Mixed lineage kinase-like protein protects against *Clostridium perfringens* infection by enhancing NLRP3 inflammasome-extracellular traps axis

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Highlight:
- MLKL is critical in limiting *Clostridium perfringens* gas gangrene and enterocolitis
- MLKL deficiency leads to impaired potassium efflux-dependent NLRP3 signaling
- MLKL mediates NLRP3 inflammasome signaling for extracellular traps formation
- MLKL-NLRP3-extracellular traps axis facilitates pathogen control in vitro and in mice

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Mixed lineage kinase-like protein protects against \textit{Clostridium perfringens} infection by enhancing NLRP3 inflammasome-extracellular traps axis

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**SUMMARY**

Despite intense research in understanding \textit{Clostridium perfringens} (\textit{C. perfringens}) pathogenesis, the mechanisms by which it is cleared from the host are largely unclarified. In \textit{C. perfringens} gas gangrene and enterocolitis model, \textit{Mlkl/C0} mice, lacking mixed lineage kinase-like protein (MLKL), are more susceptible to \textit{C. perfringens} infection. \textit{Mlkl} deficiency results in a defect in inflammasome activation, and IL-18 and IL-1β releases. Exogenous administration of recombinant IL-18 is able to rescue the susceptibility of \textit{Mlkl/C0} mice. Notably, K⁺ efflux-dependent NLRP3 inflammasome signaling downstream of active MLKL promotes bacterial killing and clearance. Interestingly, the defect of bactericidal activity is also mediated by decreased classical extracellular trap formation in the absence of \textit{Mlkl}. Our results demonstrate that MLKL mediates extracellular trap formation in a NLRP3 inflammasome-dependent manner. These findings highlight the requirement of MLKL for host defense against \textit{C. perfringens} infection through enhancing NLRP3 inflammasome-extracellular traps axis.

**INTRODUCTION**

\textit{C. perfringens} is a clinically significant opportunistic pathogen. This Gram-positive and rod-shaped bacterium is found ubiquitously in the environment, and in uncooked and processed food. \textit{C. perfringens} can cause several diseases, including gas gangrene, food poisoning, and antibiotic-associated diarrhea. Today, traumatic injury is responsible for up to 70% of the cases of clostridial gas gangrene (Ahrenholz, 1988), and radical amputation is still a favorable treatment of choice for severe \textit{C. perfringens} gas gangrene. The Centers for Disease Control and Prevention (CDC) estimates that \textit{C. perfringens} causes nearly 1 million foodborne illnesses in the United States every year (Grass et al., 2013). Generally, individuals experience abdominal pain, stomach cramps, and diarrhea within 6-24 h after consuming \textit{C. perfringens}-contaminated food. At present, the treatment recommendation is a combination of a broad-spectrum synergistic penicillin combination with clindamycin or a carbapenem, despite growing scientific concern over antibiotic resistance (Lee et al., 2019). Although there has been a great deal of research in understanding pathogenesis, the precise mechanisms by which \textit{C. perfringens} is cleared from the host remain to be unveiled, thus delaying the development of novel strategies for control of this infection.

The host innate immune system provides the first line of recognition and elimination of invading pathogens. The innate immune response to pathogen infection is initiated by the recognition of microbial-derived moieties, termed pathogen-associated molecular patterns (PAMPs), such as exotoxins, nucleic acids, lipopolysaccharide, teichoic-acid, peptidoglycan, and flagellin (Zhivaki and Kagan, 2021; Rathinam et al., 2019). Indeed, PAMPs are recognized by several families of innate receptors, including the NOD-like receptors (Tourlomousis et al., 2020), the Toll-like receptors (Mathur et al., 2012), the C-type lectin receptors (Roessner et al., 2019), the RIG-like helicases (Asaka et al., 2009), and cytosolic DNA sensors (Sisquella et al., 2017), which in turn help to detect invading pathogenic microbes and prevent disease progression. Growing evidence indicates that \textit{C. perfringens} interact with the host to drive the innate response, and Toll-like receptors and NOD-like receptors were implicated in \textit{C. perfringens} recognition (Huang et al., 2019; Yamamura et al., 2019). Hence, investigating the innate immune mechanisms of host resistance to \textit{C. perfringens} will be conducive to implementing pathogen control strategies.
The mixed lineage kinase-like protein (MLKL), is an essential necroptosis effector that operates downstream of the receptor-interacting protein kinase 1 and 3 necrosome complex (Petrie et al., 2018). The MLKL phosphorylation event is thought to trigger a molecular switch leading to the exposure of the N-terminal four-helix bundle structural domain, its oligomerization, membrane translocation, and eventually cell death (Tanzer et al., 2016). As its function as a necroptosis effector was disclosed, research on MLKL has mainly focused on its role in innate immunity when the necroptosis process is initiated. Recently, several studies including ours have demonstrated that MLKL-mediated necroptosis is predominant in serious inflammatory diseases and implicated in pathogens infection (McComb et al., 2014; Yu et al., 2017). Particularly, we have previously reported that non-hematopoietic MLKL contributes to the maintenance of intestinal integrity in a necroptosis-independent manner and protects against Salmonella mucosal infection (Yu et al., 2018). Obviously, necroptosis modulator does not seem to be the sole responsibility of active MLKL.

Here, we sought to characterize the biological implications of MLKL in response to C. perfringens infection, including the possibility that it might elicit innate host immunity. Indeed, we find that MLKL protects the host against C. perfringens gas gangrene and enterocolitis. Mlkl−/− mice exhibit higher mortality rates, more bacterial burden, and severer organ damage compared to control mice. Mechanistically, Mlk1 deficiency leads to impaired K+ efflux-dependent NLRP3 inflammasome signaling and extracellular traps formation, which facilitates bacterial growth and proliferation. Interestingly, MLKL-mediated extracellular traps release in response to C. perfringens is dependent on NLRP3 inflammasome activation. Collectively, our results reveal that MLKL is essential for host defense against C. perfringens infection through enhancing NLRP3 inflammasome-extracellular traps axis to promote bactericidal activity.

