Developmental and Comparative Immunology 131 (2022) 104377

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Chicken cathelicidin-2 promotes IL-1β secretion via the NLRP3 inflammasome pathway and serine proteases activity in LPS-primed murine neutrophils

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ARTICLE INFO

Keywords:
Chicken cathelicidins
NLRP3 inflammasome
Immunomodulation

ABSTRACT

Cathelicidins have antimicrobial and immunomodulatory activities. Previous studies have shown that chicken cathelicidin-2 (CATH-2) exerts strong anti-inflammatory activity through LPS neutralization. However, it is still unclear whether other intracellular signaling pathways are involved in CATH-2 immunomodulation. Therefore, the CATH-2-meadiated immune response was investigated in LPS-primed neutrophils. Firstly, inflammatory cytokines release was determined in LPS-primed neutrophils. The results showed that CATH-2 significantly promoted secretion of IL-1β and IL-1α while IL-6 and TNF-α were not affected. IL-1β is the key indicator of inflammasome activation. Next, NLRP3 inflammasome signaling pathway was explored using neutrophils of Nlrp3$^{-/-}$, Asc$^{-/-}$ and Casp1$^{-/-}$ mice and the results showed that the CATH-2-enhanced IL-1β release was completely abrogated, indicating it is NLRP3-dependent. Moreover, CATH-2 significantly induced activation of caspase-1 and gasdermin D (GSDMD) but did not affect LPS-induced mRNA expression of IL-1β and NLRP3, demonstrating that CATH-2 serves as the second signal activating the NLRP3 inflammasome. Furthermore, CATH-2-mediated IL-1β secretion and caspase-1 activation is dependent on potassium efflux but independent of P2X7R. In addition, other signaling pathways including JNK, ERK and SyK were investigated using different inhibitors and the results showed that these signaling pathway inhibitors partially attenuated CATH-2-enhanced IL-1β secretion, especially the JNK inhibitor. Finally, the role of serine protease in CATH-2-mediated NLRP3 inflammasome activation was investigated in neutrophils and the results showed that serine protease activity is involved in CATH-2-enhanced IL-1β secretion and caspase-1 activation. In conclusion, after LPS priming in neutrophils, CATH-2 can be an agonist of the NLRP3 inflammasome. Our study increases the understanding on immunomodulatory effects of chicken cathelicidins and provides new insight on chicken cathelicidins-mediated immune response.

1. Introduction

Cathelicidins are host defense peptides with multiple functions, such as antimicrobial, anti-tumor, anti-cancer and immunomodulatory activities, which play an important role in protecting the host from invading microorganisms and chronic inflammatory diseases (Mookherjee et al., 2020; van Harten et al., 2018). Cathelicidins exist in different species including mammals, birds, reptiles, amphibians and some fishes (van Harten et al., 2018). Cathelicidin-2 (CATH-2) is highly expressed in heterophils in chicken and exhibits a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria (van Dijk et al., 2009; Banaschewski et al., 2017; van Dijk et al., 2009; Coorens et al., 2017). Beyond antimicrobial activities, CATH-2 is found to have immunomodulatory activities. For example, CATH-2 modulates

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https://doi.org/10.1016/j.dci.2022.104377
Received 15 December 2021; Received in revised form 16 February 2022; Accepted 16 February 2022
Available online 18 February 2022

inflammatory cytokines secretion including IL-1β, IL-6 and TNF-α via the regulation of Toll-like Receptor 2 (TLR2), TLR4 and TLR9 activation (Coorens et al., 2017; Coorens et al., 2015; Peng et al., 2020). In addition to its capacity to modulate TLR signaling, it is still unclear whether other signaling pathway in the cytoplasm is involved in CATH-2 mediated inflammatory cytokines secretion.

Neutrophils are granulocytic white blood cells which are generated in the bone marrow and circulate in the blood and are considered as one of the first responder cells against invading pathogens through phagocytosis and by the release of antimicrobial compounds, such as cathelicidins (Malech et al. 2020; Mantovani et al., 2011). Neutrophils recognize pathogen associated molecular patterns (PAMPs) through intra- and extracellular receptors including TLR, NOD-like receptor (NLR), C-type lectin receptors to initiate innate immune response, resulting in inflammatory cytokines secretion and reactive oxygen species, which are critical for effective host defense against infection (Rane et al., 2018). Neutrophils as the main producer of cathelicidins not only directly kill invading pathogens, but also release cytokines, proteases and other factors that regulate the immune response (Rane et al., 2018). However, the exact mechanism of CATH-2-modulated inflammatory response in neutrophils is rarely studied. In addition, although CATH-2 is found to be produced by chicken heterophils, its broad immunomodulatory activity has been shown to be independent of species (Coorens et al., 2017; van Harten et al., 2022).

Therefore, in this study, we investigated the mechanism that CATH-2 modulates inflammatory cytokine IL-1β secretion in LPS-primed mice primary peritoneal neutrophils.

2. Methods and materials

2.1. Animals

The wild-type (WT) C57BL/6 mice were purchased from Chongqing Academy of Chinese Material Medical (Chongqing, China). Nlrp3$^{-/-}$, Asc$^{-/-}$ and Casp1$^{-/-}$ mice were kindly gifted by Dr. Feng Shao from the NIBS (National Institute of Biological Sciences, Beijing, China). All gene knockout mice were on a C57BL/6 background and maintained in Specific Pathogen Free (SPF) conditions before being used at 8–10 weeks old. All of the animal experiments were approved by the Southwest University Ethics Committee, Chongqing, China (IACUC-2019-0322-03).

2.2. Peptides

All the peptides were synthesized by China Peptides (Shanghai, China) using Fmoc-chemistry. All peptides (Table 1) were purified by reverse phase high-performance liquid chromatography to a purity >95%.

