# The tetrapeptide sequence of IL-1β regulates its recruitment and activation by inflammatory caspases

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1   **Abstract**

2   The mammalian innate immune system uses germline-encoded cytosolic pattern-recognition

3   receptors (PRRs) to detect intracellular danger signals. At least six of these PRRs are known to

4   form multiprotein complexes called inflammasomes which activate cysteine proteases known as

5   caspases.  Canonical inflammasomes recruit and activate caspase-1 (CASP1), which in turn

6   cleaves and activates inflammatory cytokines such as IL-1β and IL-18, as well as the pore

7   forming protein, gasdermin D (GSDMD), to induce pyroptotic cell death. In contrast, non-

8   canonical inflammasomes,  caspases-4/-5 (CASP4/5) in humans and caspase-11 (CASP11) in

9   mice, are activated by intracellular LPS to cleave GSDMD, but their role in direct processing of

10  inflammatory cytokines has not been established. Here we show that active CASP4/5 directly

11  cleave IL-18 to generate the active species. Surprisingly, we also discovered that CASP4/5/11

12  cleave IL-1β at D27 to generate a 27 kDa fragment that is predicted to be inactive and cannot

13  signal to the IL-1 receptor. Mechanistically, we discovered that the sequence identity of the P4-

14  P1 tetrapeptide sequence adjacent to the caspase cleavage site (D116) regulates the

15  recruitment and processing of IL-1β by inflammatory caspases to generate the bioactive

16  species. Thus, we have identified new substrates of the non-canonical inflammasomes and

17  reveal key mechanistic details regulating inflammation.

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20  **Keywords:** non-canonical inflammasomes, caspase-1, caspase-4, caspase-5, IL-1β, IL-18,

21  cytokines

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26   **Introduction**

27   The mammalian innate immune system uses germline-encoded pattern recognition receptors

28   (PRRs) to detect pathogen-associated molecular patterns (PAMPs) or damage-associated

29   molecular patterns (DAMPs)[1,2]. Upon detecting PAMPS or DAMPs, some of these PRRs rapidly

30   assemble into multi-protein complexes termed inflammasomes[1,2]. Canonical inflammasome

31   assembly typically involves recruitment of the adapter protein ASC and then pro-caspase-1

32   (pro-CASP1) to the PRR, which leads to oligomerization and  auto-proteolytic maturation of pro-

33   CASP1  into the active species. Active CASP1 then processes the inflammatory cytokines IL-1β

34   and IL-18 into their bioactive forms, as well as the pore forming protein, gasdermin D

35   (GSDMD)[3–5]. GSDMD processing liberates the N-terminus from the inhibitory C-terminal

36   fragment, allowing the N-terminus to oligomerize and form pores in the plasma membrane to

37   facilitate the release of cytokines and induce pyroptotic cell death[6–8].

38   During canonical inflammasome activation, pro-CASP1 undergoes auto-proteolysis at

39   multiple sites to generate distinct species (**Fig. 1A**)[9–12]. It is thought that IDL processing confers

40   full protease activity to the inflammatory caspases[9,12–14]. In support of this idea, recent studies

41   suggest that the CASP1 p33/10 species is the active species in cells and the p20/10 rapidly

42   loses protease activity[9]. However, ASC, which facilitates full autoproteolysis of pro-CASP1 into

43   the p20/10 species, is required for IL-1β processing for reasons that are not clear[10,11]. Current

44   structural studies also suggest that the CASP1 p20/10 is the species that binds GSDMD[15,16]. Of

45   note, CASP1 p20/10 was demonstrated to bind GSDMD through exosite interactions, but how

46   CASP1 recognizes other substrates such as IL-1β and IL-18 remains an open question.

47   In contrast to the canonical inflammasome pathway, non-canonical inflammasome

48   activation involves direct detection of cytosolic lipopolysaccharide (LPS) from gram-negative

49   bacteria by caspases-4/5 (CASP4/5) in humans and caspase-11 (CASP11) in mice[17–20]. Binding

50   of LPS induces oligomerization and autoproteolytic maturation of the non-canonical

51    inflammasomes (CASP4/5/11) to generate the active proteases[13,14,21]. Active CASP4/5/11 then

52    cleave GSDMD to induce pyroptotic cell death. Formation of GSDMD pores during non-

53    canonical inflammasome activation results in $K^+$ efflux, which activates CASP1 to induce IL-1β

54    and IL-18 processing via the canonical NLRP3 inflammasome [22,23]. Interestingly, both CASP4

55    and CASP11 have been implicated in IL-18 processing, but as the canonical pathway is

56    activated downstream, whether this is a direct processing event in cells or if CASP5, which is

57    thought to be functionally similar to CASP4, can analogously process IL-18 remains to be

58    defined[24–27]. Furthermore, like CASP1, autoproteolyzed CASP4/11 were also reported to utilized

59    exosite mediated interactions for GSDMD binding, but if and how the non-canonical

60    inflammasomes interact with other putative substrates such as IL-18 is unknown. The

61    contributions of the different inflammatory caspase species and mechanisms employed for

62    substrate recognition and processing need to be clearly defined as this has important

63    ramifications for innate immune regulation.

64         Here, we used a combination of biochemistry and chemical biology to uncover the

65    substrates cleaved by specific caspase species and to test the role of non-canonical

66    inflammasomes in cytokine maturation. We discovered that distinct inflammatory caspase

67    species interact with specific substrates with varying affinities, which likely regulates substrate

68    processing. Interestingly, we discovered that both CASP4/5 cleave IL-18 directly, but CASP11 is

69    unable to cleave IL-18. Surprisingly, we also discovered that CASP4/5/11 cleave IL-1β to

70    generate a 27 kDa fragment that was previously reported to abrogate signaling to the IL-1

71    receptor[28]. Importantly, we demonstrate that the inflammatory caspases recognize the P4–P1

72    tetrapeptide sequence adjacent to the IL-1β processing site (D116) to facilitate binding and

73    processing. Altogether, we identified new direct substrates of the non-canonical inflammasome

74    pathway and uncovered the molecular regulation governing cytokine processing by the

75    inflammatory caspases.

76   **RESULTS**

77   **The p20/10 species of human inflammatory caspases are the species that bind strongly**

78   **to inflammatory substrates.**

79   The inflammatory caspases are comprised of a CARD domain, followed by a 20 kDa large

80   catalytic subunit (LS), and a 10 kDa small subunit (SS) (**Fig. 1A**). During activation,

81   autoproteolytic maturation first occurs at the interdomain linker (IDL) which joins the LS and SS

82   to generate the active p33/10 species for CASP1, p31/10 species for CASP4, and a p38/10

83   species for CASP5 (**Fig. 1A**)[9,12]. Because IDL cleavage generates a CARD-LS that is ~35 kDa

84   when averaged, we will henceforth refer to the p35/10 when referencing them collectively for

85   conceptual ease. Next, autoproteolysis at the CARD-domain linker (CDL), which joins the

86   CARD-domain and LS, generates the fully matured p20/10 species for CASP1, p19/10 species

87   for CASP4, and p22/10 species for CASP5 (**Fig. 1A**). We will hereafter refer to them as the

88   p20/10 species when discussing them collectively. As these distinct species may have different

89   substrate specificities, we first wanted to determine at what point during activation does the

90   inflammasome interact with specific substrates. Expression of the large and small subunits of

91   inflammatory caspases as separate polypeptides results in formation of the active species[16]. We

92   first generated plasmids that allowed us to express the different subunits of CASP1/4/5 as

93   separate polypeptides using a single plasmid. This granted us the ability to express the different

94   active species of each inflammatory caspase in the absence of any stimulating ligand for

95   functional interrogation. We transiently expressed the catalytically inactive (cysteine to alanine

96   mutants) of the different CASP1/4/5 species harboring a 2xFLAG N-terminal tag in HEK 293T

97   cells ectopically expressing GSDMD-V5 and IL-1β-Myc, or HEK 293T cells expressing IL-18-V5

98   (**Fig. 1B-G**). The lysates were collected and subjected to anti-FLAG immunoprecipitation. As

99   expected, the CASP1 p20/p10 species bound GSDMD, IL-β (**Fig. 1B**), and IL-18 (**Fig. 1C**).

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100    GSDMD and IL-1β were bound weakly to CASP1 p33/10 but we could not detect any binding of

101    IL-18 to CASP1 p33/10  (**Fig. 1B,C**).

102         We then probed for binding of the human non-canonical caspases to the inflammatory

103    substrates (GSDMD, IL-1β, and IL-18). We could not detect binding of GSDMD and IL-1β to any

104    of the CASP4 species tested in this assay (**Fig. 1D**). However, we detected binding of IL-18 to

105    CASP4 p19/10 (**Fig. 1E**). During this work, we noticed that the CASP1 p12/10 antibody (Abcam)

106    had significant cross reactivity with the CASP4 p10 subunit. We therefore used this antibody to

107    check the expression of CASP4 p10 as no commercially available antibody exists that detects

108    the p10. Unfortunately, none of the CASP5 antibodies we tried detected the p10 subunit.

109    Notably, CASP4 p10, which was expressed separately, was pulled down by the p19 subunit,

110    suggesting that a functional enzyme complex was formed, but the complex did not interact

111    strongly with  GSDMD and IL-1β. Similarly, IL-18 was bound to CASP5 p22/10 and we detected

112    modest binding of GSDMD, but not IL-1β to CASP5 p22/10 (**Fig. 1F,G**). It is worth noting that no

113    processing of GSDMD, IL-1β, or IL-18 was observed with expression of the catalytically inactive

114    caspase species. Taken together, this data suggests that the auto proteolyzed p20/10 species

115    of CASP4/5 binds strongly to IL-18, but only form weak interactions with GSDMD and IL-1β.

116

117    **CASP4/5 cleave IL-1β and IL-18**

118    We next sought to investigate the functional impact of the distinct caspase species on

119    inflammatory substrate processing. We hypothesized that when the distinct species are present

120    at the same levels in cells, the p20/10 species would be the most active species given that they

121    exhibited the strongest binding to the inflammatory substrates. We thus expressed the

122    catalytically competent p35/10 and p20/10 constructs of CASP1,-4, and -5 as separate

123    polypeptides in the HEK 293T cells stably expressing the inflammatory substrates described

124    above. To prevent processing of the p35 constructs, we made the corresponding CDL mutations

125 for CASP1 (D103A, D119A), CASP4 (D59A, D80A, D104A), and CASP5 (D137A) to generate

126 constitutive p35/10 enzymes. Transient transfection of the p35/10 or p20/10 species in HEK

127 293T cells expressing GSDMD and IL-1β resulted in significant LDH release for all species (**Fig.**

128 **2A**) except for CASP5 p20/10, which was expressed at lower levels (**Fig. 2B**). Notably, the

129 CASP1 and CASP4 p35/10 and p20/10 species were expressed at relatively comparable levels,

130 but we consistently saw less  expression of CASP5 p22/10 species, suggesting CASP5 p22/10

131 may be unstable when expressed. In agreement with the p20/10 species being the species that

132 bind strongly to the inflammatory substrates, we observed slightly higher LDH release for these

133 species compared to the p35/10 species (**Fig. 2A**). Intriguingly, all the active species seemed to

134 induce some apoptosis as evidenced by PARP cleavage, but only the CASP1 p33/10 and

135 p20/10 species robustly processed IL-1β and GSDMD (**Fig. 2B**). Both the CASP4 p31/10 and

136 p19/10 species had some slight GSDMD processing, but we could not detect GSDMD

137 processing for CASP5 (**Fig. 2B**). Surprisingly, CASP4 also cleaved IL-1β to generate the active

138 17 kDa fragment, but this processing event was minor compared to CASP1 (**Fig. 2B**).