RESULTS
Mixed lineage kinase-like protein contributes to host protection against C. perfringens gas gangrene
Using C. perfringens gas gangrene model, we initially explored the possible involvement of MLKL in the host response to C. perfringens infection. WT and Mlkl−/− mice were intramuscularly infected with 5 × 10⁷ CFU of representative C. perfringens strain CP1 (a clinical isolate of C. perfringens, specific 16S rDNA gene sequence data deposited at NCBI GenBank: MW440585), and the mortality of mice were monitored over 72 h. At 22 h p.i., we observed that almost 80% of Mlkl−/− mice had died, whereas all of the WT mice remained alive (Figure 1A). Meanwhile, Mlkl−/− mice exhibited more severe clinical symptoms, demonstrated by grip loss, intense stiff, blackening, deformation, swelling, malaise, and limping (Figure 1B). The experiment was repeated with a lower infective dose of C. perfringens strain CP1 (1 × 10⁵ CFU per mouse) to study the phenotype of Mlkl−/− mice under milder conditions. In accordance with the high level of mortality, the legs of C. perfringens-infected Mlkl−/− mice also tended to be markedly red and swollen, accompanied by extensive hyperemia and hemorrhagic clots (Figure 1C). These clinical assessments were validated by the histologic analysis of muscle. Mlkl−/− mice had more severe histological damage and exacerbated tissue inflammation compared with WT mice, demonstrated by destroyed muscle architecture, elevated cell death, massive PMN infiltration, and increased expression of chemokine KC (Figures 1D–1G). To further determine whether the decreased survival and severe pathology in Mlkl−/− mice during C. perfringens infection might link to higher bacterial burdens, bacterial numbers in muscle were enumerated at 24 h p.i. As expected, significantly more bacteria were detected in the muscles of
Figure 2. Mlkl deficiency leads to increased duodenal injury during C. perfringens mucosal infection
D-4-Amino-3-isoxazolidinone-pretreated WT and Mlkl\(^{+/−}\) mice were orally infected with C. perfringens.
(A) Survival (2 \(\times\) 10\(^8\) CFU, n = 10 each group).
Mixed lineage kinase-like protein is critical in limiting *C. perfringens* enteroocolitis

Next, we asked if MLKL also regulated host defense against *C. perfringens* mucosal infection, as *C. perfringens* is one of the most prevalent foodborne pathogens. WT and Mlkl<sup>−/−</sup> mice were orally infected with 2 × 10<sup>8</sup> CFU of representative *C. perfringens* strain CP1, and animal mortality was then monitored for 72 h. Compared to WT counterparts, Mlkl<sup>−/−</sup> mice demonstrated a significantly lower survival rate. At 24 h p.i., we observed that all of Mlkl<sup>−/−</sup> mice had died, whereas almost 90% of WT mice remained alive (Figure 2A). The experiment was repeated with a sublethal dose of *C. perfringens* strain CP1 (1 × 10<sup>5</sup> CFU per mouse) to study the phenotype of Mlkl<sup>−/−</sup> mice under milder conditions (Figure 2B). In accordance with the high level of mortality, duodenal injury was more severe in Mlkl<sup>−/−</sup> mice compared with WT mice, demonstrated by extensive hyperemia and hemorrhage, complete destruction of epithelial integrity, large mucosal ulcerations, severe villous blunting, goblet cell loss, massive PMN infiltration, and elevated expression of chemokine KC (Figures 2C–2G). Epithelial barrier integrity, as measured by tissue homeostasis, and the expression of mucoproteins and tight junction proteins, was obviously disrupted in Mlkl<sup>−/−</sup> mice. Mlkl deficiency results in greatly increased numbers of TUNEL-positive epithelial cells (Figure 2H), dramatically reduced the expression of AB-PAS staining mucins and mucin 2 (Figures 2I–2K), and significantly decreased the expression of tight junction including zonula occludens (ZO)-1, occludin and claudin 3 (Figures 2L–2O) than in those of WT mice, indicating that MLKL alleviates the disruption of mucosal barrier integrity following *C. perfringens* infection.

To gain additional evidence of the role of MLKL in host defense against mucosal *C. perfringens*, infection, the pathologic changes in cecum were further determined. In accordance with the severe duodenal damage, Mlkl<sup>−/−</sup> mice also tended to lose more cecal weight compared to WT mice (Figure 3A). Meanwhile, this clinical assessment was validated by the histologic analysis of cecum. Mlkl<sup>−/−</sup> mice had severe cecal damage and exacerbated cecal inflammation, demonstrated by complete destruction of epithelial integrity, intense submucosal edema, goblet cell loss, massive inflammatory cells infiltration, and increased expression of chemokine KC (Figures 3B–3E). Similarly, the exaggerated loss of the mucosal barrier integrity in Mlkl<sup>−/−</sup> mice during *C. perfringens* infection, revealed by increased numbers of TUNEL-positive epithelial cells (Figure 3F), reduced the expression of AB-PAS staining mucins and mucin 2 (Figures 3G–3I), and decreased the expression of tight junction such as ZO-1, occludin, and claudin 3 (Figures 3J–3M). Subsequently, to further determine whether a defect in bacterial clearance contributes to the susceptible of Mlkl<sup>−/−</sup> mice during *C. perfringens* mucosal infection, the bacterial load at the mucosal site and systemic site were assessed at 24 h p.i. As compared with WT mice, Mlkl<sup>−/−</sup> mice harbored markedly elevated loads of bacteria in the duodenum, cecum, MLN, liver, and spleen (Figure 3N). Conclusively, these results suggested that MLKL is essential for the host defense against *C. perfringens*-induced enterocolitis.

**Mixed lineage kinase-like protein-mediated inflammasome activation restricts bacterial colonization**

As previous research has shown that inflammasome activation emerges as an important aspect of host defense against invading pathogens (Chudnovskiy et al., 2016; Yu et al., 2018). To confirm whether there is a
difference in inflammasome activation that can explain the increased susceptibility of Mlkl−/− mice to C. perfringens infection, we first evaluated inflammasome signaling. Strikingly, the inflammasome-dependent Caspase-1 cleavage dramatically reduced in the muscle, duodenum, and cecum tissues of infected Mlkl−/− mice relative to the levels observed in those of infected WT mice, although the expression of ASC did not appear significantly different (Figures 4A–4C). Importantly, the production of mature IL-1β and IL-18 was also significantly inhibited in the muscle, duodenum, and cecum tissues of infected Mlkl−/− mice compared with infected WT controls (Figures 4A–4F). However, TNF-α expression was comparable between both genotypes (Figures 4G–4I). Thus, Mlkl deficiency potently results in attenuated inflammasome activation following C. perfringens infection. We next asked if MLKL-mediated inflammasome activation was involved in protection against C. perfringens infection, Mlkl−/− mice were treated prophylactically (day-1 and day 0 of infection) with rIL-18 and determined whether exogenous administration of IL-18 could rescue the susceptibility to infection in the absence of Mlkl. Infected Mlkl−/− mice treated with rIL-18 showed obviously alleviated pathological damage in muscle, duodenum, and cecum tissues (Figures 4J–4L). Indeed, rIL-18 administration also strongly reduced the bacterial burden in Mlkl−/− mice, measured by the levels of viable bacteria in muscle, duodenum, cecum, MLN, liver, and spleen (Figures 4M–4R). Collectively, these results showed that inflammasome activation is critical for MLKL-facilitated pathogen control.