2.3. Preparation of neutrophils and LPS stimulation in vitro

Mice were injected intraperitoneally with 2–3 ml of 4% thioglycolate medium (Eiken, Tokyo, Japan). After 3–4 h, the mice peritoneal exudate cells were collected by peritoneal lavage and suspended with RPMI 1640 supplemented with 10% FCS or Opti-MEM (Gibco, USA) as reported previously (Zhang et al., 2019). Then, cells were seeded at 2×10^5 cells/well for 48-well plates or 5×10^5 cells/well for 12-well plates. Subsequently, suspended cells were stimulated with *E. coli* LPS (50 ng/ml) (Beyotime, China) and incubated at 37 °C with 5% CO2 for 3 h. After stimulation, peptides (5 μM) were added for an additional 9 h and ATP (1.5 mg/ml) (Beyotime, China) was added as positive control. After 12 h, supernatants and cell lysates were collected for the assays as described below. To inhibit different signaling pathways, different inhibitors were added after LPS stimulation for 30 min or 1 h. Inhibitors in this study included potassium efflux inhibitor (KCl, 5 mM and 50 mM), P2X7 inhibitor (A-740003, 100 μM) (MedChemExpress, USA), JNK inhibitor (SP600125, 40 μM) (Selleck Chemicals, USA), ERK inhibitor (FR180204, 10 μM) (Selleck Chemicals, USA), SyK inhibitor (R406, 1 μM) (Selleck Chemicals, USA), elastase inhibitor (ONO-5046, 1 mM) (Selleck Chemicals, USA) and serine protease inhibitor (PMSF, 1 mM) (Beyotime, China).

2.4. Cell viability

Cells were prepared in 48-well plates and stimulated as described above. After incubation, 10% WST-reagent was added according to manufacture's protocol. Finally, absorbance was measured at 450 nm with a FLUOstar Omega microplate reader and was corrected for absorbance at 630 nm.

2.5. Enzyme linked immunosorbent assay (ELISA)

Cells were prepared in 48-well plates and stimulated as described above. After incubation as described above, supernatants were collected and cytokines secretion level was determined by ELISA according to manufacturer's instructions. The kits used in this study included IL-1β, TNF-α, IL-1α and IL-6 (Invitrogen, CA, USA).

2.6. Western blot analysis

Cells were prepared in 12-well plates and stimulated as described above. After 12 h or 13 h incubation, supernatants were collected and concentrated using 20% (w/v) trichloroacetate, and the cells were lysed with 1 × SDS loading buffer (Beyotime, China) and then cell lysates were collected. Subsequently, concentrated supernatants and cell lysates were subjected to 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane by electroblotting. Next, the membranes were blocked with 5% nonfat dry milk and then immunoblotted with indicated antibodies (Abs) including anti-IL-1β (AF-401-NA, R&D, USA), anti-caspase-1 p20 (AG-20B-0042, AdipoGen, USA), anti-pro-IL-1β, anti-pro-caspase-1, anti-GSDMD-N, anti-GSDMD-FL (AB209845, Abcam, Cambridge, UK) and anti-β-actin (AA128, Beyotime, Beijing, China). Finally, the distinct protein bands were detected by ECL detection reagent (Biosharp, China).

2.7. Quantitative real time polymerase chain reaction (RT-PCR)

Cells were prepared in 12-well plates and stimulated as described above. After 12 h incubation, cells were lysed and total RNA were extracted by TRIzol Reagent (Life Technologies Carlsbad, CA, USA) according to the manufacturers' instructions. Then, cDNA was synthesized using PrimeScript® RT reagent Kit (Perfect Real Time) (Takara, Japan). Finally, RT-PCR was performed using the CFX96 (Bio-Rad, USA). Primers were used as follows: IL-1β forward 5′-GAA ATG CCA CCT TTT GAC AGT G and reverse 5′-TGG ATG CTC TCA TCA GGA CAG, NLRP3 forward 5′-CTT TCT GGA CTC TGA CCG GG and reverse 5′-CTC CCA TTC

Table 1
Characteristics of peptides used in this study.

Peptide	Amino acid sequence	length	charge
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF	26	+9
CATH-B1	PIRNWWIRIWEWLNGIRKRLRQRSPFYVRGHLNVTSTPQP	40	+7
CRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	34	+6

TGG CTC TTC CC, β-actin forward 5′-TGG AAT CCT GTG GCA TCC ATG AAA C and reverse 5′-TAA AAC GCA GCT CAG TAA CAG TCC G. Relative gene expression levels were normalized against the expression levels of β-actin.

2.8. Statistical analysis

Data are represented as mean ± SEM of three independent experiments for each group (n = 3). One-way ANOVA were used to analyze statistical significance among different groups. Statistical significance was shown as *p < 0.05, **p < 0.01, ***P < 0.001, ns = no significance.

3. Results

3.1. CATH-2 promotes IL-1β secretion in LPS-primed neutrophils

To investigate the immunomodulatory activity of cathelicidins, chicken cathelicidins (CATH-2 and CATH-B1) and mice cathelicidin (CRAMP, mouse homolog for human LL-37) were used in this study. Neutrophils were pretreated with *E. coli* LPS for 3 h and then 5 μM cathelicidins and ATP as positive control were added for an additional 9 h. Inflammatory cytokines secretion were determined by ELISA. As shown in Fig. 1, peptides alone did not induce inflammatory cytokines including IL-1β, TNF-α, IL-1α and IL-6 and LPS stimulation strongly increased secretion of these cytokines. Interestingly, CATH-2 significantly increased secretion of IL-1β and IL-1α in LPS-primed cells while the secretion of TNF-α and IL-6 were not affected. ATP as positive control also significantly increased IL-1β secretion and slightly IL-1α secretion. However, similar events did not occur in LPS-primed cells with the presence of CATH-B1 and CRAMP. These results indicate that CATH-2 specifically modulates signaling pathway of IL-1β secretion in LPS-primed neutrophils.

