activation. Of note, IL-1R signaling by IL-18 was unchanged between IL-1β^{WT} and IL-1β^{Δ27} (Figure 6G). IL-1β^{D27A}, which cannot generate the p27 fragment, was robustly processed into the p17 fragment and displayed the highest levels of LDH release and IL-1R signaling by both IL-1β and IL-18, but this was likely because it

was expressed at higher levels (Figure 6G). We again could not detect IL-18 processing by immunoblot, but the IL-1R assay indicated that we had significant release of active IL-18 in IL-1β^{D27A}-expressing cells compared to control cells. Taken together, our data suggest that activation of CASP4/5 induces IL-18 maturation into the bioactive cytokine but induces a deactivating cleavage event in IL-1β.

Caspase-11 does not cleave IL-18 but cleaves IL-1β to generate the p27 product

CASP11 is considered the mouse ortholog of human CASP4/5. We wondered if processing of IL-1β and IL-18 was conserved between mice and humans. To answer this, we transiently transfected mouse Gsdmd (mGsdmd), IL-1β (mIL-1β), IL-18 (mIL-18), or human IL-18 (hIL-18) into HEK293T cells expressing ΔCARD DmrB-CASP11 and activated with AP20187 (Figure 7A). Addition of AP20187 induced pyroptosis in the mGsdmd-transfected cells, as evidenced by LDH release and mGsdmd processing into the pyroptosis-inducing p30 fragment (Figure 7A). Notably, DmrB-CASP11 induced mIL-1β processing to yield the p27 fragment but not the p17 fragment (Figure 7A). Unlike CASP4/5, DmrB-CASP11 failed to process mIL-18 into the bioactive mature species, but we detected slight processing of hIL-18 (Figure 7A).

Our results with DmrB-CASP11 suggest that CASP11 cleaves mIL-1β but not mIL-18. We next wanted to determine if LPS-activated CASP11 would similarly cleave IL-1β to generate the p27 fragment but not cleave IL-18. We transiently transfected the inflammatory substrates into HEK293T cells expressing 2x-FLAG CASP11 and treated with LPS. Analogous to the DmrB-CASP11 results, LPS-activated CASP11 induced pyroptosis, as determined by GSDMD processing and LDH release (Figure 7B). Importantly, LPS-activated CASP11 processed mIL-1β to yield the p27 fragment but not the active p17 fragment and failed to process both hIL-18 and mIL-18 (Figure 7B), implying that CASP11 may not cleave IL-18 under physiologically relevant conditions.

We next sought to interrogate CASP11-mediated cytokine processing in mouse Raw 264.7 macrophages. Notably, Raw 264.7 macrophages lack the ASC adaptor protein and are unable to activate NLRP3 downstream of the non-canonical pathway to generate IL-1β p17.[3] We generated Raw 264.7 cells stably expressing ASC to allow for downstream CASP1 activation and subsequent cytokine maturation for testing. We observed CASP11-dependent GSDMD processing and LDH release in LPS-treated cells (Figure 7C). Like CASP4/5 activation in human macrophages, we observed CASP11-dependent processing of IL-1β into the p27 fragment (Figure 7C). As expected, we detected IL-1β processing into the active p17 fragment only in ASC-expressing cells. As such, although there were similar levels of LDH release (Figure 7C) and secreted IL-1β (Figure 7D), we could detect active IL-1β signaling only in ASC-expressing cells (Figure 7E), indicating that the IL-1β released by parent and *CASP1* KO cells was inactive.

Next, we wanted to uncover the differences in hIL-18 and mIL-18 processing by inflammatory caspases using *in vitro* assays. We treated lysates from HEK293T cells expressing hIL-18 or mIL-18 with equivalent activity units of recombinant inflammatory caspases. Consistent with our HEK293T data, we detected slight processing of hIL-18 into the active cytokine in CASP11-treated lysates, but no processing of mIL-18 was detected

after 24 h (Figure 7F). Notably, human CASP1 and CASP4 could robustly cleave hIL-18, but only CASP1 was able to significantly process mIL-18. We note that there was slight processing of mIL-18 by CASP4, but this was decidedly much less than CASP4 processing of hIL-18. Taken together, our data suggest that IL-18 is a direct substrate for the human but not mouse non-canonical inflammasome. However, in certain cell types that express IL-1β, non-canonical inflammasome activation leads to IL-1β processing to generate a functionally inactive p27 fragment that abrogates downstream IL-1R signaling. These data imply that the inactivation of IL-1β signaling is conserved between humans and mice, but the activation of IL-18 is species specific.

DISCUSSION

The active caspase-1 species in cells is reported to be a transient p33/10 species, and further processing into the p20/10 species generates an unstable protein that rapidly loses protease activity.[23] In agreement, several studies report that IDL processing is critical for pyroptosis,[14,15,22,23] but recent structural studies demonstrate that the p20/10 species of the inflammatory caspases utilizes an exosite to bind to GSDMD.[26,27] However, the structural basis of caspase binding to IL-1β and IL-18 remains undefined. Thus, we sought to characterize the inflammatory caspase species and their interactions with GSDMD, IL-1β, and IL-18. Our data suggest that the p20/10 species of CASP1/4/5 is the species that binds strongly to the inflammatory substrates (Figure 1). However, some notable differences exist. For example, CASP1 p20/10 binds strongly to GSDMD and IL-1β and weakly to IL-18. In contrast, CASP4/5 p20/10 binds strongly to IL-18 and weakly to GSDMD and IL-1β. The p35/10 species displayed weak interactions with the inflammatory substrates, but this was sufficient to mediate processing (Figure 2). We note that less of the p35/10 species was captured in the IP samples, and this may account for less binding of the substrates observed (Figure 1). A reciprocal experiment using GSDMD as the bait discovered that CASP4 p33/10 but not the full length bound GSDMD, consistent with our findings.[27] It is worth mentioning that less CASP4 p19/10 and CASP5 p22/10 was pulled down compared to CASP1 p20/10, but more IL-18 was bound to CASP4 and CASP5 (Figure 2G), suggesting that if a functional enzyme complex is formed, strongly bound substrates would coimmunoprecipitate with the caspase species. Indeed, a functional CASP1 p33/10 enzyme complex was formed as evidenced by the processing of substrates such as IL-18 and IL-1β and the presence ofthe p10 subunit in the immunoprecipitate (Figures 1 and 2). If CASP1 p33/10 can mediate cytokine processing, then a major question is why is ASC required? ASC acts as a signal amplifier that facilitates full autoproteolysis of pro-CASP1 into the p20/10 species,[24,25,43] precisely the species that we propose binds strongly to the cytokines. ASC may have additional functions, but we postulate that in part, it helps generate enough of the short-lived p20/10 species that recruits and processes cytokines. In support of this, recent work demonstrates that hepatocytes express low levels of ASC, and only the p32/10 species of CASP1 can be detected during inflammasome activation, which is unable to process cytokines.[44] However, overexpression of ASC results in full maturation of CASP1 into the p20/10 species concomitant with IL-1β and IL-18 maturation.[44] These differences in substrate-binding capabilities likely help to regulate inflammation by the inflammatory caspases under different pathophysiological conditions. Using the short-lived p20/10 species

for cytokine processing may be a way to limit excessive inflammation. Further studies are needed to help uncover the precise role of the distinct caspase species in innate immunity.

The non-canonical inflammasomes can cleave GSDMD to mediate pyroptosis, but whether other direct substrates exist has remained unclear. We discovered that CASP4/5, but not CASP11, can directly process IL-18 to generate the bioactive species, consistent with prior findings.[28,31,45] Notably, IL-18 is a potent activator of natural killer and T cells[46] and likely confers an advantage for mounting an immune response during infection. These findings are in line with recent genetic evidence that CASP4 is required for IL-18 maturation and secretion in epithelial cells during bacterial infection.[18,20,21] Furthermore, our data indicate that the ability of CASP4/5 to process IL-18 spans across other cell types, including macrophages (Figures 5 and 6). We also discovered that CASP1/4/5/11 can directly process IL-1β, but CASP4/5/11 primarily process IL-1β to generate a p27 fragment (Figures 2, 6, and 7) that inactivates IL-1β signaling. While this manuscript was under revision, a recent study used DmrB-CASP4 and reported processing of IL-1β to generate the mature p17 fragment.[47] We also detected modest processing of IL-1β by CASP4 to generate the p17 fragment, but this was negligible compared to the p17 generated by CASP1. Moreover, we could not detect IL-1β p17 by immunoblotting or with an IL-1R reporter assay when CASP4/5 were activated in *CASP1*-deficient macrophages (Figure 6). It is possible that the processed p17 fragment generated by CASP4/5 activity was below the limit of detection in our assays. Regardless, the fact that we could detect CASP4/5-mediated processing of IL-1β to the p27 but not the p17 fragment suggests that the former may be more physiologically relevant.

The functional impact of non-canonical inflammasome processing of IL-1β into the p27 fragment remains to be fully determined. Our data suggest that non-canonical inflammasome-mediated processing of IL-1β into IL-1β p27 is a deactivating mechanism to limit cytokine signaling, congruent with prior reports that IL-1β p27 is unable to signal to the IL-1 receptor.[41] Similarly, CASP3 was reported to cleave IL-18 at D71 to inactivate signaling.[48] Perhaps during evolution, IL-1β signaling became detrimental to the host, and the non-canonical inflammasomes evolved to induce pyroptosis and clear bacterial infections without inducing IL-1β signaling. Recent ancestral reconstruction revealed that CASP4/5 evolved after CASP1 from an ancestral caspase that cleaves IL-1β to yield the p27 fragment.[41] Notably, IL-1β and IL-18 contribute to the excessive inflammation and pathogenesis of sepsis that lead to organ failure during infections, and blocking these cytokines confers protection in acute animal models.[49] The existence of an endogenous mechanism to deactivate IL-1β signaling may provide a novel avenue for targeting IL-1β therapeutically.