NLRP3 inflammasome is required for mixed lineage kinase-like protein-mediated host defense

To further characterize the role of MLKL in host defense against C. perfringens infection, the expression of phosphorylated MLKL (p-MLKL) in the sections of muscle, duodenum, and cecum was investigated by immunohistochemical staining. Our results indicated that p-MLKL staining was predominantly located in the recruited inflammatory cells of the infected tissues (Figures 5A–5C). Subsequently, to determine whether NLRP3 inflammasome activation is involved in MLKL-mediated host defense, LPS-primed BMDMs derived from WT mice and Nlrp3−/− mice were stimulated with C. perfringens strain ATCC13124, CP1, CP3 or ATP (a conventional NLRP3 inflammasome agonist), respectively. As shown in Figures 5D–5G, 5P, and 5Q, inflammasome signaling was markedly inhibited in Nlrp3−/− macrophages vs. WT macrophages, indicated by impaired Caspase-1 and IL-1β cleavages, decreased mature IL-1β and IL-18 release, and reduced LDH activity and ASC speck formation, whereas LPS-dependent TNF-α secretion was not inhibited in the absence of Nlrp3, indicating that C. perfringens surely induced NLRP3 inflammasome activation. Recently, K⁺ efflux is widely proposed as a specific upstream requirement for NLRP3 inflammasome activation and increasing the extracellular [K⁺] inhibits NLRP3 inflammasome activation (Munoz-Planillo et al., 2013), although there is evidence that some NLRP3 activators induce NLRP3 inflammasome activation in a K⁺ efflux-independent mechanism (Groß et al., 2016). To understand the mechanism whereby C. perfringens induces NLRP3 inflammasome activation, we sought to investigate whether K⁺ efflux is required for C. perfringens-induced NLRP3 inflammasome activation. Extracellular potassium of 50 mM provides obvious inhibition of NLRP3 inflammasome activation by C. perfringens challenged, revealed by impaired Caspase-1, GSDMD, and IL-1β cleavages, and reduced mature IL-1β and IL-18 release, and decreased LDH activity, although LPS-dependent TNF-α secretion was not suppressed by high concentrations of extracellular potassium (Figures 5H–5K). Thus, these findings suggested that C. perfringens induces NLRP3 inflammasome activation in a K⁺ efflux-dependent process. We next asked
Figure 4. Inflammasome signaling downstream of MLKL confers host resistance to *C. perfringens* infection

WT and *Mlk*<sup>−/−</sup> mice were intramuscularly infected with *C. perfringens* (1 × 10<sup>7</sup> CFU, n = 10 each group) for 24 h.  
(A) The muscle tissue lysate was analyzed for Caspase-1, IL-1β, and ASC by Western blotting. GAPDH was used as a loading control.  
(D and G) The homogenate supernatant of the muscle tissue was analyzed for the presence of IL-18, IL-1β, and TNF-α protein using ELISA.  

[Graphs and images showing Western blotting and ELISA results for muscle, duodenum, and cecum from WT and *Mlk*<sup>−/−</sup> mice.]
if Mlkl deficiency impaired K⁺ efflux-dependent NLRP3 inflammasome signaling after *C. perfringens* challenge. Notably, K⁺ efflux-dependent NLRP3 inflammasome signaling was largely inhibited in the absence of Mlkl during *C. perfringens* infection, showed by impaired Caspase-1, GSDMD, and IL-1β cleavages, reduced ASC speck formation, and decreased mature IL-1β and IL-18 release, and reduced LDH activity in the treated Mlkl⁻/⁻ macrophages, whereas TNF-α secretion was not affected (Figures 5L–5Q). Interestingly, Mlkl deficiency also impaired bacterial α-toxin, heat-killed bacteria, and bacterial culture supernatants-triggered inflammasome signaling (Figures 5R–5T). Importantly, exogenous IL-18 treatment could rescue the defect of bacterial killing in Mlkl⁻/⁻, Nlrp3⁻/⁻, and Caspase-1/11⁻/⁻ macrophages, respectively (Figures 5U–5W), indicating that Mlkl deficiency leads to a defect in bacterial killing by impairing NLRP3 inflammasome activation in macrophages.

Subsequently, to further illustrate the signaling mechanism that NLRP3 inflammasome activation mediates THE protective effect of MLKL, we then utilized Nlrp3⁻/⁻ mice to evaluate the effect of NLRP3 on *C. perfringens* gas gangrene and enterocolitis. At 24 h p.i., similar to Mlkl⁻/⁻ mice, Nlrp3⁻/⁻ mice showed increased bacterial loads in muscle, duodenum, cecum, MLN, liver, and spleen (Figures 6A and 6B), indicating that there was a serious defect in the bacterial clearance of Nlrp3⁻/⁻ mice as compared with WT mice. In line with this, Nlrp3⁻/⁻ mice also demonstrated significantly decreased survival (Figures 6C and 6D), obvious clinical symptoms (Figures 6E–6H), and severe histological injury (Figures 6I–6K), suggesting that NLRP3 is beneficial to the host followed *C. perfringens* infection. Altogether, our data demonstrated that the protective effect of MLKL against *C. perfringens* infection is dependent on the capacity of MLKL to enhance NLRP3 inflammasome-mediated bacteria killing.