3.2. CATH-2 induces caspase-1 activation and IL-1β secretion via a NLRP3-dependent manner in LPS-primed neutrophils

IL-1β is a proinflammatory cytokine triggered by NLRP3 inflammasome activation which activates caspase-1 via adaptor protein ASC to process pro-IL-1β into biologically active IL-1β (Swanson et al. 2019). Therefore, to investigate whether increased IL-1β secretion induced by CATH-2 is dependent on the NLRP3 inflammasome pathway, neutrophils from WT, Nlrp3$^{-/-}$, Asc$^{-/-}$, and Casp1$^{-/-}$ mice were used in this study. The results showed that CATH-2 and ATP-mediated IL-1β secretion was completely abrogated in LPS-primed Nlrp3$^{-/-}$, Asc$^{-/-}$, and Casp1$^{-/-}$ cells while IL-6 secretion was not affected (Fig. 2A, B). Furthermore, CATH-2 and ATP significantly upregulated IL-1β and

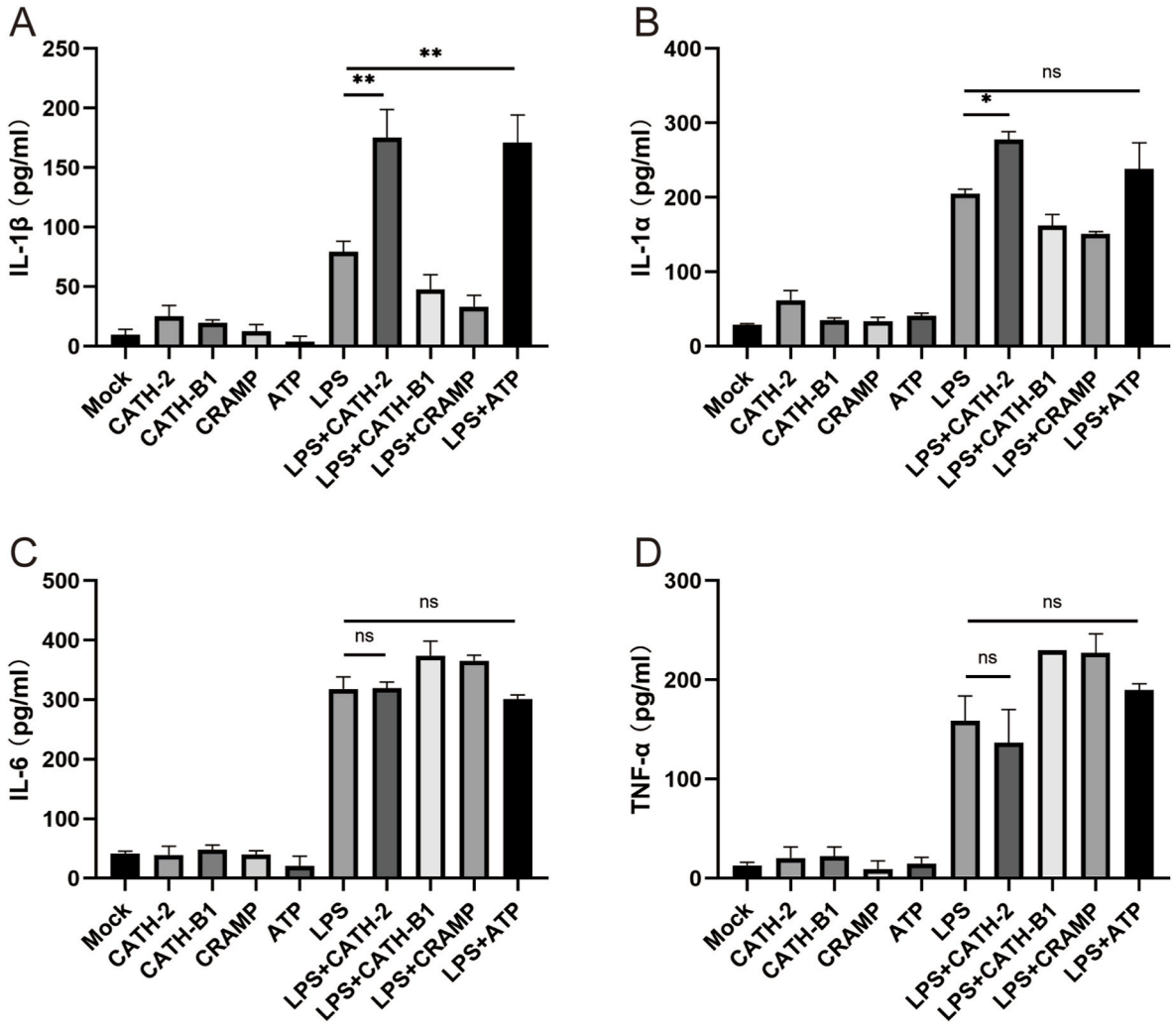

Fig. 1. CATH-2 promotes IL-1β secretion in LPS-primed neutrophils. Neutrophils were pretreated with LPS for 3 h and then peptides and ATP (as positive control) were added for an additional 9 h. After 12 h, cell supernatants were collected and cytokine levels were determined by ELISA. (A) IL-1β. (B) IL-1α. (C) IL-6. (D) TNF-α. Data are shown as mean ± SEM of three independent experiments for each group (n = 3). One-way ANOVA with Bonferroni post-test was used to analyze statistical significance. *P < 0.05, **P < 0.01, ns = no significance.