GSDMD is recognized by inflammatory caspases via an exosite, and the processing is sequence independent.[27] In contrast, we discovered that the processing of IL-18 and IL-1β is sequence dependent (Figure 3). However, the tetrapeptide sequence is not the only determinant of substrate specificity. For example, histidine 342 on CASP1 has been reported to be important for IL-1β processing.[50] The protease active sites likely also play a role in substrate recognition and binding as CASP4/5 do not bind IL-1β^{WT} but bind IL-1β^{LESD}. These differences between the caspases may account for why CASP1/4 (and likely CASP5)

employ the same exosite to recognize GSDMD[26,27] but have varying affinities and process GSDMD to varying degrees. This might also explain why CASP4/5 can cleave IL-18, but CASP11 cannot cleave mIL-18 despite mIL-18 having the conserved LESD motif and being 64% identical[51] to hIL-18 (Figure S5). Puzzlingly, neither IL-18 nor IL-1β has been identified to be a substrate of CASP4 in proteomics screens.[52,53] There are several reasons for this, including peptide abundance and the use of enzymes such as trypsin or subtiligase that may have preferences for certain sequences.

Overall, we report two new direct substrates (IL-1β and IL-18) for the non-canonical inflammasomes, expanding the repertoire of substrates beyond GSDMD. On that note, we discovered CASP4/5-dependent activation of CASP3, and CASP7 was identified as a putative substrate of CASP4,[53] suggesting there may be crosstalk between the non-canonical inflammasomes and the apoptotic pathway, similar to CASP1.[54,55] However, the mechanism remains unclear, and CASP4/5 may not directly activate CASP3, as we did not observe CASP3 activation in HEK293T cells expressing active CASP4/5 (Figure 2E). The non-canonical inflammasome likely has undiscovered substrates that play critical roles in cell death and immunity. Putative substrates may harbor LESD tetrapeptide sequences, which appear to be favored by CASP4/5. Collectively, the present study offers mechanistic insights into substrate specificities that could help design new therapies for inflammatory disorders and aid our understanding of host responses to inflammation and bacterial infections.

Limitations of this study

There are two major limitations of this study. First, we could not obtain equal expressions of the various caspase species in our immunoprecipitates, which confounds the comparisons of the substrate interactions. Second, we reconstitute the inflammasome components in HEK293T cells, and while this reductionist approach is great for gaining mechanistic insights, it does not capture the full complexity of what might be occurring during inflammasome activation in immune cells.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Cornelius Y. Taabazuing (Cornelius.taabazuing@pennmedicine.upenn.edu).

Materials availability—Materials generated in this study are available from the lead contact upon reasonable request.

Data and code availability

- Unprocessed data related to figures in this manuscript are available from the lead contact upon request.
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

HEK 293T, THP1, RAW 264.7, HeLa cells were purchased from ATCC. HEK Blue IL-1β and HEK Blue IL-18 cells were purchased form Invivogen. *CASP1* and *CASP4* KO Caco-2 cells were from the Shin lab[20] and THP1 *CASP4/5* double KO cells were a kind gift from the Bryant lab.[56] *CASP1* KO THP1 cells were previously reported[54] and *CASP4* KO HeLa cells were generated as previously described.[54,57] All cells were grown at 37°C in a 5% CO_2 atmosphere incubator and further details about the media is reported in the methods details.

METHOD DETAILS

Antibodies and reagents—Antibodies used include: GSDMD Rabbit polyclonal Ab (Novus Biologicals, NBP2-33422), FLAG M2 monoclonal Ab (Sigma, F3165), GAPDH Rabbit monoclonal Ab (Cell Signaling Tech, 14C10), CASP1 p20 Rabbit polyclonal Ab (Cell Signaling Tech, 2225s), CASP1 p12/10 Rabbit monoclonal Ab (Abcam, ab179515), CASP4 Rabbit polyclonal Ab (Cell Signaling Tech, 4450S), CASP5 Rabbit monoclonal Ab (Cell Signaling Tech, 46680S), Myc Mouse monoclonal Ab (Cell Signaling Tech, 2276S), V5 Rabbit monoclonal Ab (Cell Signaling Tech, 13202S), hIL-1β Goat Polyclonal Ab (R&D systems, AF-201-NA), hIL-18 Goat Ab (R&D systems, af2548), hIL-1α Recombinant Ab (PeproTech, 200-01A), PARP Rabbit polyclonal Ab (Cell Signaling Tech, 9542S), HA Rabbit monoclonal Ab (Cell Signaling Tech, 3724S), mIL-1β Goat polyclonal Ab (R&D systems, AF-401-NA), CASP11 Rat monoclonal Ab (Novus Biologicals, NB120-10454), FKBP12 Rabbit polyclonal Ab (Abcam, ab24373). IRDye 800CW anti-rabbit (LICOR, 925–32211), IRDye 800CW anti-mouse (LI-COR, 925–32210), IRDye 680CW anti-rabbit (LI-COR, 925–68073), IRDye 680CW anti-mouse (LI-COR, 925–68072). Other reagents used include: LPS-EB Ultrapure (Invivogen, tlrl-3pelps), VX-765 (Apexbio Technology LLC, 50-101-3604), Z-VAD-FMK (Enzo Life Sciences, NC9471015), FuGENE HD (Promega, E2311), AP20187 (Tocris 6297/5),

NP-40 Lysis Buffer Low Salt (Thomas Scientific, C994H79). Pam3CSK4 (Invivogen, tlrl-pms), phorbol 12-myristate 12-acetate (Promega, V1171), NP-40 Lysis Buffer Low Salt (Thomas Scientific, C994H79), Recombinant Human TRAIL (R&D Systems, 375-TL/CF) Staurosporine (MedChemExpress, HY-15141), Etoposide (Enzo Life Sciences, BMC-GR307), Nigericin (Cayman Chemical, 11437), Doxycycline hydrochloride (Fisher Scientific, ICN19504401), Cyquant LDH Cytotoxicity Assay (Thermo Scientific, C20300), DC Protein Assay Kit (Bio-Rad, 5000111), HEK-Blue IL-18 (Invivogen, hkb-hmil18), HEK-Blue IL-1b (Invivogen, hkb-il1bv2), Human Total IL-18/IL-1F4 Quantikine ELISA Kit (R&D Systems, DL180), Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit (R&D Systems, MLB00C), Recombinant Human Caspase-1 (Enzo Life Sciences, BML-SE168-5000), Recombinant Human Caspase-4 (Enzo Life Sciences, BML-SE176-5000), Recombinant Human Caspase-5 (Enzo Life Sciences, BML-SE171-5000), Recombinant Mouse Caspase-11 (Enzo Life Sciences, BML-SE155-5000).

HEK Blue IL-1R reporter assay—To quantify the amount of active IL-1β and IL-18 released, HEK-Blue IL-18 and HEK-Blue IL-1β (Invivogen) reporter cells were used according to manufacturer's protocols, as previously described.[42] Briefly, binding of IL-18 to the IL-18R or IL-1β to the IL-1R induces NF-κB activation downstream, which leads to dose-dependent productions of secreted embryonic alkaline phosphatase (SEAP). The amount of SEAP released can be quantified using a calorimetric assay by adding Quanti-Blue (Invivogen) and measuring the absorbance at OD620. HEK Blue cells were seeded in 96 well plates at 5 x 10^4 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 10% fetal bovine serum (FBS) to a total volume of 150 μL. 50 μL of supernatant from control or treated THP1 cells were added and the samples were incubated overnight at 37°C in a 5% CO_2 atmosphere incubator. 20 μL of supernatant containing SEAP were mixed with 180 μL of the Quanti-Blue solution and incubated at room temperature for 1 h before the absorbance at 620 nm was read on a Cytation 5 plate reader (Bio Tek). On the same plate, a serial dilution of recombinant IL-1β or IL-18 (Invivogen) was used to generate a standard curve which was used to calculate the absolute levels of active IL-1β or IL-18.

Recombinant caspase assays—For *in vitro* assays, HA-tagged IL-18 and IL-1β variants were transfected into HEK 293T cells for 24 h. The cells were then lysed in PBS and lysates were incubated with anti-HA agarose beads at 4°C overnight. The beads were then washed 3x with PBS and the cytokines were eluted using 500 μg/mL of HA peptide. The purified cytokines were then incubated in caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.) containing 1 activity unit of each indicated caspase for IL-18 assays, or 3 activity units for IL-1β assays (20 μL reactions) for 1 h or 24 h at 37°C. Samples were then mixed 1:1 with 2x sample loading buffer, boiled at 95°C for 10 min, then analyzed by SDS-PAGE and immunoblotting. We used lysates in Figure 7F instead of purified proteins.

ELISAs—THP1 cells were seeded at 8 x 10^5 cell/well in twelve-well plates and stimulated with PMA (phorbol 12-myristate 13-acetate, 50 ng/mL) for 48 h. The media was then changed to Optimem before LPS transfection (25 μg/mL). After 24 h, supernatants were harvested from LPS or vehicle treated cells and the levels of secreted cytokines were quantified by ELISA kits according to the manufacturer's instructions. Human Total IL-18/IL-1F4 Quantikine ELISA Kit (R&D Systems, DL180), Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit (R&D Systems, MLB00C).

Cell culture—HEK 293T cells, HeLa cells, and THP1 cells were purchased from ATCC. Caco-2 and THP1 knockout cell lines were previously reported.[20,54] HEK 293T and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 10% fetal bovine serum (FBS). THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 with L-glutamine and 10% fetal bovine serum (FBS). Caco-2 cells (HTB-27; American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin. All cells were grown at 37°C in a 5% CO_2 atmosphere incubator.

Generation of stable cell lines—For generating HEK 293T cells ectopically expressing GSDMD, IL-1β, IL-1α, IL-18, and caspase constructs, plasmids encoding those proteins were packaged into lentivirus by transfecting the vectors (2 μg) along with psPAX2 (2 μg), and pMD2.G (1 μg) using Fugene HD transfection reagent (Promega) into HEK 293T cells. After 2 days, the supernatants were filtered using a 0.45 μm filter, then used to infect HEK 293T cells. After 48 h, the cells expressing the indicated constructs were selected with hygromycin (200 μg/mL), blasticidin (10 μg/mL), or puromycin (1 μg/mL).