139 Unexpectedly, we also observed a 27 kDa cleavage product of IL-1β in CASP4 and CASP5

140 transfected cells (**Fig. 2B**). We note that CASP5 p22/10 was expressed less in this assay, and

141 although we could not detect the p10 subunit using an antibody, we could confirm expression

142 based on functional processing of substrates.

143     We next expressed the caspase species in HEK 293T cells stably expressing IL-18 and

144 as expected for cells lacking GSDMD[29], there was PARP processing, indicating that the

145 caspases induced apoptosis in the absence of GSDMD. We note that some PARP processing

146 also occurred in the GSDMD expressing cells, signifying that both apoptosis and pyroptosis

147 pathways can be engaged if there is enough of the functional enzyme species present. Of note,

148 a recent study identified caspase-7 as a substrate of CASP4, implying there may be crosstalk

149 between the non-canonical inflammasome pathway and the apoptotic pathway[30]. There was

150   LDH release in the GSDMD deficient cells due to secondary necrosis but it was less than the

151   LDH released in GSDMD expressing cells (**Fig. 2C**)[5,29,31]. Remarkably, all active caspase

152   species seemed to process IL-18 equivalently (**Fig. 2D**). To gain more insight into the molecular

153   basis of why CASP4/5 did not cleave IL-1β or GSDMD as efficiently as CASP1 but cleaved IL-

154   18 as efficiently, we compared the binding of these substrates to the p20/10 species, which bind

155   inflammatory substrates strongly. In agreement with our previous data, neither CASP4 p19/10

156   nor CASP5 p22/10 bound GSDMD or IL-1β as strongly as CASP1 p20/10 (**Fig. 2E**). Conversely,

157   more IL-18 was bound to both CASP4 p19/10 and CASP5 p22/10 compared to CASP1 p20/10

158   (**Fig. 2F**), suggesting that this increased binding compared to CASP1 permits processing to the

159   same degree as CASP1. This data implies that CASP4/5 may preferentially cleave IL-18 before

160   other substrates in cells.

161

162   **The P4–P1 tetrapeptide sequence of IL-1β regulates processing by inflammatory**

163   **caspases**

164   Recent *in vitro* work using recombinant CASP4/5 demonstrated that the identity of the residues

165   adjacent to the caspase cleavage site can influence the processing of substrates[32,33]. While

166   some enhancement in the catalytic efficiency of non-canonical inflammasomes for processing

167   IL-18 was achieved by substituting the P1'–P4' region with the sequence of GSDMD, these

168   substitutions in IL-1β had limited effect on catalysis[32]. This raises 3 possibilities that are not

169   mutually exclusive: 1) the P4–P1 (rather than the P1'–P4') region is the major determinant of IL-

170   1β catalysis, 2) the GSDMD sequence is not the optimal sequence for enhancing catalysis, or 3)

171   some other structural features modulate IL-1β recognition and catalysis, such as an exosite.

172   Interestingly, the processing of GSDMD by inflammatory caspases was shown to be sequence

173   independent and instead relied on recognition of an exosite[16]. We wondered if processing of IL-

174   1β was similarly sequence independent. We reasoned that processing of IL-1β at the canonical

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175    site (D116) would be sequence dependent since CASP4/5 engaged and processed IL-1β at an

176    alternative site to generate a 27 kDa (IL-1β p27) fragment, but not the p17 fragment (**Fig. 2B**).

177    To test this, we mutated the P4–P1 tetrapeptide sequence of IL-1β to match the tetrapeptide

178    sequence of known caspase substrates (**Fig. 3A**). For example, CASP1 cleaves wildtype IL-1β

179    (IL-**1β**$^{WT}$) at D116, which harbors a YVHD$_{116}$ sequence. We mutated the YVHD sequence to

180    LESD to match the tetrapeptide sequence of IL-18, to generate IL-1β$^{LESD}$. We mutated YVHD to

181    IAND (IL-1β$^{IAND}$) to match the tetrapeptide sequence of IL-1α, which was reported to be

182    processed by CASP5[34], and generated an IL-1β$^{AAAD}$ mutant which abolished all specificity. All

183    the IL-1β tetrapeptide mutants were HA-tagged at the C-terminus. We transiently co-expressed

184    the IL-1β tetrapeptide mutants with the catalytically active caspase p20/10 constructs in HEK

185    293T cells expressing GSDMD-V5 and IL-1β$^{WT}$-Myc (**Fig. 3B**). As previously observed, only

186    CASP1 p20/10 significantly processed IL-1β into the p17 fragment (**Fig. 3B**). In support of our

187    hypothesis, we observed that IL-1β$^{LESD}$ was processed by CASP1/4/5 into the active p17

188    fragment (**Fig. 3B**). Notably, processing of IL-1β$^{AAAD}$ into the p17 fragment was significantly

189    attenuated for all caspases, including CASP1. CASP1 was able to process IL-1β$^{IAND}$ to generate

190    the p17 fragment, but CASP1 also generated a substantial amount of the alternatively

191    processed p27 fragment, suggesting that processing at the canonical site (D116) was

192    suboptimal (**Fig 3B**). Intriguingly, despite CASP5 being reported to cleave IL-1α, CASP5 p22/10

193    failed to cleave IL-1β$^{IAND}$ to generate the p17 product but rather, generated the p27 product (**Fig.**

194    **3B**). These findings suggested that CASP5 may not cleave IL-1α, and indeed, we did not

195    observe processing of wildtype IL-1α by any of the caspases when activated by overexpression

196    or using an inducible system (described below) (**Supplemental Fig. S1 A,B**).

197         We next wanted to determine the molecular basis governing the processing of IL-1β$^{LESD}$

198    by the inflammatory caspases to generate the bioactive p17 fragment but not the other

199   tetrapeptide mutants. Given that wildtype IL-18 binds CASP4/5 strongly, we hypothesized that

200   IL-1β[LESD] would have increased binding to CASP4/5 compared to IL-1β[WT]. We co-transfected IL-

201   1β[LESD] with the catalytically inactive caspases and performed anti-FLAG immunoprecipitations to

202   assess binding. Consistent with our hypothesis, we did not detect binding of wildtype IL-1β to

203   CASP4/5 (**Fig. 1D,F**) but we detected binding of IL-1β[LESD] to CASP4 p19/10 and CASP5 p22/10

204   (**Fig. 3C**). Taken together, this data suggests that the tetrapeptide motif of IL-1β plays a critical

205   role in regulating its molecular interactions with inflammatory caspases to fine tune

206   inflammation.

207

208   **Caspase activation by dimerization is sufficient to cleave inflammatory substrates**

209   The consensus mechanism for initiator caspase activation involves initial dimerization, which

210   allows the caspase to gain basal activity for itself, leading to IDL autoproteolysis and

211   subsequent maturation[35]. Recently, the DmrB dimerization system has been used to induce the

212   dimerization and activation of caspases [12,13]. Briefly, replacing the CARD domain of the caspase

213   with the DmrB domain enables precise and controlled dimerization and activation in the

214   presence of the small molecule AP20187. To further probe the processing of inflammatory

215   substrates, we generated HEK 293T cells stably expressing either GSDMD-V5 or IL-18-V5

216   along with either ΔCARD DmrB-CASP1,-4, or -5 (hereafter referred to as DmrB-CASP1/4/5).

217   We treated these cells with AP20187 for 1 hour or 24 hours, and in agreement with our previous

218   data, we observed GSDMD (**Fig. 4A**) and IL-18 (**Fig. 4B**) processing. Although there was some

219   GSDMD processing within 1 h, we did not detect LDH release within this timeframe, but LDH

220   release was observed at 24 hours. Of note, DmrB-CASP1 exhibited the highest LDH release in

221   both cell lines (**Fig. 4A,B**) and was the only one that induced apoptosis that progressed to

222   secondary necrosis in cells expressing IL-18 (**Fig. 4B**). In agreement with the LDH release and

223   our prior data, DmrB-CASP1 had more GSDMD processing than DmrB-CASP4 and DmrB-

10

224  CASP5. However, DmrB-CASP5 processed IL-18 nearly to the same degree as DmrB-CASP1

225  within 1 hour, and all caspases processed IL-18 after 24 hours (**Fig. 4B**). We next assessed the

226  processing of IL-1β and the tetrapeptide mutants using the DmrB-caspases. We transiently

227  transfected wildtype or the IL-1β tetrapeptide mutants into HEK 293T cells expressing GSDMD-

228  V5 and DmrB-CASP1,-4, or -5, then treated with AP20187 for 24 hours (**Fig. 4C**). Only Dmrb-

229  CASP1 cleaved IL-1β$^{WT}$ at the canonical site to yield the p17 fragment. However, IL-1β$^{LESD}$ was

230  equivalently processed by DmrB-CASP1/4/5, and none of the caspases cleaved IL-1β$^{AAAD}$ or IL-

231  1β$^{IAND}$ (**Fig. 4C**). This orthogonal method of caspase activation confirms that CASP4/5 do not

232  process GSDMD as efficiently as CASP1, but IL-18 maturation occurs nearly as efficiently with

233  CASP4/5 as with CASP1. Furthermore, these results reaffirm that the P4-P1 tetrapeptide motif

234  of IL-1β regulates processing by inflammatory caspases.

235       CASP11 is considered the mouse  ortholog of human CASP4/5. We wondered if there

236  were differences between the mouse and human non-canonical inflammasomes in their ability

237  to process cytokines. To address this, we generated HEK 293T cells stably expressing ΔCARD

238  DmrB-CASP11 and transiently transfected mouse Gsdmd (mGsdmd), IL-1β (mIL-1β), IL-18

239  (mIL-18), or human IL-18 (hIL-18) into the cells. After 24 h post transfection, DmrB-CASP11

240  was activated with AP20187 for 24 h then samples were analyzed for LDH release and

241  immunoblotting (**Fig. 4D**). As expected, the addition of AP20187 induced pyroptosis in the

242  mGsdmd transfected cells, as evidenced by LDH release and mGsdmd processing into the

243  pyroptosis-inducing p30 fragment (**Fig. 4D**). Notably, DmrB-CASP11 induced mIL-1β

244  processing at an alternative site to yield the deactivated p27 fragment, but not the active p17

245  fragment (**Fig. 4D**). Unlike CASP4/5, DmrB-CASP11 failed to significantly process IL-18 into the

246  bioactive mature species (**Fig. 4D**), suggesting a functional divergence between mouse and

247  human non-canonical inflammasomes.