**Mlkl deficiency results in the defect of extracellular traps formation**

Because Mlkl deficiency attenuates bacterial clearance following *C. perfringens* infection, an extracellular bacterial pathogen, we delve further into the other potential mechanisms underlying MLKL in enhancing bacteria killing such as the process mediated by extracellular traps. Evidence has shown that extracellular traps release provides an extracellular site for microbial killing in the innate immune defense (Brinkmann et al., 2004; Chen et al., 2019). Factually, we find that the defect of bactericidal activity is mediated by decreased extracellular trap formation in macrophages and neutrophils in the presence of DNase I (Figures S1A and S1B). To evaluate the effect of Mlkl deficiency on extracellular traps formation, *C. perfringens*-challenged macrophages were contained with SYTOX Orange (a non-permeable dye that stains nucleic acid) and histone or MPO, pivotal components of extracellular traps. As expected, extracellular traps release was dramatically decreased in Mlkl⁻/⁻ macrophages vs. WT macrophages (Figures 7A and 7B). To quantify extracellular traps formation, extracellular DNA content was determined in the supernatants. A reduction of extracellular DNA was seen in Mlkl⁻/⁻ macrophages compared with WT macrophages (Figure 7C). Moreover, as an initial description of extracellular traps formation appeared in neutrophils (Brinkmann et al., 2004), another type of phagocyte that plays critical role in host’s defense against infection, we further determined if there is a defect of extracellular trap formation in Mlkl⁻/⁻ neutrophils. A similar defect of extracellular traps formation was observed in bacteria-infected Mlkl⁻/⁻ neutrophils vs. WT neutrophils (Figures S2A–S2C). Correspondingly, Mlkl deficiency inhibits the bacterial killing capacity of neutrophil (Figure S2D). Similarly, the expression level of histone was significantly reduced in the muscle, duodenum, and cecum tissues of infected Mlkl⁻/⁻ mice than in those of infected WT mice (Figures 7D and 7E). Thus, these observations suggested that Mlkl deficiency also resulted in the defect of extracellular traps-mediated bacteria killing.
To determine the bacterial killing capacity of BMDMs, LPS-primed WT, Mlkl−/−, and Nlrp3Δ/Δ mice were intramuscularly infected with C. perfringens (1 × 10^7 CFU) for 24 h. Representative muscle sections. p-MKL (IHC) stained brown (upper panel, magnification ×100, lower panel, magnification ×400). D-4-Amino-3-isoxazolidinone-pretreated WT and Mlkl−/− mice were orally infected with C. perfringens (1 × 10^7 CFU) for 24 h. (B and C) Representative duodenum and cecum sections. p-MKL (IHC) stained brown (upper panel, magnification ×100, lower panel, magnification ×200). LPS-primed WT and Nlrp3Δ/Δ BMDMs were stimulated with ATP (5mM, 30min), C. perfringens strain ATCC13124, CP1 or CP3 (MOI = 20, 90 min). (D) Cell supernatants and cell extracts immunoblotted for Caspase-1, IL-1β and ASC. GAPDH served as loading controls. (E and F) Culture supernatants were analyzed for IL-1β, IL-18 and TNF-α by ELISA. (G) LDH release was quantified to monitor cell lysis. Data are shown as the percentage of LDH release by Triton X-100 treated control cells. (H) Cell supernatants and cell extracts immunoblotted for Caspase-1, GSDMD, IL-1β, ASC, and GAPDH. (I and J) Culture supernatants were analyzed for IL-1β, IL-18, and TNF-α by ELISA. (K) LDH release was quantified to monitor cell lysis. Data are shown as the percentage of LDH release by Triton X-100 treated control cells. LPS-primed WT, Mlkl−/− and Nlrp3Δ/Δ BMDMs were stimulated with ATP (5mM, 30min), C. perfringens strain ATCC13124, CP1, or CP3 (MOI = 20, 90 min). (L) Cell supernatants and cell extracts immunoblotted for Caspase-1, GSDMD, IL-1β, ASC, p-MKL, and GAPDH. (M and N) Culture supernatants were analyzed for IL-1β, IL-18 and TNF-α by ELISA. (O) LDH release was quantified to monitor cell lysis. Data are shown as the percentage of LDH release by Triton X-100 treated control cells. LPS-primed WT, Mlkl−/− and Nlrp3Δ/Δ BMDMs were stimulated with ATP (5mM, 30min), C. perfringens strain ATCC13124, CP1, or CP3 (MOI = 20, 90 min). (P and Q) The cells were fixed, permeabilized, and stained for ASC (green). DAPI was used to label nuclei (blue). magnification, ×400. The percentage of cells containing ASC speckles was quantified. LPS-primed WT and Mlkl−/− BMDMs were stimulated with live bacteria (MOI = 20, 90 min), heat-killed bacteria (HK, MOI = 20, 90 min), bacterial culture supernatants (SN, 5 h) or bacterial α-toxin (20 μg/mL, 5 h). (R and T) Culture supernatants were analyzed for IL-1β, IL-18, and TNF-α by ELISA. (U–W) To determine the bacterial killing capacity of BMDMs, LPS-primed WT, Mlkl−/−, Nlrp3Δ/Δ, or Caspase-1/11−/− BMDMs were incubated with rIL-18 (1000 pg/mL) or PBS for 1 h before were infected with C. perfringens strain ATCC13124, CP1 or CP3 (MOI = 5) for 6 h, the supernatants were collected and plated on BHI agar plates to enumerate the bacteria after 24 h of anaerobic culture. Graphs are means ± SD from data pooled from four to six (E, F, G, I, J, K, M, N, O, Q, R, S, T, U, V, and W) biological replicates. Data were considered significant when *p < 0.05 or **p < 0.01.

### DISCUSSION

Our uncover the unexpected finding that MLKL promotes bacterial killing and clearance by enhancing NLRP3 inflammasome-extracellular traps axis as a host defense against C. perfringens infection. Acting as a necroptosis effector, MLKL plays significant role in innate immunity. Recently, several studies including ours showed that MLKL is implicated in sensing pathogens infection (Kitur et al., 2016; Yu et al., 2018). However, little is known about the biological implications of MLKL in the infection of C. perfringens, an extracellular bacterial pathogen. C. perfringens infection usually begins suddenly and lasts for less than 24 h, thus, it is hard to detect early and treat timely, resulting in a huge burden to public health and animal husbandry worldwide. The development of new preventive and therapeutic strategies to combat C. perfringens infection has become more urgent. The host innate immune system provides the first line of defense for the early recognition and elimination of invading pathogens. Therefore, a detailed...
Caspase-1, GSDMD, and IL-1β in C. perfringens infection. Thus, our findings show that NLRP3 promotes host survival and bacterial clearance, and enhances host defense against C. perfringens.

In vivo studies indicated that MLKL is involved in the NLRP3 inflammasome activation by bacterial stimuli. Consistent with this finding, we observed Nlrp3 deficiency obviously restricts C. perfringens infection, reflected by remarkably alleviated organs damage and decreased bacteria colonization.

Using Mlkl−/− mice, we initially demonstrate that MLKL promotes host survival and bacterial clearance during C. perfringens femoral muscle and mucosal infection, highlighting a novel role of MLKL in host defense against C. perfringens infection. To identify the potential mechanisms underlined MLKL-mediated defense, we set out to characterize the inflammasome signaling. Mlkl deficiency leads to inferior inflammasome activation in the muscle, duodenum, and cecum tissues during C. perfringens infection. Recent studies have shown that inflammasome activation participates in serious inflammatory diseases and is sufficient to protect the host against pathogens invading (Song-Zhao et al., 2014; Sellin et al., 2014). We hypothesized that the inflammasome signaling regulates MLKL-mediated host protection against C. perfringens. In line with our hypothesis, administration of exogenous IL-18 could rescue the susceptibility of Mlkl−/− mice to C. perfringens infection, reflected by remarkably alleviated organs damage and decreased bacteria colonization. Hence, it is quite possible that MLKL protects against C. perfringens infection by enhancing inflammasome activation.