CRISPR/Cas9 gene editing—*CASP4* knockout HeLa cells were generated by infecting HeLa cells with virus generates with the lentiCRISPR v2 (Addgene) plasmid coding for Cas9 and a gRNA directed at CASP4. Briefly, lentivirus was generated as described above in the generation of stable cell lines section. HeLa cells were seeded at 5 x 10^5 cells/well in 2 mL of media in 6-well tissue culture dishes. The next day, cells were infected with virus to express Cas9 and the gRNA. After 48 h, cells were transferred to a 10 cm tissue culture dish and selected with puromycin (1 μg/mL) until all control cells were no longer viable. Single cell clones were then obtained by serial dilution and confirmed by immunoblotting.

Cloning—All plasmids were cloned using Gateway technology as previously described.[22,54,57] DNA encoding the indicated proteins were inserted between the *attR* recombination sites and shuttled into modified pLEX_307 vectors (Addgene) using Gateway technology (Thermo Fisher Scientific) according to the manufacturer's instructions. Proteins expressed from these modified vectors contain an N-terminal *att*B1 linker (GSTSLYKKAGFAT) after any N-terminal tag (2xFLAG) or protein (DmrB) or a C-terminal *att*B2 linker (DPAFLYKVVDI) preceding any C-terminal tag such as V5 or HA. An internal ribosome entry site (IRES) was cloned between the large and small subunits of the caspases to allow separate expression of the two polypeptides. ΔCARD DmrB-CASP1 (residues 92–402), ΔCARD DmrB-CASP4 (residues 67–377), ΔCARD DmrB-CASP5 (residues 128–434), and ΔCARD DmrB-CASP11 (residues 71–373) were all cloned into a modified pLEX_307 vector and contained the N-terminal *att*B1 linker between the DmrB and caspase sequences. Point mutations were generated using the QuikChange II site-directed mutagenesis kit (Agilent) according to the manufacturer's instructions.

Transient transfections—HEK 293T cells were seeded in 12-well culture plates at 2.5 × 10^5 cells/well in DMEM. The following day, the indicated plasmids were mixed with a control RFP vector to a total of 1.0 μg DNA in 65 μL Opti-MEM and transfected using FuGENE HD (Promega) according to the manufacturer's protocol. Some samples were further treated (as indicated in each figure legend) with AP20187 (1 μM) or LPS (25 μg/mL)/FuGENE (0.5%). The cells were harvested at the indicated times and analyzed by LDH cytotoxicity and immunoblotting assays as described below. The amount of DNA transfected for processing assays were as follows: 0.05 μg of GFP, 0.2 μg of CASP1/4/5 p35/p10, 0.2 μg of CASP1 p20/10, 0.4 μg of CASP4 p19/p10, and 3 μg CASP5 p22/10. For Figures 2 and 0.4 μg CASP5 p22/10 was transfected. For cleavage assays that utilized the tetrapeptide mutants, 0.1 μg of IL-1β^{WT}, IL-1β^{LESD}, IL-1β^{AAAD}, or 0.8 μg of IL-1β^{IAND} was co-transfected before analysis. For assays involving CASP11 processing (Figures 4 and

5), 0.05 μg of mGsdmd, and 0.1 μg of mIL-1β, mIL-18, or hIL-18 was transfected before treatment with the indicated agents and times.

LDH cytotoxicity and immunoblotting assays—Supernatants were harvested for LDH analyses at time points indicated and analyzed using the Cyquant LDH Cytotoxicity Assay (Thermo Scientific) according to the manufacturer's protocol. LDH activity was quantified relative to a lysis control where cells were lysed using NP-40 for 30 min. For immunoblotting, protein concentrations were normalized using the DC Protein Assay Kit (Bio-Rad), separated by SDS-PAGE, transferred onto Nitrocellulose membranes (Bio-Rad), and visualized using the Odyssey M Imaging System (LI-COR Biosciences).

FLAG immunoprecipitations—HEK 293T cells were seeded in 6-well plates at 5×10^5 cells/well in DMEM for 24 h. The cells were then transiently transfected with the indicated constructs. Cells were transfected with 0.5 μg of DNA for GFP, 1 μg of CASP1 p45, 3 μg of p33/p10, 2 μg of CASP1 p20/10, 1 μg of CASP4 p43, 3 μg of CASP4 p31/10 or p19/10, 2 μg of CASP5 p50, p38/10, and 3 μg of CASP5 p22/10. For IP assays with tetrapeptide mutants, 0.5 μg of IL-1β^{LESD}, or IL-1β^{AAAD} was co-transfected. After 48 h, the cells were harvested and lysed by sonication. Lysates were clarified by centrifugation at 21,000 x g for 5 min. The soluble fractions were then normalized using the DC Protein Assay (BioRad). 100 μL of the lysates were combined with 100 μL of 2x sample loading buffer and incubated at 95°C for 10 min. Equal protein amounts of the remaining sample lysates were loaded onto Pierce Micro-Spin Columns (Thermo Scientific) containing 100 μL of anti-FLAG-M2 agarose resin (Sigma) and the samples were rotated end-over-end at 4°C overnight. The samples were then washed 3x with 1 column volume (350 μL) of PBS. Proteins were eluted by rotating the resin at room temperature for 1 h in 100 μL of PBS containing 150 ng/μL 3×-FLAG peptide (Sigma Aldrich). 100 μL of 2x sample loading buffer was added to the eluate and samples were boiled at 95°C for 10 min. Both lysates and eluates were analyzed by immunoblotting.

LPS transfections of THP-1 cells—THP-1 cells were resuspended in RPMI medium containing 50 ng/mL phorbol 12-myristate 12-acetate (PMA). Cells were plated in 12-well plates at a density of 8 x 10^5 cells/well. After 24 h, the media was replaced with fresh RPMI/10% FBS containing 5 μg/mL LPS and the cells were allowed to grow for another 24 h. The media was then replaced with Opti-MEM (0.5 mLs/well). Where indicated, cells were treated with ZVAD (40 μM)or VX765 (40 μM) 30 min before LPS transfection. The LPS solution was prepared by adding LPS (25 μg/mL final concentration) and FuGENE (0.5% final concentration) to Opti-MEM. This solution was gently mixed by flicking and incubated for 30 min at room temperature before drop-wise addition to each well. The supernatants were collected, and cells were lysed by sonication 24 h post transfection. Both supernatants and lysates were precipitated by chloroform/methanol and analyzed by immunoblotting.

Bacterial infections

Salmonella *infection of Caco-2 cells*—*Salmonella* and *Legionella* infections were carried out as previously described.[20,58,59] Caco-2 cells (HTB-27; American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were grown at 37°C in a humidified incubator with 5% CO_2. One day prior to infection, Caco-2 cells were incubated with 0.25% trypsin-EDTA (Gibco) diluted 1:1 with 1 x PBS at 37°C for 15 min to dissociate cells. Trypsin was neutralized with serum-containing medium. 24 h before infection, cells were replated in DMEM supplemented with 10% (v/v) heat-inactivated FBS without antibiotics in a 24-well plate at a density of 3 x 10^5 cells/well. 3 h before infection, the media was replaced with Opti-MEM I reduced serum medium (Thermo Fisher Scientific) containing 100 ng/mL Pam3CSK4 (Invivogen) to prime the cells. An overnight culture of wild-type (WT) *Salmonella* Typhimurium (SL1344) was diluted into LB broth containing 300 mM NaCl and then grown for 3 h at 37°C to induce SPI-1 expression. After induction, the culture was pelleted at 6,010 × g for 3 min, washed once with PBS, and then resuspended in PBS. Caco-2 cells were infected with WT *Salmonella* at a multiplicity of infection (MOI) of 60. Control cells were mock-infected with PBS. Cells were centrifuged at 290 × g for 10 min and incubated at 37°C. 1-h post-infection, infected cells were treated with 100 ng/mL Gentamicin to kill any extracellular *Salmonella* and placed back to 37°C. The supernatants were collected, and cells were lysed by sonication 6 h post infection. Both supernatants and lysates were precipitated by chloroform/methanol and analyzed by immunoblotting.

Legionella *infection of THP1 cells*—THP-1 cells in RPMI medium were plated in12-well plates at a density of 8 x 10^5 cells/well and differentiates with 40 ng/mL phorbol 12-myristate 12-acetate (PMA)for 24 h. The media was replaced with fresh RPMI/10% FBS (without PMA) and the cells were allowed to grow for another 24 h. The media was then replaced with Opti-MEM immediately before *Legionella* infection. The THP-1 cells were infected with a flagellin mutant, Δ*flaA*, of *Legionella pneumophila*,[60] which is an Lp02 strain (*rpsL, hsdR, thyA*) derived from the serogroup 1 clinical isolate Philadelphia-1. *Legionella* was grown as a stationary patch on charcoal yeast extract agar plates at 37°C. After 48 h, the bacteria were resuspended in PBS and added to the THP-1 cells at a MOI of 10. Infected THP-1s were centrifuged at 400 × g for 10 min and incubated at 37°C for 7 h. Control cells were mock-infected with PBS. The supernatants and cells were precipitated together by chloroform/methanol and analyzed by immunoblotting.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analysis was performed using GraphPad Prism 9.0 software and Microsoft Excel. Statistical significance was determined using two-sided Student's *t*-tests unless otherwise stated. Data were considered significant if the p values were less than 0.05. Graphs and error bars represent means ± SEM of three biological replicates and are representative of three independent experiments. The investigators were not blinded in all experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Human CASP4/5 directly process IL-18 at D36 to generate the activated cytokine
- CASP4/5 cleave IL-1β at D27 into a p27 species that inactivates downstream IL-1R signaling
- The tetrapeptide sequence of IL-18 and IL-1β regulates their processing by caspases
- CASP11 can process IL-1β into the deactivating p27 species but does not process IL-18