248

249 **LPS activated non-canonical inflammasomes cleave IL-1β and IL-18**

250 The non-canonical inflammasomes are activated by intracellular LPS[17–20]. We next sought to

251 determine if activation by the native ligand (LPS) would induce cytokine processing. We

252 transiently transfected LPS into HEK 293T cells expressing CASP4/5 or 2xFLAG-CASP4/5 and

253 IL-18. We detected processing of IL-18 into mature IL-18 in LPS-transfected cells (**Fig. 5A**).

254 Moreover, the presence of the N-terminal 2x-FLAG tag did not affect the activity of CASP4/5 as

255 the maturation of IL-18 was comparable to that of the untagged CASP4/5-expressing cells (**Fig.**

256 **5A**).

257 Our results with DmrB-CASP11 indicate that CASP11 cleaves IL-1β but not IL-18. We

258 wanted to determine if LPS-activated CASP11 would similarly cleave IL-1β to generate the p27

259 fragment, but not cleave IL-18. We transiently transfected the inflammatory substrates into HEK

260 293T cells stably expressing 2x-FLAG CASP11 and activated with LPS. Analogous to the

261 DmrB-CASP11 results, LPS activated CASP11 induced pyroptosis, as determined by GSDMD

262 processing and LDH release (**Fig. 5B,C**). Importantly, LPS-activated CASP11 processed mIL-

263 1β to yield the p27 fragment, but not the active p17 fragment, and failed to process IL-18 (**Fig.**

264 **5C**). Altogether, this data implies that the inactivation of IL-1β signaling is conserved between

265 humans and mice, but the activation of IL-18 is not.

266

267 **Non-canonical inflammasome activation induces IL-1β and IL-18 processing in human**

268 **macrophages and epithelial cells.**

269 The non-canonical inflammasomes are expressed in both myeloid and epithelial cells and have

270 a well-documented function in playing an important role in mediating host protection against

271 invading pathogens[17,19,24–26]. While epithelial cells express IL-18, there is limited IL-1β

272 expression even with toll-like receptor (TLR) stimulation[26,36,37]. On the other hand, monocytes

273 and macrophages robustly express IL-1β upon TLR stimulation, which can occur during

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274   infection with gram negative bacteria[36]. To gain insight into the function of non-canonical

275   inflammasomes in different cell types, we sought to determine the activity of CASP4/5 in more

276   physiologically relevant cells. We first differentiated THP1 cells into macrophages, primed with

277   LPS to ensure IL-1β expression, then transfected with LPS to induce endogenous CASP4/5

278   activation. As anticipated, LPS induced significant LDH release in control and *CASP1* knockout

279   (KO) cells, indicating CASP4/5-mediated pyroptosis (**Fig. 6A**). Indeed, LPS-transfected THP1

280   macrophages displayed GSDMD processing in both control and *CASP1* KO cells (**Fig. 6B**). To

281   delineate the contribution of CASP4/5 to IL-1β and IL-18 processing in macrophages, we

282   treated the cells with a broad-spectrum pharmacological inhibitor (ZVAD) that targets all

283   caspases, and an inhibitor that is more specific for CASP1 (VX765)[38,39]. We then transfected

284   LPS to activate CASP4/5 and 24 hours later, separated the supernatants and lysates for

285   analysis by immunoblotting. During pyroptosis, inflammatory substrates are released into the

286   supernatant through the gasdermin pores, so we expected to see the processed substrates in

287   the supernatants. In accord with this, activation of CASP4/5 in control cells led to robust

288   processing of IL-1β into the p27 fragment, which was nearly completely abrogated by ZVAD,

289   and significantly attenuated by VX765 (**Fig. 6B**). NLRP3 is activated downstream of non-

290   canonical inflammasomes, which activates CASP1 to mediate IL-1β processing into the p17

291   fragment[22,23]. Consistent with this, we also observed IL-1β maturation into the bioactive p17

292   fragment in control cells, which was completely abrogated by both inhibitors (**Fig. 6B**).

293       Importantly, we observed processing of IL-1β into the p27 fragment in *CASP1* KO cells,

294   indicating that this processing event results from CASP4/5 activity (**Fig. 6B**). We did not detect

295   the presence of the mature IL-1β p17 fragment in *CASP1* KO cells, suggesting CASP4/5 do not

296   induce significant processing of IL-1β at the canonical site in human macrophages (**Fig. 6B**).

297   Critically, the appearance of the p27 fragment was abrogated in the presence of caspase

298   inhibitors in *CASP1* KO cells (**Fig. 6B**). It should be noted that pharmacological inhibitors of

299    caspases can cross-react with other caspases and  the CASP1 inhibitor VX765 also inhibits

300    CASP4/5. Similar findings were noted for the release of mature IL-18 into the supernatant. We

301    observed CASP1-independent release of mature IL-18, which was abrogated by ZVAD and

302    VX765, signifying that CASP4/5 cleave IL-18 to generate the bioactive species in human

303    macrophages (**Fig. 6B**).

304    We next wanted to assess whether CASP4/5 mediate processing of IL-1β and IL-18 in

305    the context of a natural bacterial infection. We therefore infected THP1 macrophages with

306    *Legionella pneumophila* (hereafter called *Legionella*) and like LPS, *Legionella* infection resulted

307    in increased LDH release in control and *CASP1* KO cells compared to uninfected cells (**Fig.**

308    **6C**). *Legionella* infection induced the processing of IL-1β into both the p27 and p17 fragments in

309    control cells, but only the p27 fragment was generated in *CASP1* KO cells (**Fig. 6D**). We

310    detected a slight increase in IL-18 processing in *Legionella* infected cells. Collectively, our data

311    indicates that CASP4/5 directly process IL-1β and IL-18 in macrophages during bacterial

312    infection.

313    Because prior studies have implicated CASP4/11 in IL-18 processing in epithelial cells,

314    we wanted to determine the role of non-canonical inflammasome activation on IL-18 processing

315    in epithelial cells. We thus infected Caco-2 cells with *Salmonella* Typhimurium*,* which was

316    previously documented to activate CASP4 in Caco-2 cells[25,26]. Like CASP4/5 activation in

317    macrophages, *Salmonella* infection induced GSDMD processing and IL-18 maturation and

318    release into the supernatants (**Fig. 6E**). As previously reported, IL-18 processing was CASP1-

319    independent and CASP4-dependent (**Fig. 6E)**[25,26]. Taken together, our data suggests that IL-18

320    is a direct substrate for the non-canonical inflammasomes. However, in certain cell types that

321    express IL-1β, non-canonical inflammasome activation leads to IL-1β processing at an

322    alternative site to generate a p27 fragment that was previously reported to be functionally

323    inactive[28].

324

**CASP4/5 process IL-1β at D27 and IL-18 at D36**

326    CASP1 is known to process IL-1β at D27 to generate a fragment that is ~27 kDa[40,41]. We sought

327    to identify the alternative processing site that gives rise to IL-1β p27. We postulated that CASP1

328    prefers to cleave IL-1β at D116 to generate IL-1β p17, but CASP4/5 preferentially cleave IL-1β

329    at D27 to generate IL-1β p27. To test this hypothesis, we mutated D27 to alanine and co-

330    transfected IL-1β D27A (IL-1β$^{D27A}$) with the catalytically active species of CASP1/4/5 into HEK

331    293T cells. As anticipated, CASP1 p33/10 and p20/10 processed IL-1β$^{WT}$ and IL-1β$^{D27A}$ to

332    generate IL-1β p17 whereas CASP4 p31/10 and p19/10 primarily processed IL-1β$^{WT}$ to generate

333    the p27, and modestly generated the p17 fragment (**Fig. 7A**). Notably, CASP4 failed to cleave

334    IL-1β$^{D27A}$ to generate IL-1β p27, demonstrating that D27 is indeed the processing site for CASP4

335    (**Fig. 7A**). In agreement with our prior data, CASP5 processed IL-1β$^{WT}$ and generated only the

336    p27 fragment, which was abolished in IL-1β$^{D27A}$ transfected cells (**Fig. 7A**). We also discovered

337    that CASP5 cleaves IL-18 and wanted to confirm the site of processing. Because CASP1/4

338    process IL-18 at D36[40,41] and CASP5 processing of IL-18 generated a fragment that was the

339    same size as that of CASP1/4 processed IL-18, we hypothesized that CASP5 processed IL-18

340    at D36. Indeed, CASP1/4/5 processed IL-18$^{WT}$ to generate the mature species, which was

341    abolished in IL-18$^{D36A}$ transfected cells (**Fig. 7B**). Thus, our data reveals that the non-canonical

342    inflammasomes process IL-1β at D27 and IL-18 at D36, establishing IL-1β and IL-18 as bona

343    fide substrates of the non-canonical inflammasomes (**Fig. 7C**).

344

**Discussion**

346    All inflammatory caspases are known to cleave the effector protein GSDMD to induce

347    pyroptosis, but whether these caspases process GSDMD to the same degree when activated in

348    cells has remained unclear because ligand-mediated activation of inflammatory caspases

349    proceed with different kinetics. For example, lethal factor (LF) and the small molecule drug Val-

350    boroPro (VbP) are both activators of the NLRP1B inflammasome, but LF-induced pyroptosis

351    occurs faster than VbP-induced pyroptosis[42,43]. In this study, we developed an expression

352    system that allowed us to compare the interactions of specific caspase species with their

353    putative substrates as well as evaluate the activity in cells independent of the kinetics of ligand-

354    mediated activation. Furthermore, we utilized an orthogonal chemical biology approach to

355    inducibly dimerize and activate the inflammatory caspases that corroborates the findings from

356    our expression assays. Importantly, these findings are congruent with results obtained from

357    activating the inflammatory caspases using native ligands and pathogens in physiologically

358    relevant cells.

359        Recent studies have reported that the dominant active caspase-1 species in cells is a

360    transient p33/10 species, and further processing to the p20/10 species generates an unstable

361    protein that rapidly loses protease activity[9]. Consistent with these findings, several studies have

362    demonstrated that interdomain linker processing is critical for pyroptosis[9,12–14]. Interestingly,

363    structural studies demonstrate that the p20/10 species of the inflammatory caspases utilize an

364    exosite to bind to GSDMD[15,16]. However, the structural basis of how inflammatory caspases bind

365    to other substrates such as IL-1β and IL-18 are not fully defined. Thus, we sought to

366    characterize the inflammatory caspase species and their Interactions with the inflammatory

367    substrates, GSDMD, IL-1β, and IL-18. Our data suggests that the p20/10 species of CASP1/4/5

368    are the species that bind strongly to the inflammatory substrates (**Fig. 1**). However, some

369    notable differences exist. For example, CASP1 p20/10 binds strongly to GSDMD and IL-1β, and

370    weakly to IL-18. In contrast, CASP4/5 p20/10 binds strongly to IL-18, and weakly to GSDMD

371    and IL-1β.  Although the p35/10 species displayed weak interactions with the inflammatory

372    substrates, our data suggests that the weak interactions are sufficient to mediate processing of

373    these substrates (**Fig. 2**). We note that for CASP1, less of the p33/10 was pulled down in the IP

374  samples and this may account for why there appears to be less binding of the substrates (**Fig.**

375  **1**). However, it is worth mentioning that less CASP4 p19/10 was pulled down compared to

376  CASP1 p20/10, but more IL-18 was bound to CASP4 p19/10, suggesting that if a functional

377  enzyme complex is formed, strongly bound substrates would co-IP with the caspase species

378  (**Fig. 2F**). Indeed, a functional CASP1 p33/10 enzyme complex was formed as evidenced by the

379  processing of substrates such as IL-18 and IL-1β and the presence of the p10 in the IP samples

380  (**Fig. 1,2**). If CASP1 p33/10 can mediate cytokine processing, then a major question is why is

381  ASC required? ASC acts as a signal amplifier that facilitates full autoproteolysis of pro-CASP1

382  into the p20/10 species,[10,11,44] precisely the species that binds strongly to the cytokines. ASC

383  may have additional functions that are important for IL-1β processing, but we postulate that in

384  part, it helps generate enough of the short-lived p20/10 species that recruits and processes IL-

385  1β. These differences in substrate binding capabilities likely help to regulate inflammation by the

386  inflammatory caspases under different pathophysiological conditions. Future studies will help

387  uncover the role of ASC in cytokine maturation.