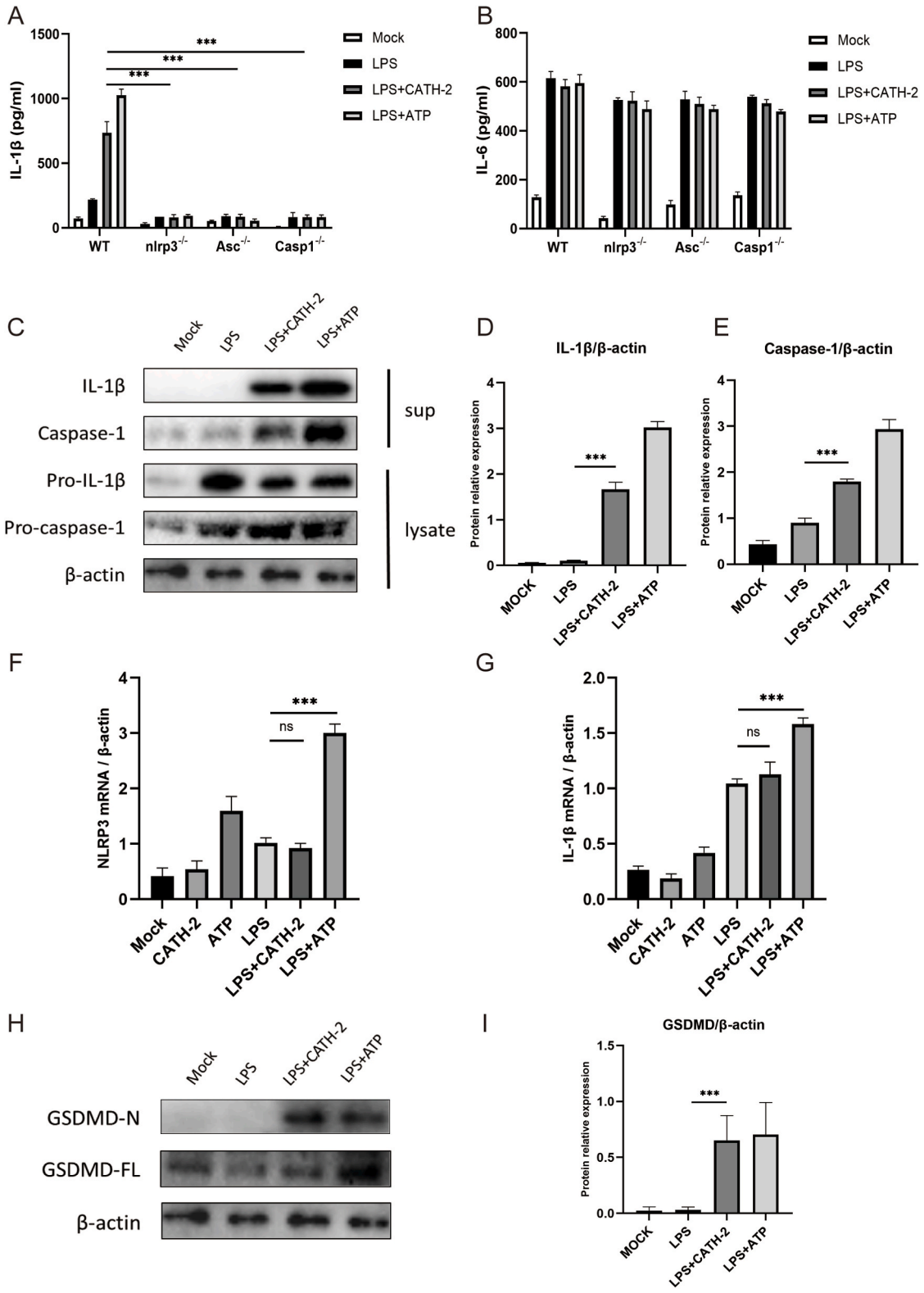

Fig. 2. CATH-2 induces caspase-1 activation and IL-1β secretion via a NLRP3-dependent manner in LPS-primed neutrophils. Neutrophils of WT, Nlrp3$^{-/-}$, Asc$^{-/-}$ and Casp1$^{-/-}$ mice were pretreated with LPS for 3 h and then peptides and ATP (as positive control) were added for an additional 9 h. After 12 h, cell supernatants (sup) and lysates were collected. The secretion level of IL-1β (A) and IL-6 (B) was determined by ELISA. Protein expression of IL-1β, caspase-1, pro-caspase-1, pro-IL-1β, β-actin (C) was detected by western blot. Ratio of IL-1β (D) and caspase-1 (E) level against β-actin level was quantified. The relative mRNA expression of NLRP3 (F) and IL-1β (G) was analyzed by RT-PCR. GSDMD (H) was detected by western blot and ratio of GSDMD level (I) against β-actin level was quantified. Relative gene expression levels were normalized against the expression levels of β-actin. One-way ANOVA with Bonferroni post-test was used to analyze statistical significance. ***$P < 0.001$, ns = no significance.

caspase-1 protein expression in LPS-primed cells and slightly decreased pro-IL-1β expression, whereas pro-caspase-1 expression was not affected (Fig. 2C, D, 2E). In addition, CATH-2 did not affect LPS-induced mRNA expression of NLRP3 and IL-1β (Fig. 2F, G). These results indicate that CATH-2 promotes IL-1β secretion via NLRP3 inflammasome activation instead of the transcription of IL-1β and NLRP3. Notably, CATH-2 also induced gasdermine D (GSDMD) secretion (Fig. 2H, I) which is cleaved by caspase-11 and inserts into the membrane, forming pores and inducing pyroptosis, suggesting the possibility that CATH-2 induces pyroptosis.

3.3. Potassium efflux modulates CATH-2-mediated caspase-1 activation and IL-1β secretion in LPS-primed neutrophils

To further investigate the mechanism by which CATH-2 could promote caspase-1 activation and IL-1β secretion in LPS-primed neutrophils, previously identified potential receptors were examined. It is widely accepted that ATP promotes IL-1β maturation via the activation of P2X purinoceptor 7 receptor (P2X7R), which is dependent on potassium efflux (Swanson et al. 2019). Our results showed that the inhibition of P2X7R dramatically abolished ATP-mediated IL-1β secretion but did not affect CATH-2-mediated IL-1β secretion in LPS-primed neutrophils (Fig. 3A), and IL-6 secretion was not affected (Fig. 3B), indicating that CATH-2-mediated IL-1β secretion is independent of P2X7R activation. Furthermore, the inhibition of potassium efflux significantly abrogated both ATP and CATH-2-mediated IL-1β secretion while IL-6 secretion was not affected (Fig. 3C, D). In addition, CATH-2-mediated caspase-1 activation was also slightly inhibited by low concentration of potassium efflux inhibitor (Fig. 3E, F, 3G). These results indicate the important role of potassium efflux in CATH-2-mediated caspase-1 activation and IL-1β secretion.