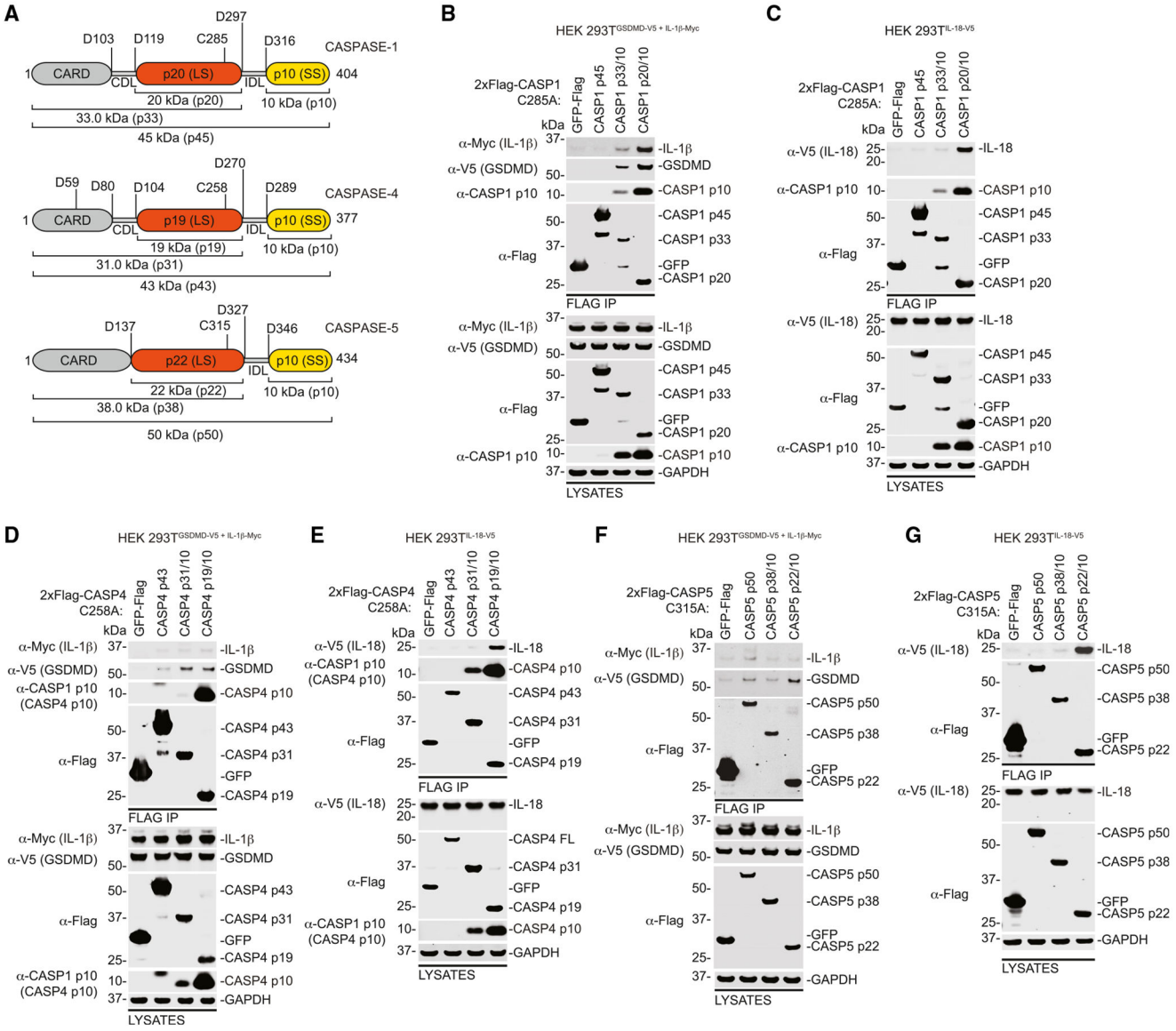

Figure 1. Autoproteolyzed inflammatory caspase species differentially bind to inflammatory substrates

(A) Schematic of human CASP1 (top), CASP4 (middle), and CASP5 (bottom) depicting the catalytic cysteines and autoproteolytic sites.

(B–G) Immunoprecipitation depicting binding of inflammatory caspase species to GSDMD and IL-1β (B, D, and F) or IL-18 (C, E, and G). Data are representative of three or more independent experiments.

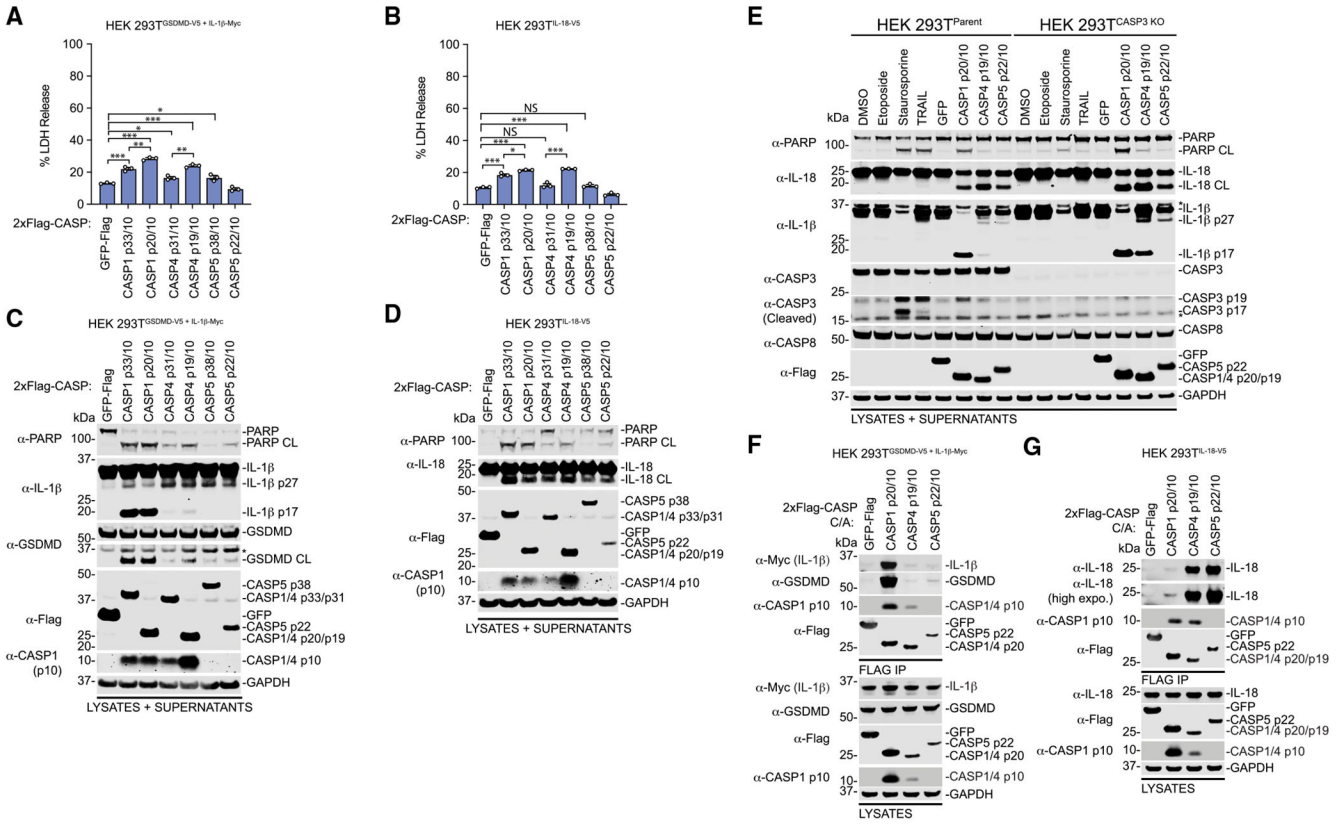

Figure 2. CASP4/5 cleave IL-1β and IL-18

(A and B) HEK293T cells expressing either GSDMD-V5 and IL-1β-Myc (A) or IL-18-V5 (B) were transiently transfected with the indicated constructs for 24 h before LDH analysis.

(C and D) HEK293T cells expressing either GSDMD-V5 and IL-1β-Myc (C) or IL-18-V5 (D) were transiently transfected with the indicated constructs for 24 h and then supernatants and lysates were combined for immunoblotting.

(E) Parent (wild-type) or *CASP3* KO HEK293T cells were transiently transfected with plasmids coding for IL-18 and IL-1β or co-transfected with the indicated caspases. After 24 h, the caspase co-transfected samples were harvested, and the remaining samples were treated with DMSO, etoposide (100 μM), staurosporine (0.5 μM), or TRAIL (150 ng/mL) for an additional 24 h before immunoblotting.

(F and G) Immunoprecipitation depicting binding of inflammatory caspase species to GSDMD and IL-1β (F) or IL-18 (G). Data are means ± SEM of three biological replicates. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by two-sided Student's t test. * = non-specific bands. Data are representative of three or more independent experiments.

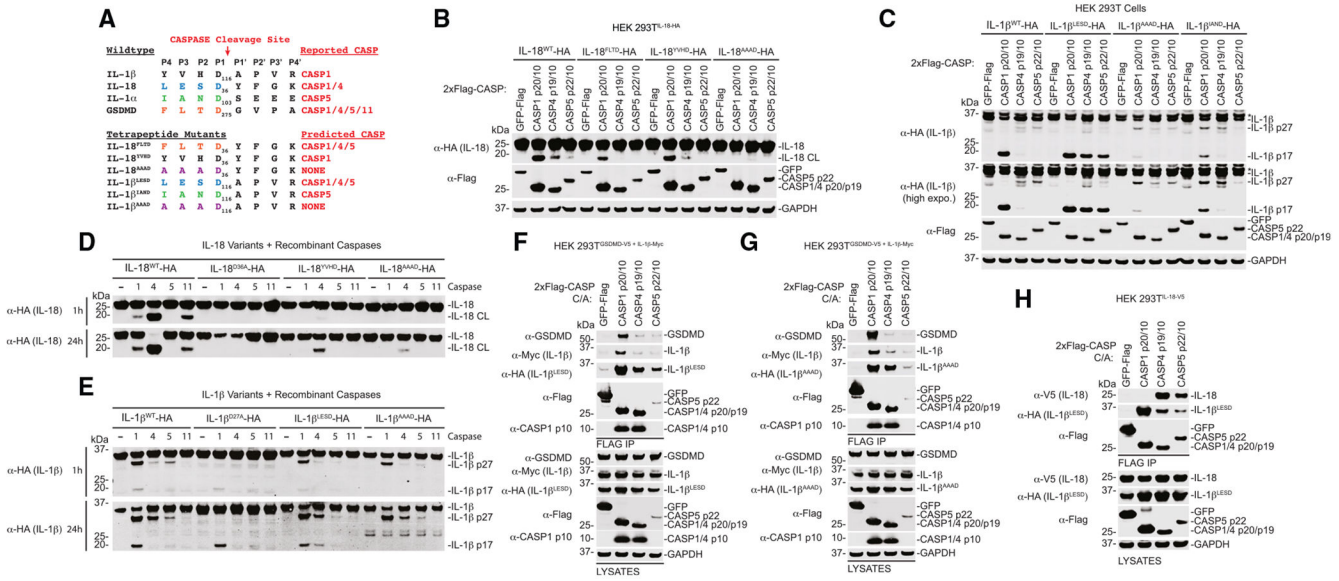

Figure 3. The P4–P1 tetrapeptide sequence of IL-18 and IL-1β regulates their recruitment and processing by inflammatory caspases

(A) Schematic depicting the sequence of wild-type IL-1β, IL-18, IL-1α, the caspases that were reported to cleave these substrates, and the tetrapeptide mutants.