388  The non-canonical inflammasomes are critical for host responses to invading gram

389  negative bacteria. When activated, CASP4/5 in humans and CASP11 in mice, are known to

390  cleave GSDMD to mediate pyroptosis, but whether other direct substrates of the non-canonical

391  inflammasomes exist has remained unclear. Here, we discovered that CASP4/5 directly process

392  IL-18 at D36 to generate the bioactive species. Notably, IL-18 is a potent activator of natural

393  killer and T cells,[45] thus, processing by CASP4/5 during infection likely confers an advantage for

394  mounting an immune response. These findings are in line with recent genetic evidence that

395  CASP4 is required for IL-18 maturation and secretion in epithelial cells during bacterial

396  infection[24–26]. Furthermore, our data indicates that the ability of CASP4/5 to process IL-18 is not

397  limited to just epithelial cells, but spans across other cell types, including macrophages (**Fig. 6**).

398  Intriguingly, while human CASP4/5 robustly process IL-18, the mouse orthologue of CASP4/5,

17

399 CASP11, is unable to process IL-18 in cells, consistent with *in vitro* reports[32,33]. Recent studies

400 also suggest CASP11 does not process IL-18 in response to non-canonical agonists,[46] implying

401 a functional divergence between humans and mice with regard to IL-18 processing. The impact

402 of this divergence on host responses to bacteria warrants future investigation.

403 Unexpectedly, although we did not detect binding of IL-1β to CASP4/5, we could detect

404 processing by both CASP4/5 to generate a p27 fragment (Fig. 2,6,7). Remarkably, the p27

405 fragment of IL-1β was the dominant species generated when CASP4/5 were activated in THP1

406 macrophages by LPS transfection or with bacterial infection (**Fig. 6**). During the preparation of

407 this manuscript, another group used DmrB-CASP4 and reported processing of IL-1β to generate

408 the mature p17 fragment[47]. We also detected modest processing of IL-1β by CASP4 to generate

409 the p17 fragment, which was significantly less than the p17 fragment generated by CASP1 in

410 HEK 293T cells (**Fig. 2**). However, we could not detect IL-1β p17 when CASP4/5 were activated

411 in human macrophages, suggesting this may not be physiologically relevant (**Fig. 6**). It is

412 possible that the processed p17 fragment generated by CASP4/5 activity was below the limit of

413 detection in our macrophage assays. Regardless, the fact that we could detect CASP4/5-

414 mediated processing of IL-1β to the p27 fragment suggests it is the more physiologically

415 relevant processing event. CASP11 also processed mouse IL-1β to generate a p27 fragment

416 (**Fig. 5**), implying that the ability to generate IL-1β p27 is at least evolutionarily conserved

417 between mice and humans – unlike IL-18 processing.

418 The functional impact of non-canonical inflammasome processing of IL-1β into the p27

419 fragment remains to be determined. It is tempting to speculate that at some point during

420 evolution, IL-1β signaling was detrimental to the host, and the non-canonical inflammasomes

421 evolved to deactivate IL-1β signaling. In support of this hypothesis, IL-1β blocking antibodies

422 were recently demonstrated to significantly reduce the incidence of lung cancer[48]. Notably, pro-

423 inflammatory cytokines, including IL-1β and IL-18, contribute to the excessive inflammation and

18

424   pathogenesis of sepsis that leads to organ failure, and blocking these cytokines confers

425   protection in acute animal models[49]. Also in support of the notion that the non-canonical

426   inflammasomes may have evolved to attenuate excessive inflammation downstream of bacterial

427   infections, recent ancestral reconstruction revealed that CASP4/5 evolved after CASP1 from an

428   ancestral caspase that cleaves IL-1β to yield the p27 fragment, which was reported to not signal

429   to the IL-1 receptor[28]. We mapped out the processing site that generates IL-1β p27 by the non-

430   canonical inflammasomes to D27 (**Fig. 7**). However, whether CASP1 can further process IL-1β

431   p27 to generate the mature, active p17 fragment remains unknown. Fascinatingly, extant

432   Carnivora harbor a CASP4 enzyme scaffold that has CASP1-like substrate specificity[50].

433   Perhaps in the Carnivora, IL-1β p17 was detrimental but later became essential for survival, and

434   thus, Carnivora CASP4 gained CASP1-like activity. Future studies are needed to delineate the

435   biological contributions of IL-1β p27 and p17 to host defense or regulating inflammation during

436   bacterial infection.

437        GSDMD is recognized by inflammatory caspases via an exosite, and the processing is

438   sequence independent[16]. In stark contrast, we discovered that the processing of IL-1β is

439   sequence dependent. When we substituted the native tetrapeptide sequence of IL-1β for that of

440   tetrapeptide sequences from other inflammatory caspase substrates, this conferred processing

441   specificity to that corresponding caspase. Specifically, we substituted the tetrapeptide sequence

442   of IL-1β (YVHD) for that of IL-18 (LESD), which we demonstrated was processed by CASP4/5,

443   the mutant IL-1β was then processed by CASP4/5 at that tetrapeptide site (**Fig. 3**). However, if

444   YVHD is replaced by AAAD, a sequence that has no specificity for a particular caspase, in

445   contrast to GSDMD, this attenuates processing at that site. Rather than processing at that site

446   to generate the bioactive p17 fragment, IL-1β$^{AAAD}$ was processed at D27 to yield a 27 kDa

447   fragment that is predicted to be inactive (**Fig. 3**). This reengineering of IL-1β substrate specificity

448   led us to discover that CASP5, which is reported to cleave IL-1α[34], does not process IL-1α (**Fig.**

19

449   **S1**). In fact, we did not observe IL-1α processing by CASP1/4/5 when activated via different

450   mechanisms. This is consistent with past and present studies that failed to detect a role for

451   inflammatory caspases in processing IL-1α, but instead identified granzyme B and calpains as

452   the proteases that mediate IL-1α processing[51,52]. The incongruent findings could arise from the

453   use of different cell types and *in vitro* vs in cell assays. Altogether, these findings indicate that

454   the tetrapeptide sequence in IL-1β regulates IL-1β recruitment and processing by CASP1/4/5.

455   We postulate that peptide inhibitors based on the LESD scaffold from IL-18 may potentially

456   serve as selective inhibitors of CASP4/5 over CASP1. Future structural studies are necessary to

457   dissect the molecular basis of distinct IL-1β and IL-18 recognition and processing by

458   inflammatory caspases.

459   In summary, report two new direct substrates (IL-1β and IL-18) for the non-canonical

460   inflammasomes. CASP4/5 cleave IL-18 at D36 to generate the bioactive fragment, but process

461   IL-1β at D27 to yield an inactive p27 fragment (**Fig. 7C**). Additionally, we discovered that

462   CASP11 similarly processes mIL-1β to generate IL-1β p27 but does not process mIL-18. Finally,

463   we found that none of the inflammatory caspases process IL-1α. Hence, the present study

464   offers mechanistic insight into substrate specificities that could help design new therapies for

465   inflammatory disorders and aid our understanding of host responses to inflammation and

466   bacterial infections.

467

468   **Materials and Methods**

469   **Antibodies and reagents**

470   Antibodies used include: GSDMD Rabbit polyclonal Ab (Novus Biologicals, NBP2-33422),

471   FLAG® M2 monoclonal Ab (Sigma, F3165), GAPDH Rabbit monoclonal Ab (Cell Signaling

472   Tech, 14C10), CASP1 p20 Rabbit polyclonal Ab (Cell Signaling Tech, 2225s), CASP1 p12/10

473   Rabbit monoclonal Ab (Abcam, ab179515), CASP4 Rabbit polyclonal Ab (Cell Signaling Tech,

474    4450S), CASP5 Rabbit monoclonal Ab (Cell Signaling Tech, 46680S), Myc Mouse monoclonal

475    Ab (Cell Signaling Tech, 2276S), V5 Rabbit monoclonal Ab (Cell Signaling Tech, 13202S), hIL-

476    1β Goat Polyclonal Ab (R&D systems, AF-201-NA), hIL-18 Goat Ab (R&D systems, af2548),

477    hIL-1α Recombinant Ab (PeproTech, 200-01A), PARP Rabbit polyclonal Ab (Cell Signaling

478    Tech, 9542S), HA Rabbit monoclonal Ab (Cell Signaling Tech, 3724S), mIL-1β Goat polyclonal

479    Ab (R&D systems, AF-401-NA), CASP11 Rat monoclonal Ab (Novus Biologicals, NB120-

480    10454), FKBP12 Rabbit polyclonal Ab (Abcam, ab24373). IRDye 800CW anti-rabbit (LICOR,

481    925-32211), IRDye 800CW anti-mouse (LI-COR, 925-32210), IRDye 680CW anti-rabbit (LI-

482    COR, 925-68073), IRDye 680CW anti-mouse (LI-COR, 925-68072). Other reagents used

483    include: LPS-EB Ultrapure (Invivogen, tlrl-3pelps), VX-765 (Apexbio Technology LLC, 50-101-

484    3604), Z-VAD-FMK (Enzo Life Sciences, NC9471015), FuGENE HD (Promega, E2311),

485    AP20187 (Tocris™ 6297/5),

486    NP-40 Lysis Buffer Low Salt (Thomas Scientific, C994H79).

487

488    **Cell Culture**

489    HEK 293T cells, HeLa cells, Caco-2 cells, and THP1 cells were purchased from ATCC.

490    Caco-2 and THP1 knockout cell lines were previously reported[25,29]. HEK 293T and HeLa cells

491    were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 10%

492    fetal bovine serum (FBS). THP-1 cells were cultured in Roswell Park Memorial Institute

493    (RPMI) medium 1640 with L-glutamine and 10% fetal bovine serum (FBS). Caco-2 cells

494    (HTB-27; American Type Culture Collection) were maintained in DMEM supplemented with 10%

495    FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin. All cells were grown at 37 °C in a 5%

496    $CO_2$ atmosphere incubator.