3.4. JNK signaling pathway is involved in CATH-2-mediated caspase-1 activation and IL-1β secretion in LPS-primed neutrophils

To further investigate whether other signaling pathways are involved in CATH-2-mediated IL-1β secretion, potential signaling pathways including JNK, ERK and SyK which can regulate the inflammatory response were explored. These experiments showed that ATP and CATH-2-mediated IL-1β secretion in LPS-primed neutrophils was significantly inhibited by inhibitors of JNK, ERK and SyK while IL-6 secretion was not affected (Fig. 4A, B). Moreover, western blot results showed that JNK inhibitor attenuated CATH-2-mediated caspase-1 activation as well as IL-1β maturation and secretion (Fig. 4C, D, 4E).

3.5. Serine protease activities regulate CATH-2-mediated caspase-1 activation and IL-1β secretion in LPS-primed neutrophils

It has been reported that some proteases in neutrophils are responsible for the processing of caspase-1, which play a critical role in caspase-1 activation to cleave pro-IL-1β into IL-1β (Demirel et al., 2020; Karmakar et al., 2012). To investigate whether serine protease activities are associated with CATH-2-mediated IL-1β secretion in LPS-primed neutrophils, cells were pretreated with specific protease inhibitors in the presence of CATH-2. The results showed that CATH-2-mediated IL-1β secretion was completely abolished by inhibitors of serine protease while IL-6 secretion was not affected (Fig. 5A, B). In addition, caspase-1 activation was also significantly inhibited by these two inhibitors while pro-caspase-1 and pro-IL-1β protein expression were not affected (Fig. 5C, D, 5E).

4. Discussion

Cathelicidins are host defense peptide components of the innate immune system, essential to protect the host from microbial infection through directly killing pathogens and modulating immune responses. CATH-2 is identified in chicken and shows a broad spectrum of antibacterial activity (Cuperus et al., 2016; Schneider et al., 2017). For example, CATH-2 provides protection *in vivo* against *pseudomonas aeruginosa* and avian pathogenic *E. coli* infection (Cuperus et al., 2016; Coorens et al., 2017). From the immunomodulatory perspective, CATH-2 has strong capacity to neutralize bacterial LPS or LTA, resulting in the silencing of the inflammatory response via the inhibition of TLR2 and TLR4 activation (van Dijk et al., 2016; Coorens et al., 2017), which helps the host to prevent excessive inflammation in response to microorganism. In addition, it has been shown that CATH-2 exerts its immunomodulatory activity in different species, such as human peripheral blood mononuclear cells (PBMCs), chicken macrophages, mice macrophages and porcine macrophages (van Dijk et al., 2009; van Dijk et al., 2016; Coorens et al., 2015; van Harten et al., 2022), indicating CATH-2 performs its activity in a species-independent manner. However, it is still unclear whether CATH-2 exerts "real" immunomodulatory activity independent of LPS neutralization. Therefore, the immunomodulatory function of CATH-2 was investigated in neutrophils pre-treated with LPS, in which inflammatory cytokines transcription was been induced.

Maturation and secretion of IL-1β requires two signals (Swanson et al. 2019). Bacterial or pathogenic molecules such as lipoprotein and LPS as first signal activate the NF-κB signaling pathway and produce pro-IL-1β, the transcriptional and post-translational modification of NLRP3. Then, a bacterial toxin or ATP as second signal activates the NLRP3 inflammasome through the adaptor proteins ASC to recruit caspase-1 which processes pro-IL-1β into mature IL-1β. Our data showed that CATH-2 promotes IL-1β secretion and caspase-1 activation dependent on NLRP3 inflammasome pathway in LPS-primed neutrophils. However, due to the possibility of some structural and sequence differences among these three peptides (Table 1) as well as different cell types tested in this study, CATH-B1 and CRAMP failed to induce IL-1β secretion and caspase-1 activation. This indicates that it is likely that CATH-2 plays a similar role as ATP as the second signal to activate the NLRP3 inflammasome. However, in contrast to ATP, whose function as 2nd signal is dependent on P2X7R (Karmakar et al., 2016), CATH-2 does not directly act as an agonist of the P2X7R, since in our experiments the P2X7R inhibitor did not significantly affect CATH-2-mediated IL-1β secretion. This is compatible with the latest observation that human cathelicidin LL-37 also induces IL-1β release via a P2X7R-independent mechanism in human monocytes and mice peritoneal macrophages (McHugh et al., 2019). These results confirm the capacity of CATH-2 to promote IL-1β secretion, but the specific receptor of CATH-2 still needs to be studied further.

So far, inflammasomes have been widely investigated in immune cells, particularly macrophages and neutrophils. Recent research has shown the importance of the inflammasome-mediated response against pathogens (Fang et al., 2019). Knodler et al. reported that inflammasomes activation and pyroptosis are important in the gut defense against enteric bacterial infection (Knodler et al., 2014). Our data showed that CATH-2 induced activation of caspase-1 and gasdermin-D, indicating that CATH-2 mediates cell deaths via canonical and non-canonical pathways. However, it is still unclear whether this cell death can be described as a form of neutrophils pyroptosis because cell metabolic activities are not affected after 12 h incubation (Supplementary Fig. 1). Nevertheless, CATH-2 enhanced NLRP3 inflammasome activation resulted in strong inflammatory response and programmed cell death, which indicates that CATH-2 plays a critical role in amplifying the inflammatory response and would contribute to the host defense against pathogens of immune evasion.