Owing to MLKL mainly located in the recruited inflammatory cells, BMDMs were used to delve into the molecular mechanisms of MLKL-mediated inflammasome signaling. Increased evidence showed necroptotic stimuli-activated MLKL can promote NLRP3 inflammasome activation in a cell-intrinsic manner (Conos et al., 2017; Gutierrez et al., 2017). Although NLRP3 inflammasome can be initiated by C. perfringens (Yamamura et al., 2019), the understanding of regulatory mechanisms of NLRP3 inflammasome activation is limited. Consistent with this finding, we observed Nlrp3 deficiency obviously restricts C. perfringens-triggered inflammasome signaling. To date, three mechanisms for NLRP3 inflammasome activation have been proposed (Bueer et al., 2014), including K+ efflux (Munoz-Planillo et al., 2013), reactive oxygen species (ROS) generation (Zhou et al., 2011), and lysosomal destabilization (Hornung et al., 2008). Strikingly, we further found that C. perfringens elicits NLRP3 inflammasome activation in a K+ efflux-dependent manner. Subsequently, we characterized whether MLKL modulates C. perfringens-stimulated K+ efflux-dependent NLRP3 inflammasome activation. Consistent with in vivo data, Mlkl deficiency significantly impaired C. perfringens-induced inflammasome signaling via the NLRP3-ASC axis, demonstrated by eliminated Caspase-1, GSDMD, and IL-1β cleavages, decreased ASC speck formation, and reduced mature IL-1β and IL-18 release and LDH activity compared with WT macrophages. More importantly, Mlkl−/−, Nlrp3−/−, and Caspase-1/11−/− macrophages are unable to effectively control of multiplication of C. perfringens, and exogenous IL-18 treatment could rescue the defect in bacteria control. Used Nlrp3−/− mice, we further show that NLRP3 promotes host survival and bacterial clearance, and enhances host defense against C. perfringens infection. Thus, our in vivo and in vitro findings show that NLRP3 inflammasome is required for the protective effects of MLKL during bacterial infection. Interestingly, inflammasome signaling induced by bacterial α-toxin, heat-killed bacteria, or bacterial culture supernatants, was prominently inhibited in the absence of Mlkl, we suspect that active MLKL signaling may lead to enhancing the state of host immune response and limiting C. perfringens pathogenesis, although perfringolysin O (PFO), a key virulence factor of C. perfringens, was reported as an essential bacterial factor for triggering NLRP3 inflammasome activation (Yamamura et al., 2019). Although understanding the underlying mechanism of MLKL signaling and NLRP3 inflammasome signaling activation by C. perfringens awaits future investigation, this study extends our understanding of the biological implication of MLKL in host intrinsic immune responses.
Figure 7. Extracellular traps formation contributes to MLKL-mediated host defense

LPS-primed WT and Mlkl<sup>−/−</sup> BMDMs were infected with C. perfringens strain ATCC13124, CP1, or CP3 (MOI = 20, 90 min).

(A) DNA decorated with histone and MPO within the extracellular traps structures was detected by immunofluorescence. Histone (green), MPO (green) and DNA (orange/blue). magnification, ×400.
Extracellular traps, a process by which innate myeloid cells such as neutrophils (Brinkmann et al., 2004), macrophages (Chow et al., 2010), basophils (Morshed et al., 2014), eosinophils (Yousefi et al., 2008), and mast cells (von Kockritz-Blickwede et al., 2008) kill invading pathogens, is the indispensable host intrinsic defense mechanism that differs from phagocytosis and degranulation. Activated innate myeloid cells release these web-like structures composed of decondensed chromatin and several bactericidal cellular proteins that provide extracellular sites for trapping and inhibiting a broad range of microorganisms. Meanwhile, we find that a defect in bacterial killing by impairing extracellular trap formation in macrophages and neutrophils. As MLKL is essential for the host defense against bacterial infection through enhancing bactericidal activity. We speculate whether MLKL also participates in the regulation of extracellular traps-dependent bacterial killing process. As expected, Mlkl deficiency significantly impaired C. perfringens-triggered extracellular traps release in macrophages and neutrophils. In line with this, a defect of extracellular traps formation was observed in bacteria-infected muscle, duodenum, and cecum tissues of C. perfringens infection. As master of key hub in enhancing the NLRP3 inflammasome-extracellular traps axis to strengthen bacterial eradication and host defense.

Raf-MEK-ERK signaling has been proved to be required for several stimuli-elicited extracellular traps formation (Hakkim et al., 2011). Raf-MEK-ERK pathway is also induced by C. perfringens. We hypothesized that the defect of raf-MEK-ERK signaling may lead to impairing C. perfringens-triggered extracellular traps release in the absence of Mlk1. Unexpectedly, Mlk1 deficiency does not impair C. perfringens-induced raf-MEK-ERK signaling, indicating that raf-MEK-ERK signaling is dispensable for MLKL-mediated extracellular traps formation. Recently, extracellular traps formation is driven by inflammasome-dependent IL-1β and IL-18 (Huang et al., 2020). Additionally, noncanonical inflammasome signaling can also elicit extracellular traps release via GSDMD activation (Chen et al., 2018). Here, we report a novel discovery of MLKL in the context of C. perfringens infection, which promotes classical extracellular trap formation in a NLRP3 inflammasome-dependent manner, in turn, enhances NLRP3 inflammasome-extracellular traps axis to strengthen bacterial control and host defense.

In summary, in the present study, we provide the first evidence demonstrating the importance of MLKL in mediating a protective innate immune response and preventing severe C. perfringens gas gangrene and enterocolitis. This information increases our understanding of the beneficial role of MLKL in host defense against C. perfringens infection. As master of key hub in enhancing the NLRP3 inflammasome-extracellular traps axis, MLKL improved host survival, induced the resolution of organs damage and inflammation, and accelerated bacterial eradication, supporting that it will be of interest to dissect whether pharmacological and vaccine activation of the MLKL-NLRP3 inflammasome-extracellular traps pathway confers protection in pathogens invading.

Limitations of the study

Our study provides an unexpected discovery of MLKL in the context of C. perfringens infection, which promotes NLRP3 inflammasome-extracellular traps axis to strengthen bacterial eradication and host defense. We provided a certain clue that NLRP3 inflammasome signaling is involved in MLKL-mediated extracellular traps formation, but the detailed downstream mechanism remains unclear. As MLKL is the critical terminal executor of necroptosis, it is worthwhile to investigate the biological significance of necroptosis following C. perfringens infection in future studies. Also, our results showed that C. perfringens α-toxin may serve as an agonist of MLKL signaling. Although additional studies are needed to better explore the relationship between C. perfringens α-toxin and active MLKL signaling, and which is critical for the design of potential therapeutic interventions.
Figure 8. Blocking NLPR3 inflammasome signaling attenuates MLKL-mediated extracellular traps formation following C. perfringens challenge

LPS-primed WT and Mlkl−/− BMDMs were infected with C. perfringens strain ATCC13124, CP1, or CP3 (MOI = 20, 90 min).

(A) Cell extracts immunoblotted for p-P38, P38, p-JNK, JNK, p-ERK1/ERK2, ERK1/ERK2, and GAPDH. Cells stimulated with zymosan (1 mg/mL) were used as positive controls. LPS-primed WT, Mlkl−/−, Nlrp3−/−, and Caspase-1/11−/− BMDMs were treated with or without rIL-18 (1000 pg/mL) for 1 h before were infected with C. perfringens strain ATCC13124, CP1 or CP3 (MOI = 20, 90 min).
ETICS STATEMENT
All animal studies were conducted according to experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Inner Mongolia University ([2020] 022).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105121.