(B and C) HEK293T cells expressing IL-18-HA were transiently co-transfected with the indicated active caspase and IL-18 tetrapeptide mutants (B), or HEK cells were co-transfected with the indicated caspases and IL-1β tetrapeptide mutants (C) for 24 h prior to immunoblot analysis.

(D–H) The indicated IL-18 (D) or IL-1β (E) constructs were purified from HEK293T cells and incubated with one activity unit (for IL-18) or three activity units (for IL-1β) of the indicated recombinant caspases for 1 h or 24 h prior to immunoblot analysis. HEK293T cells stably expressing GSDMD-V5 and IL-1β-Myc (F and G) or IL-18-V5 (H) were transiently co-transfected with the indicated catalytically inactive (C/A) caspase constructs and the IL-1β tetrapeptide mutants, IL-1β^{LESD} (F and H) or IL-1β^{AAAD} (E), for 48 h before immunoprecipitation and immunoblot analysis. * = non-specific bands. Data are representative of three or more independent experiments. High expo. = high exposure.

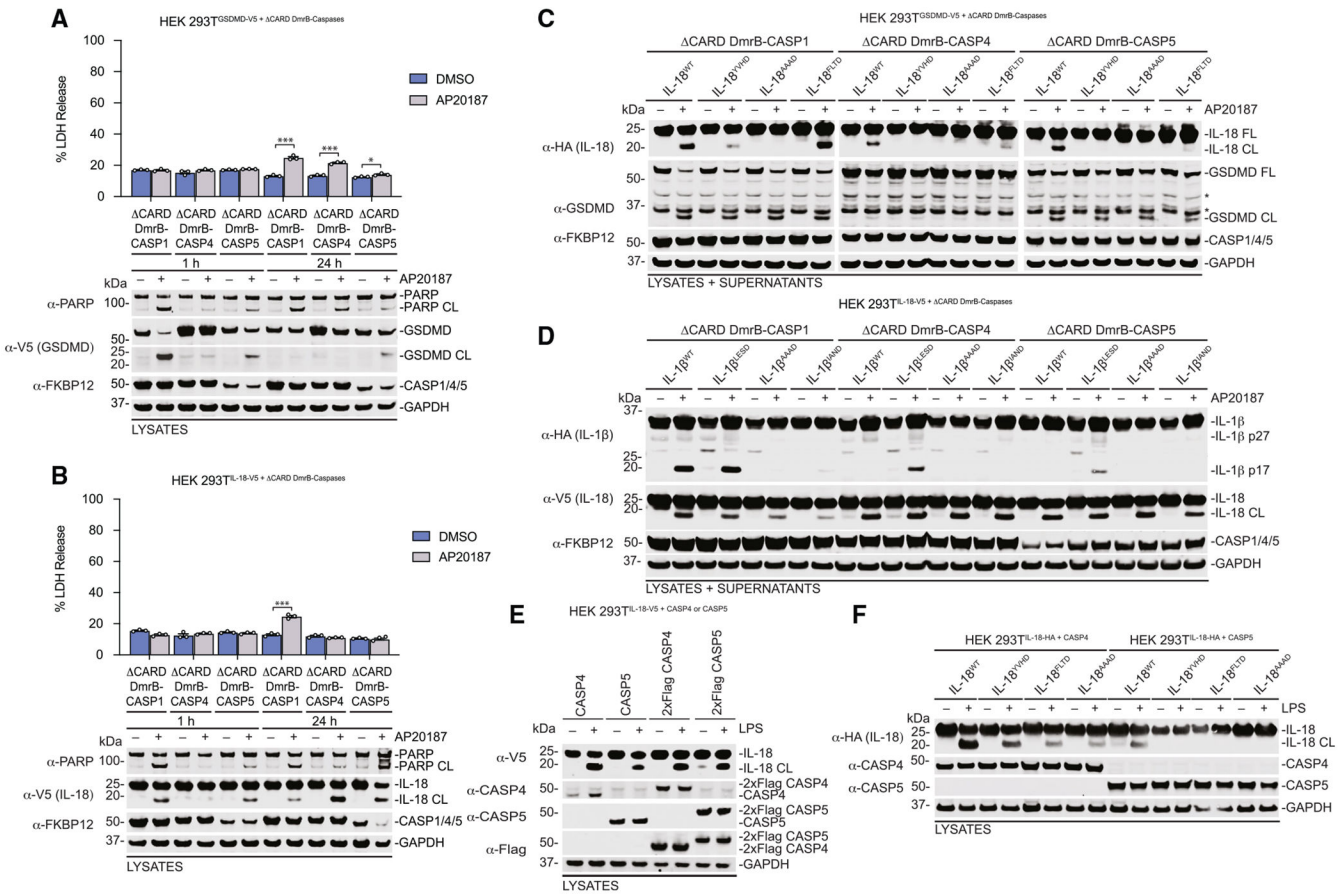

Figure 4. Dimerization and LPS-activated CASP1/4/5 induce processing of inflammatory substrates

(A and B) HEK293T cells stably expressing ΔCARD DmrB-CASP1, -4, or -5 and GSDMD-V5 (A) or IL-18-V5 (B) were treated with AP20187 (1 μM) for 1 h or 24 h before LDH and immunoblot analysis.

(C and D) HEK293T cells stably expressing ΔCARD DmrB-CASP1, -4, or -5 and GSDMD-V5 (C) or ΔCARD DmrB-CASP1, -4, or -5 and IL18-V5 (D) were transiently transfected with the indicated IL-18 (C) or IL-1β (D) constructs for 24 h before the addition of 1 μM AP20187 for 3 h (C) or 24 h (D). Lysates and supernatants were pooled and analyzed by immunoblotting.

(E) HEK293T cells stably expressing IL-18-V5 and either CASP4 or CASP5 were transfected with LPS (25 μg/mL) for 24 h before samples were analyzed by immunoblotting.

(F) HEK293T cells stably expressing the indicated IL-18 variants and either CASP4 or CASP5 were transfected with LPS (25 μg/mL) for 24 h and then analyzed by immunoblotting. Data are means ± SEM of three biological replicates. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by two-sided Student's t test. Data are representative of three or more independent experiments.

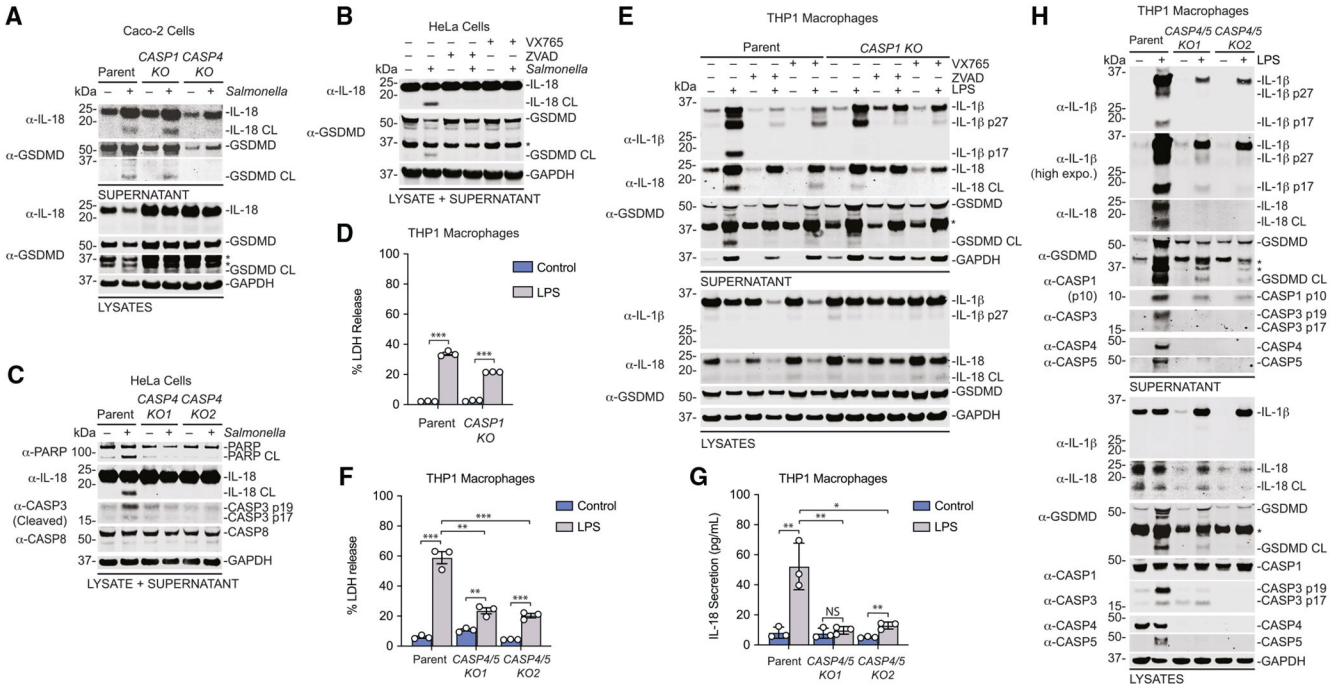

Figure 5. Cytosolic LPS and pathogenic infections induce non-canonical inflammasome-mediated processing of inflammatory substrates in human macrophages and epithelial cells

(A) Caco-2 cells were primed with 100 ng/mL Pam3CSK4 for 3 h and then infected with *Salmonella* Typhimurium (multiplicity of infection [MOI] = 60) for 6 h. Cells and their supernatants were collected separately and analyzed by immunoblotting.