497

498    **Generation of stable cell lines.**

499  For generating HEK 293T cells ectopically expressing GSDMD, IL-1β, IL-1α, IL-18, and caspase

500  constructs, plasmids encoding those proteins were packaged into lentivirus by transfecting the

501  vectors (2 µg) along with psPAX2 (2 µg), and pMD2.G (1 µg) using Fugene HD transfection

502  reagent (Promega) into HEK 293T cells. After 2 days, the supernatants were filtered using a

503  0.45 µm filter, then used to infect HEK 293T cells. After 48 hours, the cells expressing the

504  indicated constructs were selected with hygromycin (200 µg/mL), blasticidin (10 µg/mL), or

505  puromycin (1 µg/mL).

506

507  **Cloning**

508  All plasmids were cloned using Gateway technology as previously described[12,29,53]. DNA

509  encoding the indicated proteins were inserted between the *attR* recombination sites and

510  shuttled into modified pLEX_307 vectors (Addgene)  using Gateway technology (Thermo

511  Fisher Scientific) according to the manufacturer's instructions. Proteins expressed from these

512  modified vectors contain an N-terminal *att*B1 linker (GSTSLYKKAGFAT) after any N-terminal

513  tag (2xFLAG) or protein (DmrB) or a C-terminal *att*B2 linker (DPAFLYKVVDI) preceding any

514  C-terminal tag such as V5 or HA. An internal ribosome entry site (IRES) was cloned between

515  the large and small subunits of the caspases to allow separate expression of the two

516  polypeptides. ΔCARD DmrB-CASP1 (residues 92 – 402), ΔCARD DmrB-CASP4 (residues 67

517  – 377), ΔCARD DmrB-CASP5 (residues 128 – 434), and ΔCARD DmrB-CASP11 (residues

518  71 – 373) were all cloned into a modified pLEX_307 vector and contained the N-terminal

519  *att*B1 linker between the DmrB and caspase sequences. Point mutations were generated

520  using the QuikChange II site-directed mutagenesis kit (Agilent) according to the

521  manufacturer's instructions.

522

523  **Transient transfections**

22

524    HEK 293T cells were seeded in 12-well culture plates at $2.5 \times 10^5$ cells/well in DMEM. The

525    following day, the indicated plasmids were mixed with a control RFP vector to a total of 1.0 µg

526    DNA in 65 µL Opti-MEM and transfected using FuGENE HD (Promega) according to the

527    manufacturer's protocol. Some samples were further treated (as indicated in each figure legend)

528    with AP20187 (1 µM) or LPS (25 µg/mL)/FuGENE (0.5%). The cells were harvested at the

529    indicated times and analyzed by LDH cytotoxicity and immunoblotting assays as described

530    below.

531

532    **LDH cytotoxicity and immunoblotting assays**

533    Supernatants were harvested for LDH analyses at time points indicated and analyzed using the

534    Cyquant LDH Cytotoxicity Assay (Thermo Scientific) according to the manufacturer's protocol.

535    LDH activity was quantified relative to a lysis control where cells were lysed using NP-40 for 30

536    minutes. For immunoblotting, protein concentrations were normalized using the DC Protein

537    Assay Kit (Bio-Rad), separated by SDS-PAGE, transferred onto Nitrocellulose membranes (Bio-

538    Rad), and visualized using the Odyssey M Imaging System (LI-COR Biosciences).

539

540    **FLAG immunoprecipitations**

541    HEK 293T cells were seeded in 6-well plates at $5 \times 10^5$ cells/well in DMEM for 24 h. The cells

542    were then transiently transfected with the indicated constructs. After 48 h, the cells were

543    harvested and lysed by sonication. Lysates were clarified by centrifugation at 21,000 x g for 5

544    minutes. The soluble fractions were then normalized using the DC Protein Assay (BioRad).  100

545    µL of the lysates were combined with 100 µL of 2x sample loading buffer and incubated at 95 °C

546    for 10 minutes. Equal protein amounts of the remaining sample lysates were loaded onto Pierce

547    Micro-Spin Columns (Thermo Scientific) containing 100 µL of anti-FLAG-M2 agarose resin

548    (Sigma) and the samples were rotated end-over-end at 4°C overnight. The samples were then

549    washed 3x with 1 column volume (350 µL) of PBS. Proteins were eluted by rotating the resin at

550    room temperature for 1 hour in 100 µL of PBS containing 150 ng/ µL 3×-FLAG peptide (Sigma

551    Aldrich). 100 µL of 2x sample loading buffer was added to the eluate and samples were boiled

552    at 95 °C for 10 minutes. Both lysates and eluates were analyzed by immunoblotting

553

554    **LPS transfections of THP-1 cells**

555    THP-1 cells were resuspended in RPMI medium containing 50 ng/ml phorbol 12-myristate 12-

556    acetate (PMA). Cells were plated in 12-well plates at a density of 8 x $10^5$ cells/well. After 24

557    hours, the media was replaced with fresh RPMI/10% FBS containing 5 µg/ml LPS and the cells

558    were allowed to grow for another 24 h. The media was then replaced with Opti-MEM (0.5

559    mls/well). Where indicated, cells were treated with ZVAD (40 µM) or VX765 (40 µM) 30 minutes

560    before *LPS  transfection. The LPS solution was prepared by adding LPS (25 µg/mL final*

561    *concentration) and FuGENE (0.5% final concentration) to Opti-MEM. This solution was gently*

562    *mixed by flicking and  incubated for 30 mins at room temperature before drop-wise addition to*

563    *each well.*  The supernatants were collected, and cells were lysed by sonication 24 h post

564    transfection. Both supernatants and lysates were precipitated by chloroform/methanol and

565    analyzed by immunoblotting.

566

567    **Bacterial Infections**

568    ***Salmonella* infection of Caco-2 cells**

569    *Salmonella* and *Legionella* infections were carried out as previously described[25,54,55]. Caco-2

570    cells (HTB-27; American Type Culture Collection) were maintained in Dulbecco's modified

571    Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum

572    (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were grown at 37°C in a

573    humidified incubator with 5% $CO_2$. One day prior to infection, Caco-2 cells were incubated with

24

574    0.25% trypsin-EDTA (Gibco) diluted 1:1 with 1 x PBS at 37°C for 15 min to dissociate cells.

575    Trypsin was neutralized with serum-containing medium. 24 hours before infection, cells were

576    replated in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS without antibiotics in a

577    24-well plate at a density of 3 x $10^5$ cells/well. 3 hours before infection, the media was replaced

578    with Opti-MEM I reduced serum medium (Thermo Fisher Scientific) containing 100 ng/ml

579    Pam3CSK4 (Invivogen) to prime the cells. An overnight culture of wild-type

580    (WT) *Salmonella* Typhimurium (SL1344) was diluted into LB broth containing 300 mM NaCl and

581    then grown for 3 hours at 37°C to induce SPI-1 expression. After induction, the culture was

582    pelleted at 6,010 × g for 3 minutes, washed once with PBS, and then resuspended in PBS.

583    Caco-2 cells were infected with WT *Salmonella* at a multiplicity of infection (MOI) of 60. Control

584    cells were mock-infected with PBS. Cells were centrifuged at 290 × g for 10 min and incubated

585    at 37°C. 1-hour post-infection, infected cells were treated with 100 ng/mL Gentamicin to kill any

586    extracellular *Salmonella* and placed back to 37°C. The supernatants were collected, and cells

587    were lysed by sonication 6 h post infection. Both supernatants and lysates were precipitated by

588    chloroform/methanol and analyzed by immunoblotting.

589    ***Legionella* infection of THP1 cells**

590    THP-1 cells in RPMI medium were plated in12-well plates at a density of 8 x $10^5$ cells/well and

591    differentiates with 40 ng/ml phorbol 12-myristate 12-acetate (PMA) for 24 h. The media was

592    replaced with fresh RPMI/10% FBS (without PMA) and the cells were allowed to grow for

593    another 24 h. The media was then replaced with Opti-MEM immediately before *Legionella*

594    infection. The THP-1 cells were infected with a flagellin mutant, Δ*flaA*, of *Legionella*

595    *pneumophila*[56], which is an Lp02 strain (*rpsL, hsdR, thyA*) derived from the serogroup 1 clinical

596    isolate Philadelphia-1. *Legionella* was grown as a stationary patch on charcoal yeast extract

597    agar plates at 37°C. After 48 hours, the bacteria were resuspended in PBS and added to the

598    THP-1 cells at a MOI of 10. Infected THP-1s were centrifuged at 400 × g for 10 min and

599    incubated at 37°C for 7 hours. Control cells were mock-infected with PBS. The supernatants

600    and cells were precipitated together by chloroform/methanol and analyzed by immunoblotting.

601

602    **Data analysis and statistics**

603    Statistical analysis was performed using GraphPad Prism 9.0 software and Microsoft Excel.

604    Statistical significance was determined using two-sided Student's *t*-tests.

605

606    **Author Contributions**

607    CYT conceived and directed the project, performed experiments, analyzed data, and wrote the

608    manuscript. PE, CH, BD performed experiments and analyzed data. MSE, and JZ performed

609    bacterial infections in Caco-2 cells. JL and TS performed cloning and generated plasmids. All

610    authors read and approved the manuscript.

611

620

621    **References**

622    1.    Broz, P. & Dixit, V. M. Inflammasomes: Mechanism of assembly, regulation and
623          signalling. *Nat Rev Immunol* **16**, 407–420 (2016).
624    2.    Lamkanfi, M. & Dixit, V. M. Mechanisms and functions of inflammasomes. *Cell* **157**,
625          1013–1022 (2014).

3. Shi, J. *et al.* Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **526**, 660–665 (2015).

4. Kayagaki, N. *et al.* Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* **526**, 666–671 (2015).

5. He, W. T. *et al.* Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. *Cell Res* **25**, 1285–1298 (2015).

6. Ding, J. *et al.* Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature* **535**, 111–116 (2016).

7. Liu, X. *et al.* Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153–158 (2016).

8. Sborgi, L. *et al.* GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death . *EMBO J* **35**, 1766–1778 (2016).

9. Boucher, D. *et al.* Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity. *Journal of Experimental Medicine* **215**, 827–840 (2018).

10. Guey, B., Bodnar, M., Manié, S. N., Tardivel, A. & Petrilli, V. Caspase-1 autoproteolysis is differentially required for NLRP1b and NLRP3 inflammasome function. *Proc Natl Acad Sci U S A* **111**, 17254–17259 (2014).

11. Broz, P., von Moltke, J., Jones, J. W., Vance, R. E. & Monack, D. M. Differential requirement for caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* **8**, 471–483 (2010).

12. Ball, D. P. *et al.* Caspase-1 interdomain linker cleavage is required for pyroptosis. *Life Sci Alliance* **3**, e202000664 (2020).

13. Ross, C., Chan, A. H., von Pein, J., Boucher, D. & Schroder, K. Dimerization and auto-processing induce caspase-11 protease activation within the non-canonical inflammasome. *Life Sci Alliance* **1**, (2018).