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AUTHOR CONTRIBUTIONS

Y.L., L.-H.X., and S.-X.Y. designed experiments. Y.L., L.-H.X., F.-X.L., N.W., Y.-Z.M., J.-W.L., Y.-J.W., J.L., Y.-X.L., X.-Y.W., and F.-H.M. performed the experiments and analyzed the data. Y.L. wrote the article. Y.-J.Y., G.-P.L., X.W., and S.-X.Y. revised the article. All authors read and approved the final article.
DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects.

We worked to ensure diversity in experimental samples through the selection of the cell lines.

We worked to ensure diversity in experimental samples through the selection of genomic datasets.

While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-ASC            | Adipogen | Cat # A29151803f |
| Alexa Fluor® 488-conjugated anti-rabbit IgG secondary antibody | Abcam | Cat # ab150077 |
| Anti-MPO            | Biorbyt | Cat # orb16003; RRID: AB_10752135 |
| Anti-ELANE          | AbClonal | Cat # A13015; RRID: AB_2759862 |
| Anti-histone        | AbClonal | Cat # A2348 |
| Anti-Ly-6G/Ly-6c    | BioLegend | Cat # 108419; RRID: AB_493480 |
| Anti-F4/80          | BioLegend | Cat # 123119; RRID: AB_893491 |
| Anti-mucin 2        | Santa Cruz | Cat # sc-515032; RRID: AB_2815005 |
| Anti-claudin 3      | Abcam | Cat # A2946 |
| Anti-p-MLKL         | Abcam | Cat # ab196436 |
| Anti-Caspase-1      | Adipogen | Cat # A28881708 |
| Anti-IL-1β          | Adipogen | Cat # PRP1119 |
| Anti-GSDMD          | Santa Cruz | Cat # sc-393656 |
| Anti-P38 MAPK       | AbClonal | Cat # A4771; RRID: AB_2863345 |
| Anti-p-P38 MAPK     | AbClonal | Cat # APO526 |
| Anti-JNK            | Proteintech | Cat # 66210-1-lg |
| Anti-p-JNK          | AbClonal | Cat # APO276 |
| Anti-ERK1/2         | AbClonal | Cat # A10613 |
| Anti-p-ERK1/2       | AbClonal | Cat # APO472 |
| Anti-GADPH          | Roteintech | Cat # 60004-1-lg |
| **Bacterial and virus strains** | | |
| C. perfringens strain ATCC13124 | Guangdong Huankai Microbial Sci.&Tech.CO.,Ltd | FSCC137003 |
| C. perfringens strain CP1 | This paper | NCBI GenBank: MW440585 |
| C. perfringens strain CP3 | This paper | NCBI GenBank: MW440587 |
| **Chemicals, peptides, and recombinant proteins** | | |
| RPMI1640 medium     | Gibco | Cat # 31800-022 |
| FBS fetal bovine serum | Gibco | Cat # A31608-02 |
| Penicillin          | Gibco | Cat # 15140-122 |
| Streptomycins       | Gibco | Cat # 15140-122 |
| D-4-Amino-3-isoxazolidinone | Coolaber | Cat # CA1662 |
| Recombinant IL-18   | Novoprotein | Cat # CX06 |
| LPS                 | Invivogen, Inc. | Cat # tbl-3鹈ps |
| Potassium chloride  | Sigma-Aldrich | Cat # 109360250 |
| ATP                 | Sigma | Cat # A2383 |
| DAPI                | Solarbio | Cat # C0065 |
| Sytox Orange        | Invitrogen | Cat # S-11368 |
| Hoechst 33342       | Wanleibio | Cat # WLA042a |
| Sytox Green         | Invitrogen | Cat # S7020 |
| Formalin solution   | Macklin | Cat # P804536 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Hematoxylin & eosin | Solarbio | Cat # SL7050-500 |
| Triton X-100        | Sigma-Aldrich | Cat # 93443 |
| Complete protease inhibitor cocktail | Sigma-Aldrich | Cat # 9036-19-5 |
| TRI-reagent         | Sigma-Aldrich | Cat # T9424 |
| SYBR Green          | Roche | Cat # 04913914001 |
| Tris-HCl            | Sigma-Aldrich | Cat # T9424 |
| NaCl                | Sigma-Aldrich | Cat # S3014 |
| Na<sub>3</sub>VO<sub>4</sub> | Sigma-Aldrich | Cat # S 6508 |

### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse bone marrow neutrophils isolation kit® | TianJin 371 HaoYang Biological Manufacture Co | Cat # LZS1100 |
| AB-PAS Stain Kit   | Solarbio, | Cat # 1285 |
| LDH Cytotoxicity Assay kit® | Beyotime Biotechnology | Cat # C0017 |
| TUNEL staining kit | KeyGEN BioTECH | Cat # KGA7061 |
| TNF-α ELISA Kit    | R&D | Cat # MTA00B |
| IL-1β ELISA Kit    | R&D | Cat # ML800C |
| IL-18 ELISA Kit    | Solarbio | Cat # SEKM-0019 |
| BCA Protein Quantification Kit | Thermo | Cat # 23225 |

### Experimental models: Cell lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse BMDM          | This paper | N/A |
| Mouse bone marrow-derived neutrophils | This paper | N/A |

### Experimental models: Organisms/strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: Wildtype:CS7BL/6J | Jackson Laboratory | Strain # 000664 RRID:IMSR_JAX:000664 |
| Mouse: Mlk1<sup>−/−</sup> | (Wu et al., 2013) Gift by Dr. Jia-Huai Han (Xiamen University, China) | N/A |
| Mouse: Nlrp3<sup>−/−</sup> | Jackson Laboratory | Strain # 021302 RRID:IMSR_JAX:021302 |
| Mouse: Caspase1/11<sup>−/−</sup> | (Thurston et al., 2016) Gift by Dr. Feng Shao (National Institute of Biological Sciences, Beijing, China) | N/A |

### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GADPH-F: CACCCCGACAGGACACTGAGCAAG | This paper | N/A |
| GADPH-R: GGGGGTTCTGGGATGGAATTTGAG | This paper | N/A |
| Occludin-F: CAGCCCTTCGCTCTCATCG | This paper | N/A |
| Occludin-R: GTCGGGTCCACTCCCATTA | This paper | N/A |
| ZO-1-F: GACCTTGAGCAGCCGTCTATA | This paper | N/A |
| ZO-1-R: CGGTAGGGGATGTGTCATAGGT | This paper | N/A |
| Claudin 3-F: CCTAGGAACTGTCCAAGCCG | This paper | N/A |
| Claudin 3-R: CCC- GTTCATGTTTGCCGTC | This paper | N/A |

### Software and algorithms

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Prism9              | GraphPad Software | [https://www.graphpad.com/](https://www.graphpad.com/) |
| ImageJ(Fiji)        | [Schindelin et al., 2012](https://fiji.sc) | [https://fiji.sc](https://fiji.sc) |
RESOURCE AVAILABILITY

Lead contact

Further information and requests for data should be directed to and will be fulfilled by the lead contact, Shui-Xing Yu (shuixingyu@imu.edu.cn).