Inflammasome activation includes multiple upstream signals, such as potassium (K^+) efflux. It has been known that potassium efflux is essential for inflammasome activation (Muñoz-Planillo et al., 2013). Our study showed that disruption of K^+ efflux attenuates CATH-2-mediated IL-1β secretion and caspase-1 activation, which is consistent with the findings that K^+ efflux is the common trigger of NLRP3 inflammasome activation (Rivers-Auty and Brough 2015). Therefore, it can be

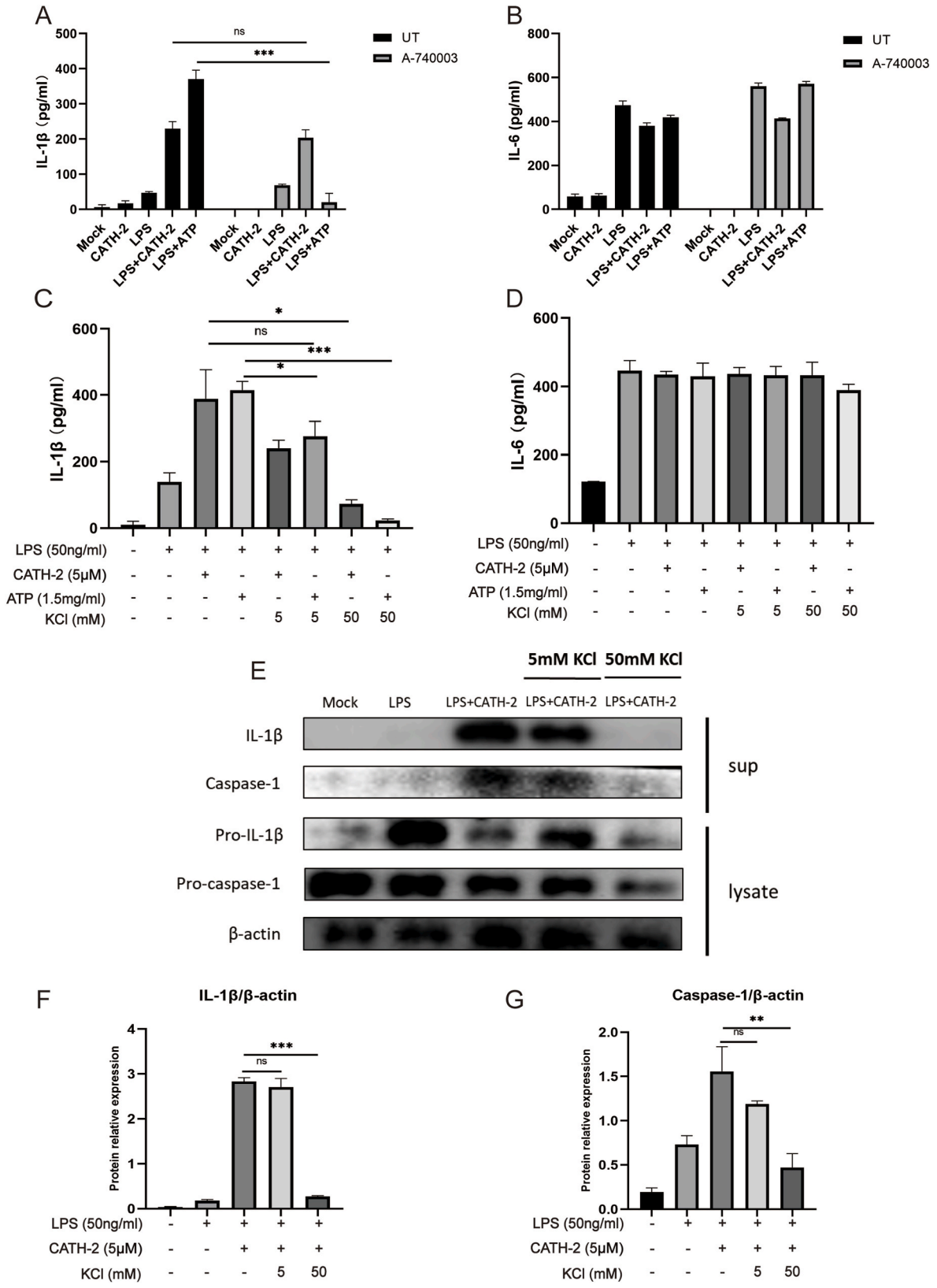

Fig. 3. Potassium efflux modulates CATH-2-mediated caspase-1 activation and IL-1β secretion in LPS-primed neutrophils. Neutrophils were pretreated with LPS for 3 h. Then, cells were untreated (UT) or treated with inhibitors of P2X7R (A-740003) and K^+ efflux (KCl) for 30 min and 1 h, respectively. Subsequently, CATH-2 was added for an additional 9 h. After incubation, cell supernatants (sup) and lysates were collected. The secretion level of IL-1β (A, C) and IL-6 (B, D) in P2X7R and KCl-treated cells was detected by ELISA. Protein expression of IL-1β, caspase-1, pro-caspase-1, pro- IL-1β and β-actin (E) was detected by western blot. Ratio of IL-1β (F) and caspase-1 (G) level against β-actin level was quantified. One-way ANOVA with Bonferroni post-test was used to analyze statistical significance. *P < 0.05, ***P < 0.001, ns = no significance.