14. Lee, B. L. *et al.* Caspase-11 auto-proteolysis is crucial for noncanonical inflammasome activation. *Journal of Experimental Medicine* **215**, 2279–2288 (2018).

15. Liu, Z. *et al.* Caspase-1 Engages Full-Length Gasdermin D through Two Distinct Interfaces That Mediate Caspase Recruitment and Substrate Cleavage. *Immunity* **53**, 106-114.e5 (2020).

16. Wang, K. *et al.* Structural Mechanism for GSDMD Targeting by Autoprocessed Caspases in Pyroptosis. *Cell* **180**, 941-955.e20 (2020).

17. Casson, C. N. *et al.* Human caspase-4 mediates noncanonical inflammasome activation against gram-negative bacterial pathogens. *Proc Natl Acad Sci U S A* **112**, 6688–6693 (2015).

18. Shi, J. *et al.* Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* **514**, 187–192 (2014).

19. Hagar, J. A., Powell, D. A., Aachoui, Y., Ernst, R. K. & Miao, E. A. Cytoplasmic LPS activates caspase-11: Implications in TLR4-independent endotoxic shock. *Science (1979)* **341**, 1250–1253 (2013).

20. Kayagaki, N. *et al.* Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science (1979)* **341**, (2013).

21. Akuma, D. C. *et al.* Noncanonical inflammasome assembly requires caspase-11 catalytic activity and intra-molecular autoprocessing. *bioRxiv* 2022.09.21.508824 (2022) doi:10.1101/2022.09.21.508824.

22. Kayagaki, N. *et al.* Non-canonical inflammasome activation targets caspase-11. *Nature* **479**, 117–121 (2011).

23. Rühl, S. & Broz, P. Caspase-11 activates a canonical NLRP3 inflammasome by promoting K+efflux. *Eur J Immunol* **45**, 2927–2936 (2015).

24. Wandel, M. P. *et al.* Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms. *Nat Immunol* **21**, 880–891 (2020).

677    25.    Naseer, N. *et al.* Salmonella enterica Serovar Typhimurium Induces NAIP/NLRC4- And
678           NLRP3/ASC-Independent, Caspase-4-Dependent Inflammasome Activation in Human
679           Intestinal Epithelial Cells. *Infect Immun* **90**, (2022).
680    26.    Knodler, L. A. *et al.* Noncanonical inflammasome activation of caspase-4/caspase-11
681           mediates epithelial defenses against enteric bacterial pathogens. *Cell Host Microbe* **16**,
682           249–256 (2014).
683    27.    Kobayashi, T. *et al.* The Shigella OspC3 effector inhibits caspase-4, antagonizes
684           inflammatory cell death, and promotes epithelial infection. *Cell Host Microbe* **13**, 570–583
685           (2013).
686    28.    Bibo-Verdugo, B. *et al.* Resurrection of an ancient inflammatory locus reveals switch to
687           caspase-1 specificity on a caspase-4 scaffold. *J Biol Chem* **298**, 101931 (2022).
688    29.    Taabazuing, C. Y., Okondo, M. C. & Bachovchin, D. A. Pyroptosis and Apoptosis
689           Pathways Engage in Bidirectional Crosstalk in Monocytes and Macrophages. *Cell Chem
690           Biol* **24**, 507–514 (2017).
691    30.    Davies, C. W. *et al.* Discovery of a caspase cleavage motif antibody reveals insights into
692           noncanonical inflammasome function. *PNAS* **118**, e2018024118 (2021).
693    31.    Silva, M. T. Secondary necrosis: The natural outcome of the complete apoptotic program.
694           *FEBS Letters* vol. 584 4491–4499 Preprint at
695           https://doi.org/10.1016/j.febslet.2010.10.046 (2010).
696    32.    Bibo-Verdugo, B., Snipas, S. J., Kolt, S., Poreba, M. & Salvesen, G. S. Extended subsite
697           profiling of the pyroptosis effector protein gasdermin D reveals a region recognized by
698           inflammatory caspase-11. *Journal of Biological Chemistry* **295**, 11292–11302 (2020).
699    33.    Gonzalez Ramirez, M. L. *et al.* Extensive peptide and natural protein substrate screens
700           reveal that mouse caspase-11 has much narrower substrate specificity than caspase-1.
701           *Journal of Biological Chemistry* vol. 293 7058–7067 Preprint at
702           https://doi.org/10.1074/jbc.RA117.001329 (2018).
703    34.    Wiggins, K. A. *et al.* IL-1α cleavage by inflammatory caspases of the noncanonical
704           inflammasome controls the senescence-associated secretory phenotype. *Aging Cell* **18**,
705           (2019).
706    35.    Riedl, S. J. & Salvesen, G. S. The apoptosome: Signalling platform of cell death. *Nature
707           Reviews Molecular Cell Biology* vol. 8 405–413 Preprint at
708           https://doi.org/10.1038/nrm2153 (2007).
709    36.    Puren, A. J., Fantuzzi, G. & Dinarello, C. A. Gene expression, synthesis, and secretion of
710           interleukin 18 and interleukin 1β are differentially regulated in human blood mononuclear
711           cells and mouse spleen cells. *Proc Natl Acad Sci U S A* **96**, 2256–2261 (1999).
712    37.    Lin, X. Y., Choi, M. S. K. & Porter, A. G. Expression analysis of the human caspase-1
713           subfamily reveals specific regulation of the CASP5 gene by lipopolysaccharide and
714           interferon-γ. *Journal of Biological Chemistry* **275**, 39920–39926 (2000).
715    38.    Por□ba, M., Strózyk, A., Salvesen, G. S. & Dra□g, M. Caspase substrates and inhibitors.
716           *Cold Spring Harb Perspect Biol* **5**, (2013).
717    39.    Wannamaker, W. *et al.* (S)-1-((S)-2-{[1-(4-amino-3-chloro-phenyl)-methanoyl]-amino}-3,
718           3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2R,3S)-2-ethoxy-5-oxo- tetrahydro-
719           furan-3-yl)-amide (VX-765), an orally available selective interleukin (IL)-converting
720           enzyme/caspase-1 inhibitor, exhibits potent anti-inflammatory activities by inhibiting the
721           release of IL-1β and IL-18. *Journal of Pharmacology and Experimental Therapeutics* **321**,
722           509–516 (2007).
723    40.    Chan, A. H. & Schroder, K. Inflammasome signaling and regulation of interleukin-1 family
724           cytokines. *Journal of Experimental Medicine* **217**, (2020).
725    41.    Afonina, I. S., Müller, C., Martin, S. J. & Beyaert, R. Proteolytic Processing of Interleukin-
726           1 Family Cytokines: Variations on a Common Theme. *Immunity* vol. 42 991–1004
727           Preprint at https://doi.org/10.1016/j.immuni.2015.06.003 (2015).

728    42.    Taabazuing, C. Y., Griswold, A. R. & Bachovchin, D. A. The NLRP1 and CARD8
729            inflammasomes. *Immunol Rev* **297**, 13–25 (2020).
730    43.    Chui, A. J. *et al.* N-terminal degradation activates the NLRP1B inflammasome. *Science*
731            *(1979)* **364**, 82–85 (2019).
732    44.    Dick, M. S., Sborgi, L., Rühl, S., Hiller, S. & Broz, P. ASC filament formation serves as a
733            signal amplification mechanism for inflammasomes. *Nat Commun* **7**, 1–12 (2016).
734    45.    Poznanski, S. M. *et al.* Combined Stimulation with Interleukin-18 and Interleukin-12
735            Potently Induces Interleukin-8 Production by Natural Killer Cells. *J Innate Immun* **9**, 511–
736            525 (2017).
737    46.    Ming Man, S. *et al.* Differential roles of caspase-1 and caspase-11 in infection and
738            inflammation. *Sci Rep* **7**, (2017).
739    47.    Chan, A. H. *et al.* Caspase-4 dimerisation and D289 auto-processing elicits an
740            interleukin-1β converting enzyme. *bioRxiv* 2023.01.05.522955 (2023)
741            doi:10.1101/2023.01.05.522955.
742    48.    Ridker, P. M. *et al.* Effect of interleukin-1β inhibition with canakinumab on incident lung
743            cancer in patients with atherosclerosis: exploratory results from a randomised, double-
744            blind, placebo-controlled trial. *The Lancet* **390**, 1833–1842 (2017).
745    49.    van der Poll, T., van de Veerdonk, F. L., Scicluna, B. P. & Netea, M. G. The
746            immunopathology of sepsis and potential therapeutic targets. *Nature Reviews*
747            *Immunology* vol. 17 407–420 Preprint at https://doi.org/10.1038/nri.2017.36 (2017).
748    50.    Devant, P., Cao, A. & Kagan, J. C. *Evolution-inspired redesign of the LPS receptor*
749            *caspase-4 into an interleukin-1β-converting enzyme. Sci. Immunol* vol. 6
750            https://www.science.org (2021).
751    51.    Afonina, I. S. *et al.* Granzyme B-dependent proteolysis acts as a switch to enhance the
752            proinflammatory activity of IL-1α. *Mol Cell* **44**, 265–278 (2011).
753    52.    Tsuchiya, K. *et al.* Gasdermin D mediates the maturation and release of IL-1α
754            downstream of inflammasomes. *Cell Rep* **34**, (2021).
755    53.    Johnson, D. C. *et al.* DPP8/DPP9 inhibitor-induced pyroptosis for treatment of acute
756            myeloid leukemia. *Nat Med* **24**, 1151–1156 (2018).
757    54.    Naseer, N. *et al.* Human NAIP/NLRC4 and NLRP3 inflammasomes detect Salmonella
758            type III secretion system activities to restrict intracellular bacterial replication. *PLoS*
759            *Pathog* **18**, (2022).
760    55.    Zhang, J., Brodsky, I. E. & Shin, S. Yersinia Type III-Secreted Effectors Subvert
761            Caspase-4-dependent Inflammasome Activation in Human Cells. *bioRxiv*
762            2023.01.24.525473 (2023) doi:10.1101/2023.01.24.525473.
763    56.    Ren, T., Zamboni, D. S., Roy, C. R., Dietrich, W. F. & Vance, R. E. Flagellin-deficient
764            Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS*
765            *Pathog* **2**, 0175–0183 (2006).
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770    **FIGURES**
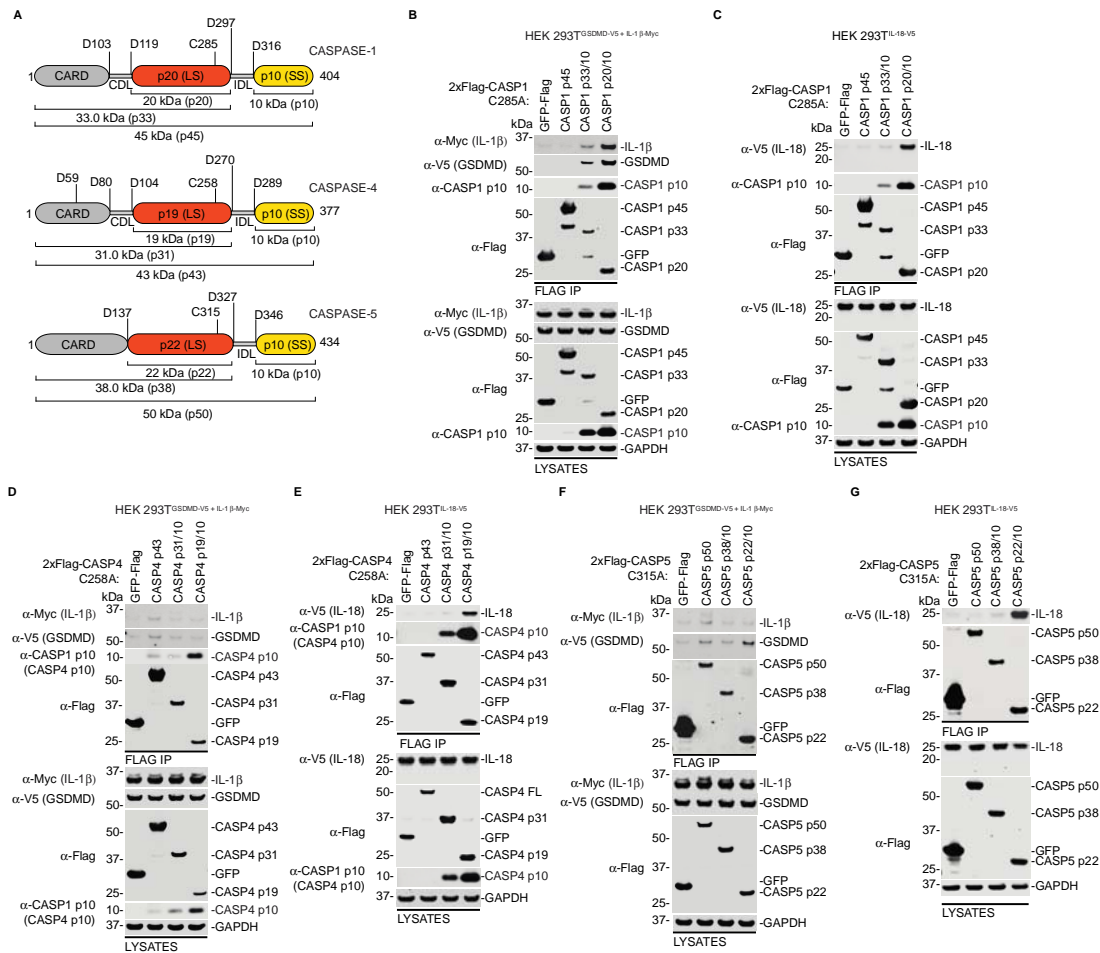
**Figure 1. The p20/10 species of human inflammatory caspases are the species that bind strongly to inflammatory substrates** (**A**) Schematic of human CASP1 (top), CASP4 (middle) and CASP5 (bottom) depicting the catalytic cysteines and autoproteolytic sites that give rise to the distinct caspase species. (**B,D,F**) HEK 293T cells stably expressing C-terminally V5-tagged GSDMD (GSDMD-V5) and Myc-tagged IL-1β (IL-1β-Myc) were transiently transfected with the indicated catalytically inactive caspase constructs. After 48 h, the cells were harvested and subjected to anti-FLAG IP followed by immunoblot analysis. GFP control was C-terminally FLAG tagged (GFP-FLAG) and the catalytically dead caspases all harbored an N-terminal 2xFLAG tag. (**C,E,G**) HEK 293T cells stably expressing C-terminally V5-tagged IL-18 (IL-18-V5) were transiently transfected with the indicated constructs as in B,D, and F for 48 h before the cells