Materials availability

This study did not generate new materials or reagents.

Data and code availability

- Information on the C. perfringens clinical isolates have been deposited in NCBI GenBank and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All data produced or analyzed for this study are included in the published article and its supplementary files, and will be shared by the lead contact upon request.

- This paper does not report original code.

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

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mikil−/− mice were a gift from Dr. Jia-Huai Han (Xiamen University, China) (Wu et al., 2013). Nlrp3−/− mice (Jackson Laboratory, Strain #:021302 RRID: IMSR_JAX:021302) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Caspase-1/11−/− mice were kindly provided by Dr. Feng Shao (National Institute of Biological Sciences, Beijing, China) (Thurston et al., 2016). They were subsequently backcrossed onto the C57BL/6J (Jackson Laboratory, Strain #:000664 RRID: IMSR_JAX:000664) background for eight generations and heterozygous breeding pairs were used to generate WT mice. The mice were housed in a pathogen-free facility, with sterile food and water in the animal house of the Laboratory Animal Center of Inner Mongolia University. All animal studies were conducted according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Inner Mongolia University ([2020]022).

Cell lines

Bone marrow-derived macrophages (BMDMs) were prepared and cultured as previously described (Yu et al., 2015). In brief, BMDMs were isolated from mouse femurs of six to eight week old mice and cultured in RPMI1640 medium (Gibco, #31800-022) containing 10% FBS fetal bovine serum (Gibco, #A31608-02), 25% L929 cell–conditioned medium, 100 U/mL penicillin (Gibco, #15140-122), and 100 U/mL streptomycins (Gibco, #15140-122), at 37°C in a humidified atmosphere containing 5% CO2. Matured BMDMs were harvested for assays at day 7 of differentiation. Neutrophils were isolated by using the commercially available mouse bone marrow neutrophils isolation kit® (TianJin HaoYang Biological Manufacture Co., #LZS1100, China) according to the manufacturer’s instructions.

C. perfringens strain CP1 for establishment of a mouse model of gas gangrene

For the gas gangrene model, representative C. perfringens strain CP1 (a clinical isolate of C. perfringens, specific 16S rDNA gene sequence data deposited at NCBI GenBank: MW440585) was grown to exponential phase in brain heart infusion broth (BHI, Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co., Ltd, #HB8297-5, China) under anaerobic conditions at 37°C. Six to eight weeks old sex-matched mice were intramuscularly infected with 1 x 10^7 colony-forming units (CFUs) of C. perfringens strain CP1 diluted in PBS in a total volume of 100 μL. At 24 h.p.i., mice were euthanized to collect infected leg muscle for quantification of bacterial burden. For the survival study, mice were intramuscularly challenged with 5 x 10^7 CFUs of C. perfringens strain CP1. C. perfringens gas gangrene was assessed in a blinded manner using an amended version of a previously described scoring criteria (Awad et al., 2001). In brief, the severity of grip, deformation, stiffness, blackening, swelling, limping, and malaise pathology parameters were scored as either 0 (no discernible pathology), 0.5 (some demonstrable pathology), 1 (mild pathology), 1.5 (clearly noticeable pathological changes), 2 (moderate pathology), 2.5 (marked pathology apparent) or 3 (near death).
EnteroColitis model
To induce enterocolitis, following the administration of 0.4 mg of D-4-Amino-3-isoxazolidinone (Coolaber, #CA1662) per mouse, six to eight weeks old sex-matched mice were orally challenged with 1 x 10^8 CFUs of representative C. perfringens strain CP1 diluted in PBS in a total volume of 100 μL. At 24 h p.i., mice were euthanized to collect duodenum, cecum, spleen, liver, and MLN for quantification of bacterial burden. Tissue samples of duodenum and cecum were fixed in buffered formalin solution (4%) and embedded in paraffin. Sections (5 μm thick) were then stained with hematoxylin and eosin (H&E, Solarbio, #SL7050-500). C. perfringens-induced enterocolitis was assessed in a blinded manner using an amended version of a previously described score (Erben et al., 2014; Lei et al., 2016). In brief, duodenum histopathology scores for damage were determined as follows: inflammatory cell infiltrate (scores 0 to 4), mucosal ulcerations (scores 0 to 4), goblet cell loss (scores 0 to 4) and villous blunting (scores 0 to 4). Cecum pathology scores were determined as follows: submucosal edema (scores 0 to 3), goblet cell depletion (scores 0 to 3), epithelial integrity (scores 0 to 3), and polymorphonuclear leukocyte (PMN) infiltration (scores 0 to 4). For the survival study, mice were orally infected with 2 x 10^8 CFUs and 1 x 10^8 CFUs of C. perfringens strain CP1, respectively.

METHOD DETAILS
Administration of recombinant IL-18
Milkil−/− mice were i.p. injected recombinant IL-18 (Novoprotein, #CK06) at a dose of 1.0 μg per mouse in 100 μL PBS on day-1 and day 0. The mice were intramuscularly or orally infected with 1 x 10^7 or 1 x 10^8 CFUs of representative C. perfringens strain CP1 on day 0. At 24 h p.i., tissue samples of muscle, duodenum and cecum were fixed in 4% neutral buffered formalin and sections were stained with H&E to examine morphologic changes. Meanwhile, aseptically excised tissues were homogenized mechanically in cold PBS (at a ratio of 4 mL per gram tissue). Serial dilutions of tissue homogenates were plated on agar plates and Log CFUs per organ was determined after 24 h of anaerobic culture.

Inflammasome assays
Matured BMDMs were primed with LPS (500 ng/mL, Invivogen, #tlrl-3pelps) for 4 h in serum-free RPMI-1640 medium and then washed twice with RPMI-1640 medium. The cells were then treated with or without potassium chloride (Sigma-Aldrich, #1049360250) 30 min before stimulation with C. perfringens strain ATCC13124 (Guangdong Huankai Microbial Sci. & Tech. CO., Ltd, #FSCC137003), CP1 or CP3 (clinical isolates of C. perfringens, specific 16S rDNA gene sequence data deposited at NCBI GenBank: MW440585 and MW440587) at a multiplicity of infection (MOI = 20) for 90 min, or one of the following stimulators: ATP (5 mM, 30 min, Sigma, #A2383), heat-killed bacteria (HK, MOI = 20, 90 min), bacterial culture supernatants (SN, 5 h), and bacterial α-toxin (20 μg/mL, 5 h), respectively. After treatment, the cell supernatants and lysates were collected for ELISA and western blotting assay. The LDH activities in the supernatants were determined by the LDH Cytotoxicity Assay kit® (Beyotime Biotechnology, #C0017) according to the manufacturer’s protocols. Percent LDH release was calculated as (mean OD value of sample – mean OD value of blank)/(mean OD value of 1% TritonX-100 control sample – mean OD of blank) x 100. To analyze ASC speckles, the cells were stained with ASC primary antibody (Adipogen, #A29151803) and Alexa Fluor® 488-conjugated anti-rabbit IgG secondary antibody (Abcam, #ab150077). The nuclei were stained with DAPI (2 μg/mL), Solarbio, #C0065). ASC speckles were visualized on a Laser Co-focus light microscopy (Nikon) and images were taken. To determine the bacterial killing capacity of BMDMs, LPS-primed BMDMs were incubated with rIL-18 (1000 pg/mL) or PBS for 1 h before infected with C. perfringens strain ATCC13124, CP1 or CP3 (MOI = 5) for 6 h, the supernatants were collected and plated on BHI agar plates to enumerate the bacteria after 24 h of anaerobic culture.