(B and C) Parent (B) and *CASP4 KO* (C) HeLa cells were infected with *Salmonella* Typhimurium (MOI = 60) for 6 h. Cell lysates and supernatants were combined and analyzed by immunoblotting.

(D and E) THP1 cells were differentiated into macrophages with phorbol 12-myristate 12-acetate (PMA) (50 ng/mL) for 24 h and primed with LPS (5 μg/mL) for another 24 h. Where indicated, THP1 macrophages were treated with ZVAD (40 μM) or VX765 (40 μM) 30 min before LPS transfections. Cells were transfected with LPS (25 μg/mL) for 24 h, and then samples were analyzed for LDH release (D) and immunoblotting (E).

(F–H) THP1 cells were differentiated into macrophages with PMA (50 ng/mL) for 48 h and then transfected with LPS (25 μg/mL) for 24 h. Samples were then analyzed for LDH release (F), IL-18 secretion (G), and immunoblotting (H). Data are means ± SEM of three biological replicates. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by two-sided Student's t test. * = non-specific bands. Data are representative of three or more independent experiments.

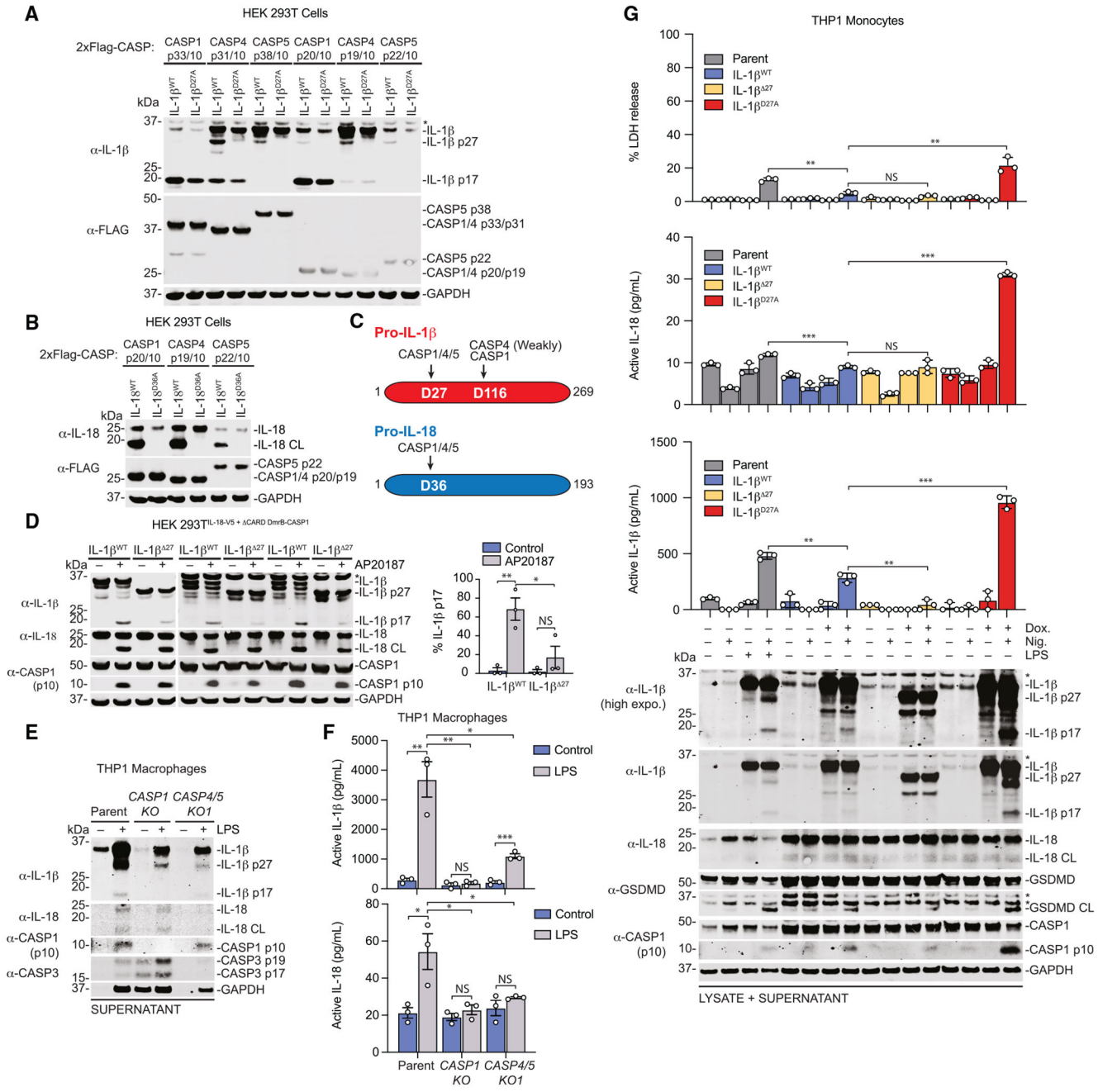

Figure 6. CASP4/5 preferentially cleave IL-1β at D27 to deactivate IL-1R signaling and IL-18 at D36 to activate cytokine signaling

(A and B) HEK293T cells were transiently co-transfected with the indicated constructs for 24 h and then analyzed by immunoblotting.

(C) Schematic of inflammatory substrates and representative cleavage sites by inflammatory caspases.

(D) HEK293T cells stably expressing IL-18-V5 and ΔCARD DmrB-CASP1 were transiently transfected with IL-1β^{WT} or IL-1β$^{\Delta 27}$ for 24 h before the addition of AP20187 (1 μM) for 1 h. Three independent experiments were then analyzed by immunoblotting, and the amount

of cleaved IL-1β p17 was quantified by taking the ratio of the intensity of the cleaved IL-1β p17 to uncleaved IL-1β^{WT} or IL-1$\beta^{\Delta 27}$ respectively.

(E and F) THP1 cells were differentiated into macrophages with PMA (50 ng/mL) for 48 h. Cells were then transfected with LPS (25 μg/mL) for 24 h, and then supernatants were analyzed by immunoblotting (E), or the amount of active cytokines was quantified by measuring IL-1R stimulation (F).

(G) Parent THP1 cells were primed with LPS (5 μg/mL, 4 h, parent cells only) and then treated with nigericin (Nig., 5 μM) for 1 h before supernatants were analyzed for LDH release and IL-1 receptor activation by IL-18 and IL-1β. THP1 monocytes stably expressing dox-inducible IL-1β^{WT}, IL-1$\beta^{\Delta 27}$, or IL-1β^{D27A} were unprimed and were treated with doxycycline (Dox., 10 μg/mL, 24 h) and then treated with nigericin (Nig., 5 μM) for 1 h before analysis. Lysates and supernatants were then combined and analyzed by immunoblotting. Data are means ± SEM of three biological replicates. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by two-sided Student's t test. Data are representative of three or more independent experiments. * = non-specific bands.

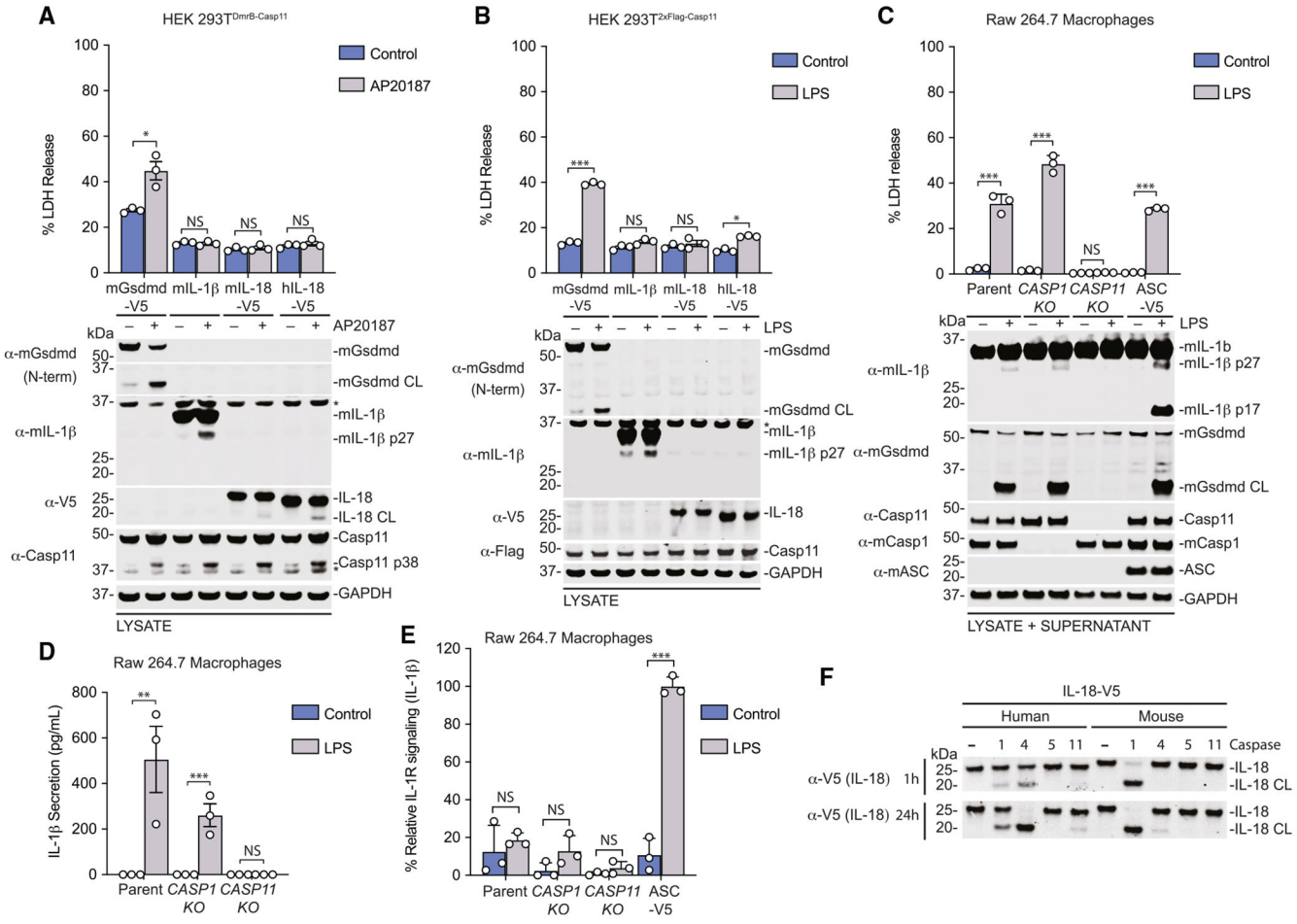

Figure 7. Caspase-11 does not cleave IL-18 but cleaves IL-1β to generate the p27 product