782    were harvested and subjected to anti-FLAG IP followed by immunoblotting. Data are

783    representative of three or more independent experiments.

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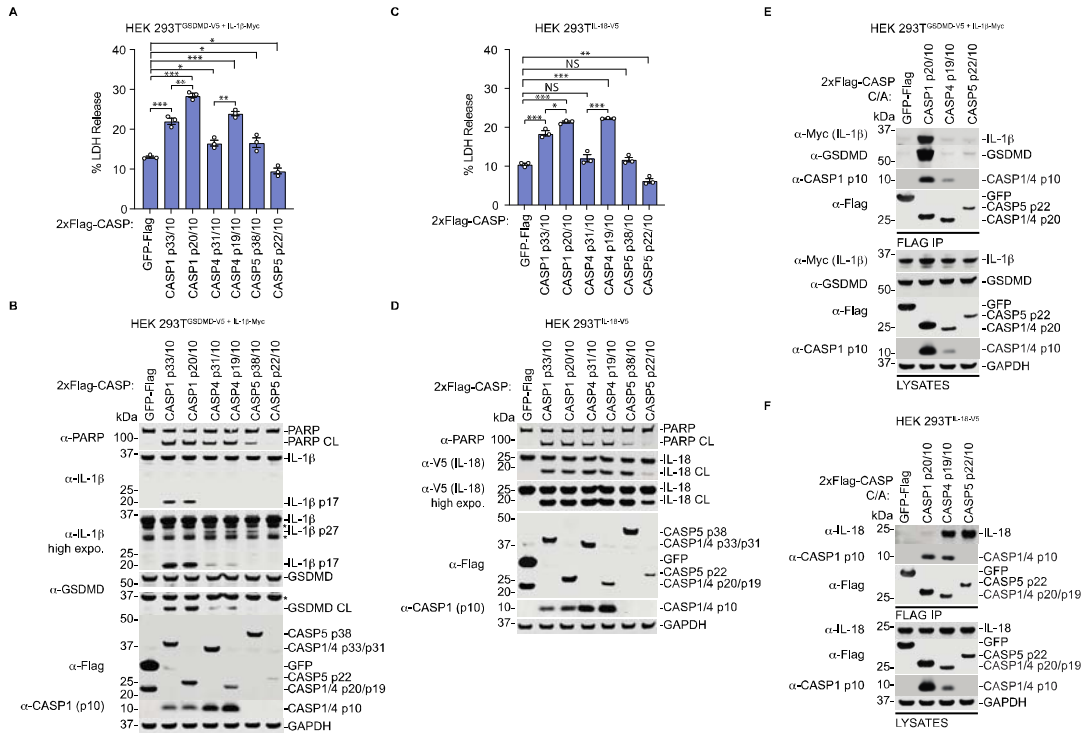
**Figure 2. CASP4/5 cleave IL-1β and IL-18.** (**A,B**) HEK 293T cells stably expressing GSDMD-V5 and IL-1β-Myc were transiently transfected with the indicated constructs. After 24 h, samples were analyzed for LDH release (**A**) and immunoblotting (**B**). (**C,D**) HEK 293T cells stably expressing IL-18-V5 were transiently transfected with the indicated constructs. After 24 h, samples were analyzed for LDH release (**C**) and immunoblotting (**D**). (**E,F**) HEK 293T cells stably expressing GSDMD-V5 and IL-1β-Myc (**E**) or IL-18-V5 (**F**) were transfected with the indicated catalytically inactive caspase constructs. After 48 h, the cells were harvested and subjected to anti-FLAG IP followed by immunoblot analysis. 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 compared with control. *Represents non-specific bands in immunoblots. Data are representative of three or more independent experiments.
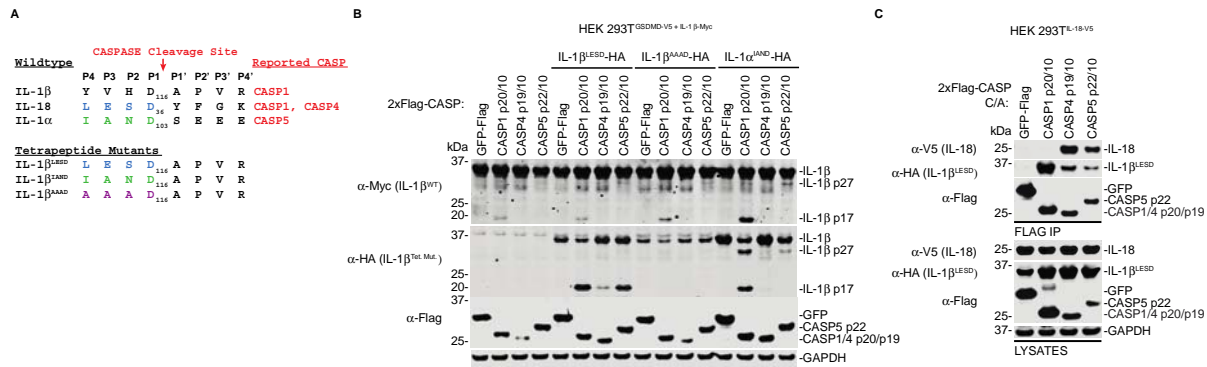
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**Figure 3. The P4 – P1 tetrapeptide sequence of IL-1β regulates processing by CASP1/4/5.**