Extracellular traps formation assays
The matured BMDMs were seeded on 12-mm 0.01% poly-L-lysine–coated coverslips in 24 well-plates and then were incubated with LPS for 4 h before being infected with C. perfringens strain ATCC13124, CP1 or CP3 at a multiplicity of infection (MOI = 20) for 90 min, respectively. Cells were counterstained with Sytox Orange (5 μM, Invitrogen, #S-11368) and Hoechst 33342 (2 μM, Wanleibio, #WLA042a), and then were incubated with MPO primary antibody (Biorbyt, #orb16003), ELANE Polyclonal antibody (ABclonal, #A13015) or histone primary antibody (Abclonal, #A2348) and Alexa Fluor® 488-conjugated anti-rabbit IgG secondary antibody (Abcam, #ab150077). Extracellular traps were visualized on a Laser Co-focus light microscopy (Nikon) and images were analyzed (Schindelin et al., 2012). To quantify extracellular traps formation,
extracellular DNA content in the supernatants were determined. LPS primed-BMDMs were stimulated with bacteria (MOI = 20), Sytox Green (5 μM, Invitrogen, #S7020) was added after 90 min. The cell supernatants were collected for fluorescence assay using a Varioskan Flash plate reader (Thermo Scientific).

**Tissue histology and immunostaining**

For histology, tissue samples of muscle, duodenum and cecum were fixed in 4% buffered formalin (Macklin, #P804536) solution and sections were stained with H&E to examine morphologic changes. For immunohistochemistry, tissue sections were stained with Ly-6G/Ly-6c (BioLegend, #108419), F4/80 (BioLegend, #123119), mucin 2 (Santa Cruz, #sc-515032), claudin 3 (Abcam, #A2946), and p-MLKL (Abcam, #ab196436) antibodies. For immunofluorescence, sections were stained with histone primary antibody (Abclonal, #A2348) and Alexa Fluor® 488-conjugated anti-rabbit IgG secondary antibody (Abcam, #ab150077). Meanwhile, apoptotic cell death in tissues were analyzed by TUNEL staining using a commercial kit (KeyGEN BioTECH, #KGA7061) following the manufacturer’s instructions. DAPI (2 μg/mL) was used to stain nuclei. After sequential excitation, images were acquired utilizing a Laser Co-focus light microscopy (Nikon). For mucins secretion and glycosylation patterns assays, duodenal and cecal tissues were collected and stained with alcian blue as well as periodic acid–Schiff’s reagent (AB-PAS staining, Solarbio, #1285) according to the manufacturer’s manual.

**Cytokine release analysis**

Aseptically excised tissues were homogenized mechanically in cold PBS (at a ratio of 4 mL per gram tissue) containing 1% Triton X-100 (Sigma-Aldrich, #93443) and complete protease inhibitor cocktail (Sigma-Aldrich, #9036-19-5). Concentrations of various cytokines/chemokines in tissue homogenates or cell culture supernatants were measured by an ELISA assay following the R&D systems instruction.

**Real-time PCR**

Total RNA was extracted using TRI-reagent (Sigma-Aldrich, #T9424) according to the manufacturer’s instruction. Subsequently, Quantitative real-time PCR assays were performed using SYBR Green (Roche, #04913914001) on an IQ5 Real-Time PCR Detection System (Bio-Rad). Gene expression levels were calculated using the 2^{-ΔΔCt} method. The following primer sequences were used: GAPDH sense 5’-CACCCCA GCAAGGACACTGAGGAG-3’, antisense 5’-GGGGTCTGGGATGGAAATTGTGAG-3’. Ocludin sense 5’-CAGCCTTTGCTCTACCAGC-3’, antisense 5’-GTCGGGTTCACTCCCATTA-3’. ZO-1 sense 5’-GACCT TGAGCAGCCGTCATA-3’, antisense 5’-CCGTAGGCGATGGTCATAGTT-3’. Claudin 3 sense 5’-CCTAGGCAGATGGTCATAGT-3’, antisense 5’-CCCCTTTTCATGTTTGCTG-3’. ERK1/2 sense 5’-ACACGGAGATTGGTCTCTG-3’, antisense 5’-GTTGTAAGCAATGGATTC-3’.

**Immunoblotting**

Stimulated BMDMs or tissues were homogenized in lysis buffer solution [1% Triton X-100, 50 mM Tris-HCl (pH 7.4, Sigma-Aldrich, #1081284001), 150 mM NaCl (Sigma-Aldrich, #S3041), 0.1 mM Na3VO4 (Sigma-Aldrich, #S6508)] supplemented with a complete protease inhibitor cocktail, and supernatants of stimulated BMDMs were harvested and precipitated by methanol chloroform extraction to collect the cell lysate. The total lysates were separated by SDS-PAGE and transferred to PVDF membrane. The membranes were blotted with primary antibodies against Caspase-1 (Adipogen, #A28881708), IL-1β (Adipogen, #PRP119119-20μg), ASC (Adipogen, #A29151803), GSDMD (Santa Cruz, #sc-393656), P38 MAPK (Abclonal, #A4771), p-P38 MAPK (Abclonal, #AP0526), JNK (Proteintech, #66210-1-lg), p-JNK (Abclonal, #AP0276), ERK1/2 (Abclonal, #A10613), p-ERK1/2 (Abclonal, #AP0472), and GADPH (Roteintech, #60004-1-lg).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Each experiment included 10 mice per group. Age-matched mice of both genders were included and randomly assigned to groups. Date are represented as mean ± SD. Differences between mean values of normally distributed data were assessed with one-way ANOVA (Dunnett’s t-test) and two-tailed Student’s t-test. Log-rank test was used for statistical analysis of animal mortality. *p < 0.05 and **p < 0.01 compared with control group. Statistical analysis was performed using Prism9 (GraphPad Software, La Jolla, CA, USA). The statistical values and details of the experiments can be found in the results, figure legends, and respective figures.