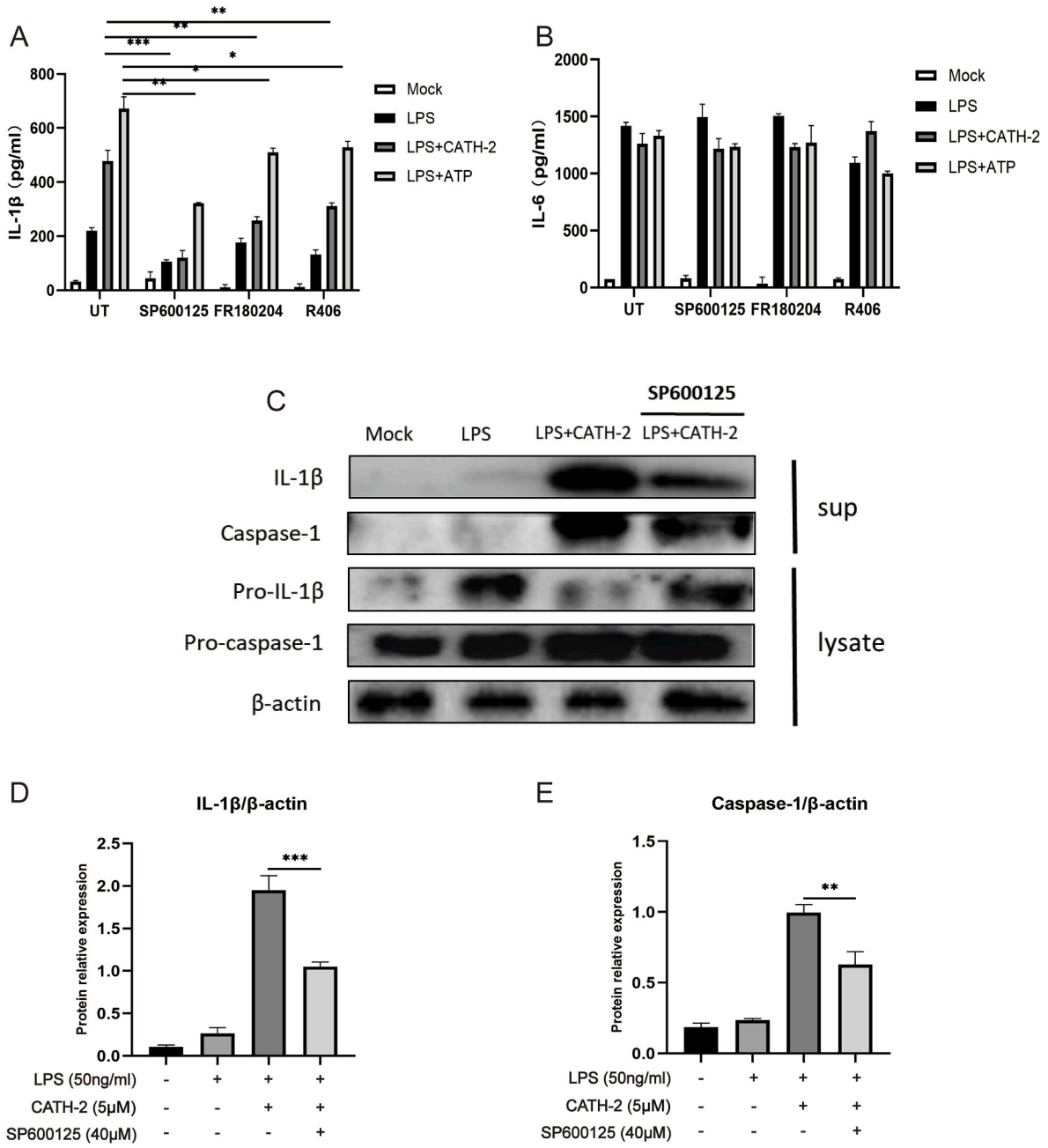

Fig. 4. JNK signaling pathway is involved in CATH-2-mediated caspase-1 activation and IL-1β secretion in LPS-primed neutrophils. Neutrophils were pretreated with LPS for 3 h. Then, cells were untreated (UT) or treated with inhibitors of JNK (SP600125), ERK (FR180204) and SyK (R406) for 1 h. Subsequently, CATH-2 were added for additional 9 h. After incubation, cell supernatants (sup) and lysates were collected. The secretion level of IL-1β (A) and IL-6 (B) was detected by ELISA. Protein expression of IL-1β, caspase-1, pro-caspase-1, pro- IL-1β and β-actin (C) was detected by western blot. Ratio of IL-1β (D) and caspase-1 (E) level against β-actin level was quantified. One-way ANOVA with Bonferroni post-test was used to analyze statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001.

reasonably speculated that CATH-2 induces K^+ efflux. Although CATH-2-enhanced NLRP3 inflammasome activation is dependent on K^+ efflux, it is still unclear which specific factor drives K^+ efflux. Recently, it has been identified that the two-pore domain K^+ channel (K_{2P}) TWIK2 as the K^+ efflux channel triggers NLRP3 inflammasome activation (Di et al., 2018). Therefore, whether CATH-2 drives K^+ efflux via its interaction with TWIK2 needs to be further investigated.

Besides TLRs activation, activation of other intracellular or extracellular signaling pathways can lead to cytokine secretion. It has been shown that kinases including JNK, SyK and ERK can regulate NLRP3 inflammasome activation (Lin et al., 2015; Okada et al., 2014; Chei et al., 2020). LL-37 was found to exhibit pro-inflammatory effects through ERK and JNK activation (Niyonsaba et al., 2005; Chotjumlong et al., 2013). Similarly, our present study showed that inhibitors of JNK, SyK and ERK reduce CATH-2-mediated IL-1β secretion. These results indicate that cathelicidins from different species can show similar functional property, but not all cathelicidins act similarly since CRAMP and CATH-B1 were inactive in our experimental setup.

High level of IL-1β production in neutrophils has been reported to be both inflammasome and protease dependent. Demirel et al. found that NLRP3 and serine protease-dependent release of IL-1β contributes to antimicrobial activity of neutrophils against uropathogenic *E. coli* (Demirel et al., 2020), indicating the synergetic effect of serine protease and inflammasomes on IL-1β secretion. Pires et al. confirmed that serine protease-mediated IL-1β secretion is both inflammasome-dependent and independent in response to *Staphylococcus aureus* (Pires and Parker