(**A**) Schematic depicting the sequence of wildtype IL-1β, IL-18, IL-1α, the caspases that were reported to cleave these substrates, and the tetrapeptide mutants. (**B**) HEK 293T cells stably expressing GSDMD-V5 and IL-1β-Myc were transiently co-transfected with the indicated catalytically active caspase constructs and IL-1β tetrapeptide mutants (Tet. Mut.) for 24 h prior to immunoblot analysis. (**C**) HEK 293T cells stably expressing IL-18-V5 were transiently co-transfected with the indicated catalytically inactive (C/A) caspase constructs and the IL-1β mutant, in which the tetrapeptide sequence was substituted for the sequence found in IL-18 (IL-1β$^{LESD}$). 48 h post transfection, samples were subjected to anti-FLAG immunoprecipitation followed by immunoblot analysis. Data are representative of two or more independent experiments.
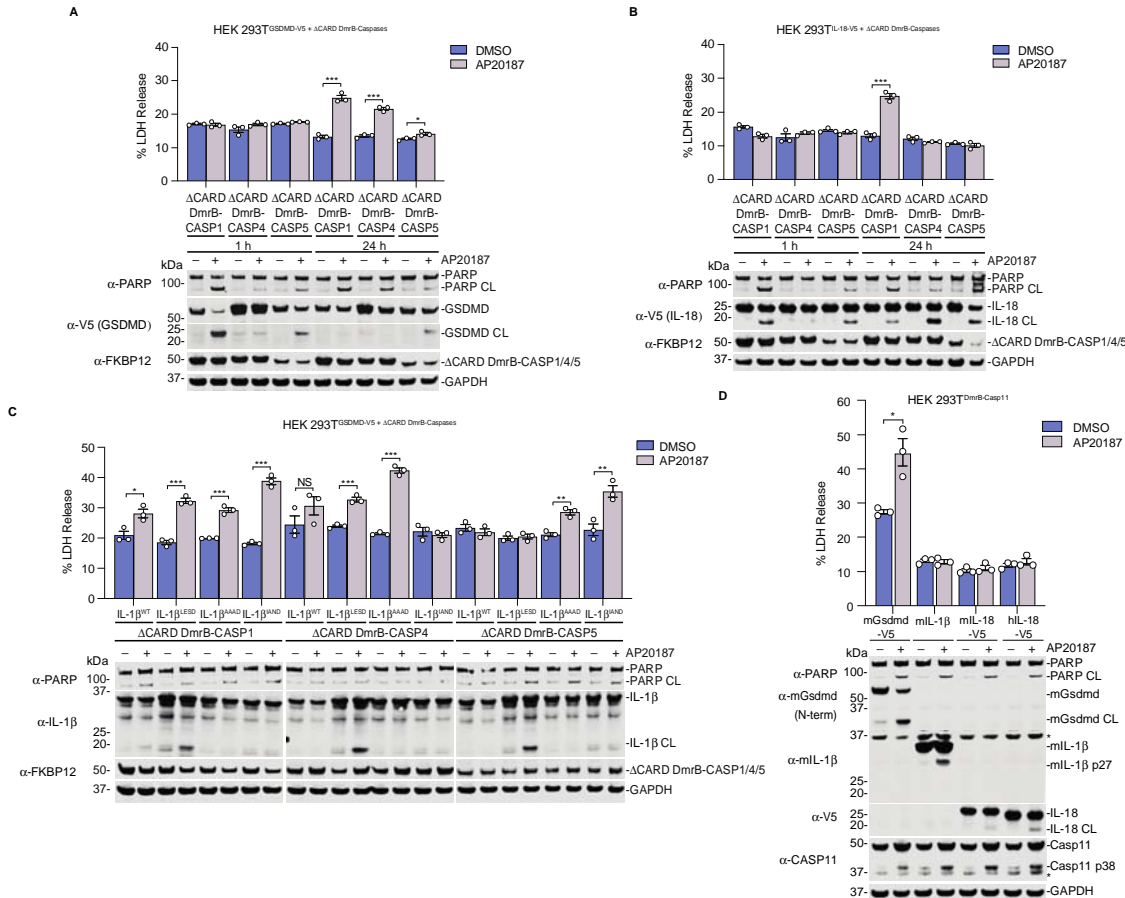
**Figure 4. Dimerization of ΔCARD DmrB-CASP1/4/5/11 mediates processing of inflammatory substrates.** (**A,B**) HEK 293T Cells stably expressing ΔCARD DmrB-CASP1,-4, or -5 and  GSDMD-V5 (**A**) or IL-18-V5 (**B**) were treated with AP20187 (1 μM) for 1 h or 24 h. Cell death was measured by LDH and samples were analyzed by immunoblotting. (**C**) HEK 293T cells stably expressing ΔCARD DmrB-CASP1,-4, or -5 and  GSDMD-V5 were transiently transfected with the indicated IL-1β constructs for 24 h before the addition of AP20187 (1 μM) for 24 h. Cell death was measured by LDH and samples were analyzed by immunoblotting. (**D**) HEK 293T cells stably expressing ΔCARD Dmrb-CASP11 were transiently transfected with the indicated constructs. After 24 h, samples were treated with AP20187 (1 μM) for 24 h then analyzed for LDH release and 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 compared with

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835    control. *Represents non-specific bands in immunoblot. Data are representative of two or more

836    independent experiments.

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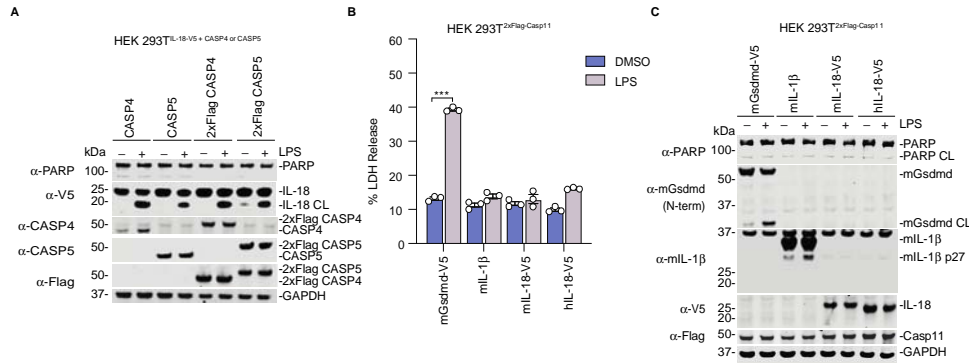843

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**Figure 5. LPS activated non-canonical inflammasomes cleave IL-1β and IL-18.** (**A**) HEK 293T cells stably expressing IL-18-V5 and either CASP4 or CASP5 were transfected with LPS (25 μg/mL) for 24 h before samples were collected and analyzed by immunoblotting. (**B,C**) HEK 293T cells stably expressing 2xFLAG-CASP11 were transiently transfected with the indicated constructs. After 24 h, samples were transfected with LPS (25 μg/mL) for 24 h then analyzed for LDH release (**B**) and immunoblotting (**C**). 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 compared with control. Data are representative of two or more independent experiments. \*Represents non-specific bands in immunoblot. The small m or h represents mouse or human versions of the proteins respectively.

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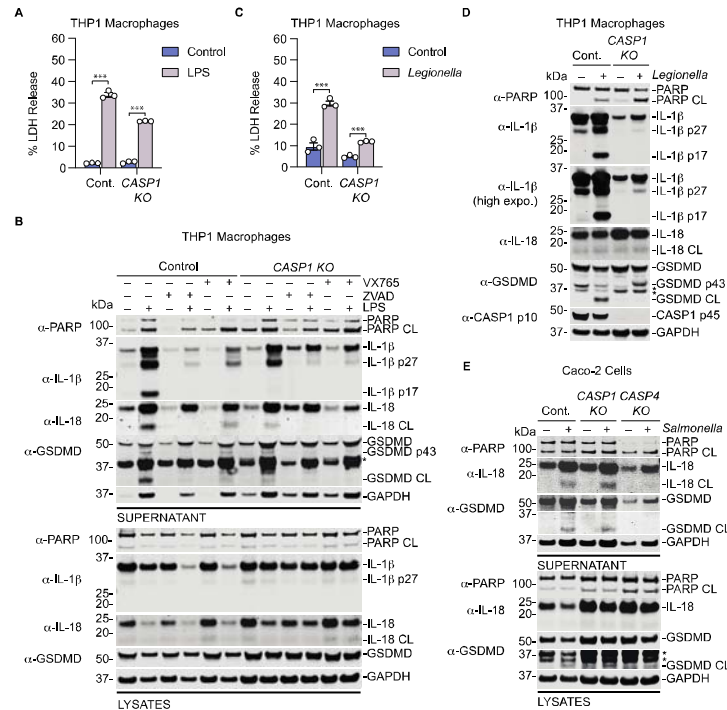
**Figure 6. Cytosolic LPS and pathogenic infections induce non-canonical inflammasome mediated processing of inflammatory substrates in human macrophages and epithelial cells.** (**A,B**) THP1 cells were terminally differentiated into macrophages with phorbol 12-myristate 12-acetate (50 ng/mL) for 24 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 minutes before LPS transfections. Cells were then transfected with LPS (25 μg/mL). 24 h after LPS transfection, samples were analyzed for LDH release (**A**) and immunoblotting (**B**). (**C,D**) THP1 cells were terminally differentiated into macrophages with phorbol 12-myristate 12-acetate (40 ng/mL) for 48 h. Cells were then treated with *Legionella pneumophila* (MOI = 20) for 7 h before the supernatants were analyzed for LDH release (**C**) and then combined with the lysates for immunoblotting (**D**). € Caco-2 cells were primed with 100 ng/ml Pam3CSK4 for 3 h, then infected with *Salmonella* Typhimurium (MOI = 60) for 6 h. Cells and their supernatants were collected separately, samples were precipitated, and analyzed by immunoblotting. Data are means ± SEM of three biological replicates. ***P < 0.001 and **P < 0.01 by two-sided

882    Student's *t*-test compared with control. *Represents non-specific bands in immunoblot. Data are

883    representative of two or more independent experiments.

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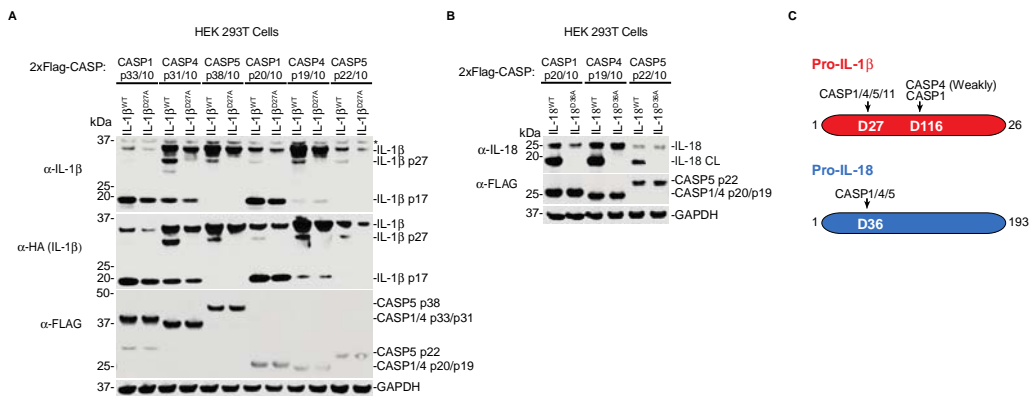886

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**Figure 7. CASP4/5 preferentially cleave IL-1β at D27 and IL-18 at D36.** (**A,B**) HEK 293T cells were transiently co-transfected with the indicated constructs. After 24 h, samples were harvested and analyzed by immunoblotting. (**C**) Schematic of inflammatory substrates and representative cleavage sites by inflammatory caspases. Data are representative of three or more independent experiments. *Represents non-specific bands in immunoblot.
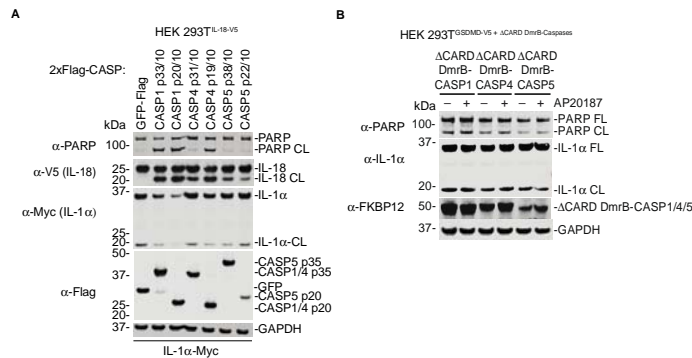
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922    **SUPPLEMENTAL FIGURES**

923    **Supplemental Figure S1.**



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925    **Figure S1. CASP1/4/5 do not cleave IL-1α.** (**A**) HEK 293T cells stably expressing IL-18-V5

926    were transiently transfected with the indicated constructs. After 24 h, samples were then

927    harvested and analyzed by immunoblotting. (**B**) HEK 293T cells stably expressing GSDMD-V5

928    and either ΔCARD DmrB-CASP1,-4, or -5 were transiently transfected with a plasmid coding for

929    wildtype IL-1α for 24 h. Samples were then treated with AP20187 (1 μM) for 24 h and analyzed

930    by immunoblotting. Data are representative of three or more independent experiments.