(A and B) HEK293T cells stably expressing ΔCARD Dmrb-CASP11 (A) or 2xFLAG-CASP11 (B) were transiently transfected with the indicated constructs. After 24 h, samples were treated with AP20187 (1 μM) (A) or LPS (25 μg/mL) (B) for 24 h and then analyzed for LDH release and immunoblotting.

(C and E) Raw 264.7 macrophages were primed with LPS (5 μg/mL) for 4 h and then transfected with LPS (25 μg/mL) for 6 h before LDH release and immunoblot analysis (C) and IL-1 receptor activation by IL-1β (E).

(D) Raw 264.7 macrophages were treated as in (C) and then analyzed for IL-1β release by ELISA.

(F) HEK293T cell lysates expressing either human IL-18 or mouse IL-18 were incubated with one activity unit of the indicated recombinant caspases for 1 h or 24 h and then analyzed by immunoblotting. Data are means ± SEM of three biological replicates. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ by two-sided Student's t test. Data are representative of three or more independent experiments. * = non-specific bands. The small m or h represents mouse or human proteins, respectively.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GSDMD Rabbit polyclonal Ab	Novus Biologicals	Cat#NBP2-33422; RRID:AB_2687913
FLAG® M2 Mouse monoclonal Ab	Sigma	Cat# F3165; RRID:AB_259529
GAPDH Rabbit monoclonal Ab	Cell Signaling Tech	Cat#2118S; RRID:AB_561053
CASP1 p20 Rabbit polyclonal Ab	Cell Signaling Tech	Cat#2225s; RRID:AB_2243894
CASP1 p12/10 Rabbit monoclonal Ab	Abcam	Cat#ab179515; RRID:AB_2884954
CASP4 Rabbit polyclonal Ab	Cell Signaling Tech	Cat#4450S; RRID:AB_1950386
CASP5 Rabbit monoclonal Ab	Cell Signaling Tech	Cat#46680S; RRID:AB_2799306
Myc Mouse monoclonal Ab	Cell Signaling Tech	Cat#2276S; RRID:AB_331783
V5 Rabbit monoclonal Ab	Cell Signaling Tech	Cat#13202S; RRID:AB_2687461
hIL-1β Goat Polyclonal Ab	R&D systems	Cat# AF-201-NA; RRID:AB_354387
hIL-18 Goat Polyclonal Ab	R&D systems	Cat# AF2548; RRID:AB_562603
hIL-1α Rabbit Recombinant Ab	PeproTech	Cat#200-01A; “NA”
PARP Rabbit polyclonal Ab	Cell Signaling Tech	Cat#9542S; RRID:AB_2160739
HA Rabbit monoclonal Ab	Cell Signaling Tech	Cat#3724S; RRID:AB_1549585
mIL-1β Goat polyclonal Ab	R&D systems	Cat#AF-401-NA; RRID:AB_416684
mGSDMD Rabbit monoclonal Ab	Abcam	Cat# ab209845; RRID:AB_2783550
mCASP1 Mouse monoclonal Ab	AdipoGen	Cat# AG-20B-0042-C100; RRID:AB_2755041
mIL-18 Rabbit Recombinant Ab	Abcam	Cat# ab207323; RRID:AB_2895063
CASP11 Rat monoclonal Ab	Novus Biologicals	Cat#NB120-10454; RRID:AB_2259600
FKBP12 Rabbit polyclonal Ab	Abcam	Cat#ab24373; RRID:AB_732383
Cleaved Caspase-3 (Asp175) (5A1E) Rabbit monoclonal Ab	Cell Signaling Tech	Cat# 9664; RRID:AB_2070042
Caspase 8 Rabbit monoclonal Ab	Abcam	Cat# ab108333; RRID:AB_10866391
Caspase-8 (1C12) Mouse monoclonal Ab	Cell Signaling Tech	Cat# 9746; RRID:AB_2275120
Caspase-3 Rabbit Polyclonal Ab	Cell Signaling Tech	Cat# 9662; RRID:AB_331439
Caspase-8 (D35G2) Rabbit monoclonal Ab	Cell Signaling Tech	Cat# 4790; RRID:AB_10545768
IRDye 800CW anti-rabbit	LI-COR	Cat#926–32213; RRID:AB_621848
IRDye 800CW anti-mouse	LI-COR	Cat#926–32212; RRID:AB_621847
IRDye 800CW anti-goat	LI-COR	Cat#926–32214; RRID:AB_621846
IRDye 800CW anti-rat	LI-COR	Cat#925–32219; RRID:AB_2721932
IRDye 680CW anti-rabbit	LI-COR	Cat#926–68073; RRID:AB_10954442
IRDye 680CW anti-mouse	LI-COR	Cat#926–68072; RRID:AB_10953628
IRDye 680CW anti-goat	LI-COR	Cat#926–68074; RRID:AB_10956736
IRDye 680CW anti-rat	LI-COR	Cat#926–68076; RRID:AB_10956590
Bacterial and virus strains		
Salmonella Typhimurium (SL1344)	Sunny Shin	*NA*
Legionella pneumophila	Sunny Shin	*NA*