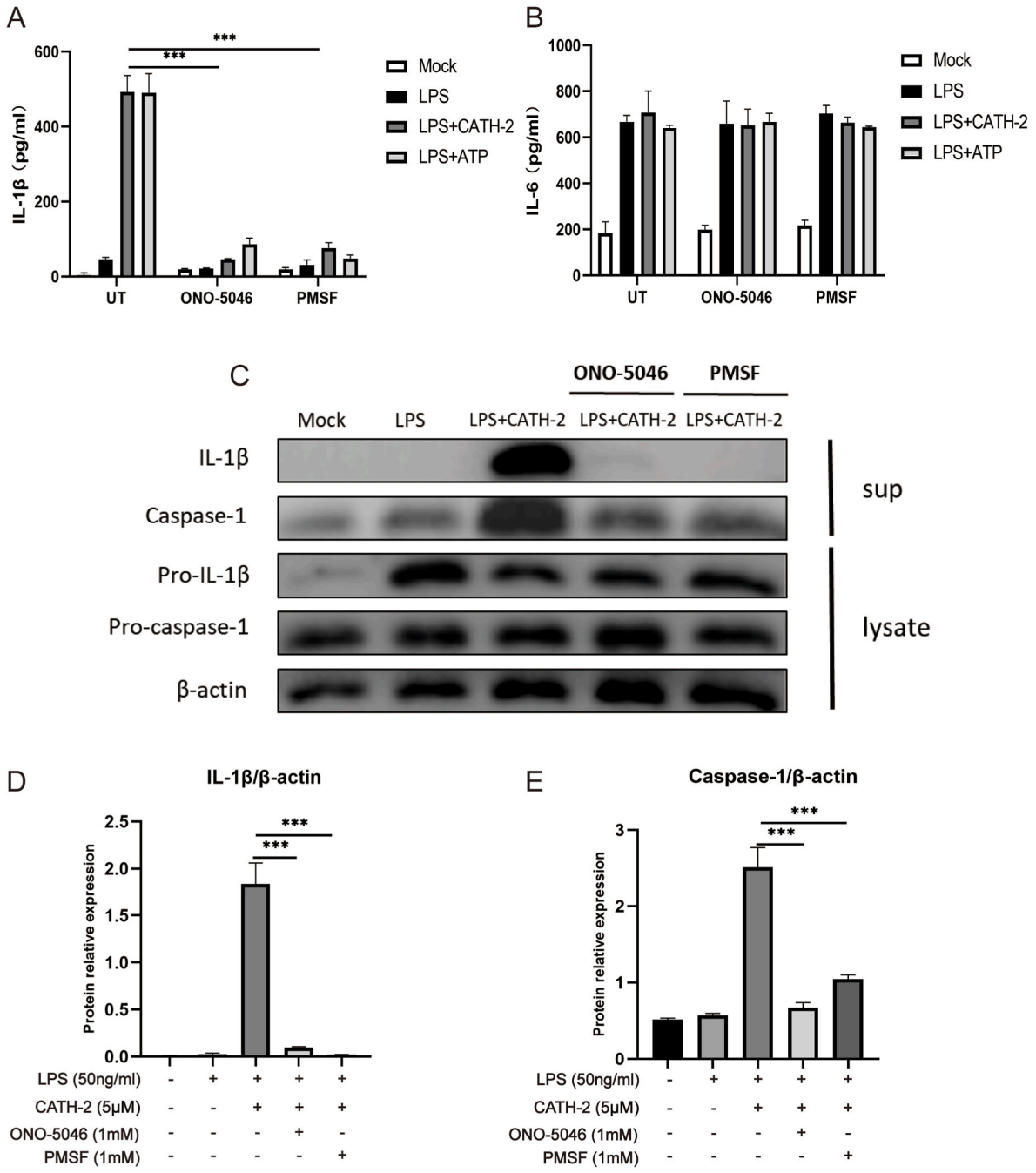

Fig. 5. Serine protease activities regulate CATH-2-mediated caspase-1 activation and IL-1β secretion in LPS-primed neutrophils. Neutrophils were pretreated with LPS for 3 h. Then, cells were untreated (UT) or treated with proteases inhibitors (PMSF, ONO-5046) for 1 h. Subsequently, CATH-2 were added for additional 9 h. After incubation, cell supernatants (sup) and lysates were collected. The secretion level of IL-1β (A) and IL-6 (B) was detected by ELISA. Protein expression of IL-1β, caspase-1, pro-caspase-1, pro- IL-1β and β-actin (C) was detected by western blot. Ratio of IL-1β (D) and caspase-1 (E) level against β-actin level was quantified. One-way ANOVA with Bonferroni post-test was used to analyze statistical significance. ***$P < 0.001$.

2018). Generally, inflammasome activation induces caspase-1 activation to cleave off pro-IL-1β. However, Karmakar et al. reported that during *Pseudomonas aeruginosa* infection inflammasome NLRC4 and caspase-1 are not involved in IL-1β secretion (Karmakar et al., 2012). Our data showed that both deletion of caspase-1 and serine protease completely abrogate IL-1β secretion in LPS-primed neutrophils, demonstrating caspase-1-mediated IL-1β secretion is dependent of serine protease activity.

In conclusion, in this study, we investigated the mechanism of CATH-2-mediated IL-1β maturation and secretion in LPS-primed neutrophils. CATH-2 significantly promoted IL-1β release and caspaspe-1 activation via NLRP3 pathway and this process is dependent on potassium efflux but independent of P2X7R. In addition, SyK and serine protease are involved in CATH-2-mediated IL-1β release and caspaspe-1 activation.

Fundings

This study was supported by National Natural Science Foundation of China (32102684, 32172850, 31902256), Chongqing Science and Technology Commission (cstc2021jcyj-msxm2218, cstc2021jcyj-msxmX0504), the Fundamental Research Funds for the Central Universities (SWU120057) and the Foundation for Innovation Research

Groups in Chongqing Universities (CXQT20004).

Data availability

All data is available within the article and from the corresponding author on reasonable request.

Declaration of competing interest

There is no conflict of interest in this manuscript.

Acknowledgements

The authors would like to thank Dr. Feng Shao from NIBS (National Institute of Biological Sciences, Beijing) to provide the Casp1$^{-/-}$, Asc$^{-/-}$ and Nlrp3$^{-/-}$ mice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2022.104377.

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