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Tocris™ AP 20187	Fisher Scientific	Cat#62–975
LPS-EB Ultrapure	Invivogen	Cat#tlrl-3pelps
VX-765	Apexbio Technology LLC	Cat#50-P101-3604
Z-VAD-FMK	Enzo Life Sciences	Cat#NC9471015
FuGENE HD	Promega	Cat#2311
Pam3CSK4	Invivogen	Cat#tlrl-pms
phorbol 12-myristate 12-acetate (PMA)	Promega	Cat#V1171
Recombinant Human TRAIL	R&D Systems	Cat#375-TL/CF
Staurosporine	MedChemExpress	Cat#HY-15141
Etoposide	Enzo Life Sciences	Cat#BMC-GR307
Nigericin (sodium salt)	Cayman Chemical	Cat#11437
Doxycycline hydrochloride	Fisher Scientific	Cat#ICN19504401
Caspase-1 (human), (recombinant)	Enzo Life Sciences	Cat#BML-SE168-5000
Caspase-4 (human), (recombinant)	Enzo Life Sciences	Cat#BML-SE176-5000
Caspase-5 (human), (recombinant)	Enzo Life Sciences	Cat#BML-SE171-5000
Caspase-11 (mouse), (recombinant)	Enzo Life Sciences	Cat# BML-SE155-5000
Critical commercial assays		
Cyquant LDH Cytotoxicity Assay	Thermo Scientific	Cat#C20300
DC Protein Assay kit	Bio-Rad	Cat#5000111
HEK-Blue™ IL-18	Invivogen	Cat#hkb-hmil18
HEK-Blue™ IL-1β	Invivogen	Cat#hkb-il1bv2
Human Total IL-18/IL-1F4 Quantikine ELISA Kit	R&D Systems	Cat#DL180
Mouse IL-1 beta/IL-1F2 Quantikine ELISA Kit	R&D Systems	Cat#MLB00C
Experimental models: Cell lines		
HeLa	ATCC	RRID:CVCL_0030
HeLa CASP4 KO	This Study	N/A
THP1	ATCC	RRID:CVCL_0006
THP1	Clare Bryant[56]	N/A
THP1 CASP4/CASP5 KO	Clare Bryant[56]	N/A
THP1 CASP1 KO	Taabazuing et al. 2017[54]	N/A
THP1 pCW57.1 IL-1β	This Study	N/A
THP1 pCW57.1 IL-1β$^{\Delta 27}$	This Study	N/A
THP1 pCW57.1 IL-1β^{D27A}	This Study	N/A
Caco-2	Naseer et al. 2022[20]	N/A
Caco-2 CASP1 KO	Naseer et al. 2022[20]	N/A
Caco-2 CASP4 KO	Naseer et al. 2022[20]	N/A
Raw 264.7 Cas9	ATCC	RRID:CVCL_0493
Raw 264.7 CASP1 KO	Taabazuing et al. 2017[54]	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Raw 264.7 CASP11 KO	Daniel Bachovchin	N/A
Raw 264.7 ASC	Daniel Bachovchin	N/A
HEK 293T	ATCC	RRID:CVCL_0063
HEK293T CASP3 KO	This Study	N/A
HEK293T GSDMD/IL-1β	This Study	N/A
HEK293T IL-18	This Study	N/A
HEK293T GSDMD/DmrB_ΔCARD-CASP1	This Study	N/A
HEK293T GSDMD/DmrB_ΔCARD-CASP4	This Study	N/A
HEK293T GSDMD/DmrB_ΔCARD-CASP5	This Study	N/A
HEK293T IL-18/DmrB_ΔCARD-CASP1	This Study	N/A
HEK293T IL-18/DmrB_ΔCARD-CASP4	This Study	N/A
HEK293T IL-18/DmrB_ΔCARD-CASP5	This Study	N/A
HEK293T DmrB_ΔCARD-CASP11	This Study	N/A
HEK293T IL-18/CASP4	This Study	N/A
HEK293T IL-18/CASP5	This Study	N/A
HEK293T IL-18/2xFLAG-CASP4	This Study	N/A
HEK293T IL-18/2xFLAG-CASP5	This Study	N/A
HEK293T 2xFLAG-CASP11	This Study	N/A
HEK293T IL-18^{AAAD}	This Study	N/A
HEK293T IL-18^{YVHD}	This Study	N/A
HEK293T IL-18^{FLTD}	This Study	N/A
HEK293T IL-18^{AAAD}/CASP4	This Study	N/A
HEK293T IL-18^{YVHD}/CASP4	This Study	N/A
HEK293T IL-18^{FLTD}/CASP4	This Study	N/A
HEK293T IL-18^{AAAD}/CASP5	This Study	N/A
HEK293T IL-18^{YVHD}/CASP5	This Study	N/A
HEK293T IL-18^{FLTD}/CASP5	This Study	N/A
Oligonucleotides		
IL-1β D27A F:5′-ggatgacttgttctttgaagctgccggccctaaacagatgaagtgc-3’	This Study	N/A
IL-1β D27A R: 5′-gcacttcatctgtttagggccggcagcttcaaagaacaagtcatcc-3’	This Study	N/A
IL-18 D36A F: 5′-gatgatgaaaacctggaatcaGCCtactttggcaagcttgaatc-3’	This Study	N/A
IL-18 D36A R: 5′-gattcaagcttgccaaagtaGGCtgattccaggttttcatcatc-3’	This Study	N/A
IL-1β YVHD to AAAD F: 5′-cttcttcgacacatgggataacgaggctgctgcggccgatgcacctgtacg-3’	This Study	N/A
IL-1β YVHD to AAAD R: 5′-cgtacaggtgcatcggccgcagcagcctcgttatcccatgtgtcgaagaag-3’	This Study	N/A
IL-1β YVHD to LESD F: 5′cgacacatgggataacgaggctttagagagcgatgcacctgtacgatcac-3’	This Study	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IL-1β YVHD to LESD R: 5′-gtgatcgtacaggtgcatcgctctctaaagcctcgttatcccatgtgtcg-3’	This Study	N/A
IL-1β YVHD to IAND F: 5′-gtacaggtgcatcgttcgcaatagcctcgttatcccatgtgtcgaagaaga-3’	This Study	N/A
IL-1β YVHD to IAND R: 5′-tcttcttcgacacatgggataacgaggctattgcgaacgatgcacctgtac-3’	This Study	N/A
hCASP1 C285A F: 5′-ggctgtcaccacgggcggcctggatgatga-3’	This Study	N/A
hCASP1 C285A R: 5′-tcatcatccaggccgcccgtggtgacagcc-3’	This Study	N/A
hCASP1 D103N F: 5′-aacatctggaaattaccttaatatgcaaaactctcaaggagtacttt-3’	This Study	N/A
hCASP1 D103N R: 5′-aaagtactccttgagagttttgcatattaaggtaatttccagatgtt-3’	This Study	N/A
hCASP1 D119N F: 5′-cctcaggcagtgcagaacaacccagctatgc-3’	This Study	N/A
hCASP1 D119N R: 5′-gcatagctgggttgttctgcactgcctgagg-3’	This Study	N/A
hCASP4 C258A F: 5′-catcattgtccaggccgccagaggtgcaaaccgt-3’	This Study	N/A
hCASP4 C258A R: 5′-acggtttgcacctctggcggcctggacaatgatg-3’	This Study	N/A
hCASP4 D59N F: 5′-ttctcttgcatagagtttgccatgacccgaactttg-3’	This Study	N/A
hCASP4 D59N R: 5′-caaagttcgggtcatggcaaactctatgcaagagaa-3’	This Study	N/A
hCASP4 D80N F: 5′-acaaatgcttcttcaaacctttttaacataaaccaaatatcccccaa-3’	This Study	N/A
hCASP4 D80N R: 5′-ttgggggatatttggtttatgttaaaaaaggtttgaagaagcatttgt-3’	This Study	N/A
hCASP4 D104N F: 5′-gagtcaggagaatctacaaatgccctcaagctttgt-3’	This Study	N/A
hCASP4 D104N R: 5′-acaaagcttgagggcatttgtagattctcctgactc-3’	This Study	N/A
hCASP5 C315A F: 5′-gtcatcattgtccaggccgccagaggtgaaaaacatgg-3’	This Study	N/A
hCASP5 C315A R: 5′-ccatgtttttcacctctggcggcctggacaatgatgac-3’	This Study	N/A
hCASP5 D137N F: 5′-cccaaacacttctcaatatgaaccaaaagatcaccagtgta-3’	This Study	N/A
hCASP5 D137N R: 5′-tacactggtgatcttttggttcatattgagaagtgtttggg-3’	This Study	N/A
hCASP3F_CR1: CACCGGGAGAACACTGAAAACTCAG	This Study	N/A
hCASP3R_CR1: AAACCTGAGTTTTCAGTGTTCTCCC	This Study	N/A
hCASP4F_CR1 (HeLa KO1, 2): CACCGTATGCAAGAGAAGCAACGTA	This Study	N/A
hCASP4R_CR1(HeLa KO1, 2): AAACTACGTTGCTTCTCTTGCATAC	This Study	N/A
IL-18 LESD to AAAD F: 5′-gctgaagatgatgaaaacgctgcggccgattactttggcaagcttgaatc-3’	This Study	N/A
IL-18 LESD to AAAD R: 5′-gattcaagcttgccaaagtaatcggccgcagcgttttcatcatcttcagc-3’	This Study	N/A
IL-18 LESD to YVHD F: 5′gctgaagatgatgaaaactatgtgcacgattactttggcaagcttgaatc-3’	This Study	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IL-18 LESD to YVHD R: 5′-gattcaagcttgccaaagtaatcgtgcacatagttttcatcatcttcagc-3’	This Study	N/A
IL-18 LESD to FLTD F: 5′-gctgaagatgatgaaaacttcctgacagattactttggcaagcttgaatc-3’	This Study	N/A
IL-18 LESD to FLTD R: 5′-gattcaagcttgccaaagtaatctgtcaggaagttttcatcatcttcagc-3’	This Study	N/A
Recombinant DNA		
pLEX_307_HA	Daniel Bachovchin	N/A
2xFlag-N-pLEX_307_Myc	This Study	N/A
DmrB-N-pLEX_307_Flag	This Study	N/A
pLEX_307 GFP_Flag	This Study	N/A
pLEX_307 2XFlag_CASP1 p45 C285A	This Study	N/A
pLEX_307 2XFlag_CASP1 p33/p10	This Study	N/A
pLEX_307 2XFlag_CASP1 p33/p10 C285A	This Study	N/A
pLEX_307 2XFlag_CASP1 p20/p10	This Study	N/A
pLEX_307 2XFlag_CASP1 p20/p10 C285A	This Study	N/A
pLEX_307 DmrB_ΔCARD-M92 CASP1	This Study	N/A
pLEX_307 2XFlag_CASP4 p43 C258A	This Study	N/A
pLEX_307 2XFlag_CASP4 p31/p10	This Study	N/A
pLEX_307 2XFlag_CASP4 p31/p10 C258A	This Study	N/A
pLEX_307 2XFlag_CASP4 p19/p10	This Study	N/A
pLEX_307 2XFlag_CASP4 p19/p10 C258A	This Study	N/A
pLEX_307 2XFlag_CASP4	This Study	N/A
pLEX_307 CASP4	This Study	N/A
pLEX_307 DmrB_ΔCARD-M67 CASP4	This Study	N/A
pLEX_307 2XFlag_CASP5 p50 C315A	This Study	N/A
pLEX_307 2XFlag_CASP5 p38/p10	This Study	N/A
pLEX_307 2XFlag_CASP5 p38/p10 C315A	This Study	N/A
pLEX_307 2XFlag_CASP5 p22/p10	This Study	N/A
pLEX_307 2XFlag_CASP5 p22/p10 C315A	This Study	N/A
pLEX_307 2XFlag_CASP5	This Study	N/A
pLEX_307 CASP5	This Study	N/A
pLEX_307 DmrB_ΔCARD-M128 CASP5	This Study	N/A
pLEX_307 2XFlag_CASP11	This Study	N/A
pLEX_307 DmrB_ΔCARD-M71 CASP11	This Study	N/A
pLEX_307 GSDMD_V5	This Study	N/A
pLEX_304 IL-18_V5	This Study	N/A
pLEX_307 IL-1β_Myc	This Study	N/A
pLEX_307 IL-1α_Myc	This Study	N/A
pLEX_307 IL-1β_HA	This Study	N/A
pLEX_307 IL-1β^{LESD}_HA	This Study	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pLEX_307 IL-1β^{AAAD}_HA	This Study	N/A
pLEX_307 IL-1β^{IAND}_HA	This Study	N/A
pLEX_307 DmrB_ΔCARD-M128 CASP5	This Study	N/A
pLEX_307 DmrB_ΔCARD-M71 CASP11	This Study	N/A
pLEX_307 mGSDMD_V5	This Study	N/A
pLEX_304 mIL-1β_V5	This Study	N/A
pLEX_304 mIL-18_V5	This Study	N/A
pLEX_307 IL-1β^{D27A}_HA	This Study	N/A
pLEX_307 IL-18^{D36A}_HA	This Study	N/A
pLEX_307 IL-1β^{Δ27}_HA	This Study	N/A
pCW57.1 IL-1β	This Study	N/A
pCW57.1 IL-1β^{Δ27}	This Study	N/A
pCW57.1 IL-1β^{D27A}	This Study	N/A
pLEX_307 IL-18_HA	This Study	N/A
pLEX_307 IL-18^{YVHD}_HA	This Study	N/A
pLEX_307 IL-18^{AAAD}_HA	This Study	N/A
pLEX_307 IL-18^{FLTD}_HA	This Study	N/A
Software and algorithms		
GraphPad Prism Version 9	GraphPad Software	GraphPad Prism (RRID:SCR_002798)
Empiria Studio	Li-Cor Inc.	Empiria Studio (RRID:SCR_022512)