Kaempferol-Driven Inhibition of Listeriolyisin O Pore Formation and Inflammation Suppresses *Listeria monocytogenes* Infection

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**ABSTRACT** *Listeria monocytogenes* remains a nonnegligible cause of foodborne infection, posing a critical threat to public health. Under the global antibiotic crisis, novel alternative approaches are urgently needed. The indispensable role of listeriolyisin O (LLO) in the intracellular life cycle, barrier penetration, colonization, and systemic dissemination of *L. monocytogenes* renders it a potent drug target, which means curbing *L. monocytogenes* via interfering with LLO-associated pathogenic mechanisms. Here, we identified kaempferol, a natural small molecule compound, as an effective LLO inhibitor that engaged the residues Glu437, Ile468, and Tyr469 of LLO, thereby suppressing LLO-mediated membrane perforation and barrier disruption. Moreover, we found that kaempferol also suppressed host-derived inflammation in a distinct way independent of LLO inhibition. The in vivo study revealed that kaempferol treatment significantly reduced bacterial burden and cytokine burst in target organs, thereby effectively protecting mice from systemic *L. monocytogenes* infection. Our findings present kaempferol as a potential therapeutic application for *L. monocytogenes* infection, which is less likely to induce drug resistance than antibiotics because of its superiority of interfering with the pathogenesis process rather than exerting pressure on bacterial viability.

**IMPORTANCE** Currently, we are facing a global crisis of antibiotic resistance, and novel alternative approaches are urgently needed to curb *L. monocytogenes* infection. Our study demonstrated that kaempferol alleviated *L. monocytogenes* infection via suppressing LLO pore formation and inflammation response, which might represent a novel antimicrobial-independent strategy to curb listeriosis.

**KEYWORDS** *Listeria monocytogenes*, anti-infection, anti-inflammation, kaempferol, listeriolyisin O (LLO) inhibitor

*L. monocytogenes* is an opportunistic pathogen responsible for listeriosis, a sporadic disease associated with the consumption of highly contaminated food, especially ready-to-eat food products. In China, the foodborne proportion of *L. monocytogenes* is up to 99% (1). *L. monocytogenes* can rapidly replicate in spoiled food products because of the increased alkalinity (2). In addition, the microorganism can survive at refrigeration temperatures and high salt concentrations; thus, it is ubiquitous in water, soil, refrigerators, and the food processing industry, as well as silage and livestock environments (3), posing a serious threat to public health. Although the cases of *L. monocytogenes* infection per year are much lower than those of other foodborne illnesses (about 23,150 cases worldwide), the pathogen stands out for its high mortality among infected patients (20 to ~30%) (4). For most normal adults who are healthy and immunocompetent without specific medical conditions or pregnancy, the ingestion of severely contaminated food commonly leads to mild to severe gastroenteritis symptoms that may resolve spontaneously. However, equal or even lower levels of food contamination can cause systematic clinical syndromes with high risk of death in aging populations.
newborns, and immunocompromised persons, most frequently meningitis and sepsis. In particular, the infection of pregnant individuals can cause abortion and other complications related to pregnancy (5). In ruminants, *L. monocytogenes* can cause invasive infection in fetuses and the central and nervous system, resulting in abortion and circling disease, respectively (6).

The combination of ampicillin or amoxicillin with the aminoglycoside antibiotic gentamicin is the standard clinical therapy for severe listeriosis (2). In nonmeningeal infections, vancomycin is the best choice, while erythromycin is more applicable for pregnant women. Compared with other resistant bacteria such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Enterococcus faecalis*, *L. monocytogenes* retains a higher rate of susceptibility to broad-spectrum antibiotics at present. However, the efficiency of antibiotics against *L. monocytogenes* is also declining owing to the frequent emergence of resistant strains that acquire resistance gene from other donor organisms (7). Notably, commensal microbes in the host intestine provide the first-line defense against *L. monocytogenes* infection via occupying the intestinal surface, producing diverse products with antimicrobial activity, consuming nutrients, or modulating immune defense pathways (8, 9). Thus, the side effect of antibiotics on the host microbiota may likely facilitate the expansion and traversing of *L. monocytogenes* in the intestine and therefore lead to treatment failure, especially in immunocompromised individuals and in cases with intestinal disease. Given the global crisis of antibiotic resistance that we are facing and the limitations of antibiotic therapy for *L. monocytogenes* infection, continuous studies of other alternative therapeutic strategies are needed.

The successful adaptation of *L. monocytogenes* to diverse mammals and birds relies on the evolutions of the interaction between relevant virulence determinants and host defense systems. Among these virulence factors, the cholesterol-dependent pore-forming toxin listeriolysin O (LLO) plays a central role in *L. monocytogenes* pathogenicity. Unlike other cholesterol-dependent cytolysins (CDCs) that act like a bazooka to stimulate lytic cell death and tissue damage (10), LLO has been characterized as a phagosome-specific lysin owing to its optional activity under low-acid conditions (pH < 6) of mature phagosome and its decisive contribution to phagosome escape of *L. monocytogenes* into cytoplasm (11). Intriguingly, accumulating evidence reveals that LLO is more likely to function like a Swiss army knife, because it is not limited to allowing intracellular replication and spreading, the unique CDC toxin also has many other potential activities that depend not on large pores but, rather, on small membrane perforations caused by incomplete pores (12, 13). LLO is most known for its function in phagosome escape by perforating the phagosomal membrane (14). Interestingly, LLO secreted by *L. monocytogenes* within the phagosome also hinders lysosome killing of bacteria through suppressing reactive oxygen species (ROS) production (15). In addition, LLO pore formation in the cytosol of host cells would promote *L. monocytogenes* infection via causing SUMOylation-associated protein degradation (16). Importantly, similar to other CDCs, the extracellular LLO also perforates the host cell membrane and therefore causes intricate cellular events that benefit *L. monocytogenes* infection. For example, LLO has been reported to promote *L. monocytogenes* internalization before cell entry through activating a Ca\(^{2+}\)-dependent cPKC/Rac1/Arp2/3 signaling pathway (17). Extracellular LLO can also evoke an inflammatory response by perturbing ionic homeostasis, especially K\(^+\) and Ca\(^{2+}\) (18, 19). Notably, the alteration of Ca\(^{2+}\) homeostasis subsequent to LLO pore formation would favor infection via remodeling the mitochondrial network and endoplasmic reticulum (ER) response. Surprisingly, it has been proposed that the host cell signaling activated by the pore formation of extracellular LLO has a pronounced impact on subsequent vacuolar escape of *L. monocytogenes*, which might be related to the effects of ionic homeostasis on the endosomal network (20). The multifaceted effects of LLO pore formation on the host cell highlight its extraordinary contribution to *L. monocytogenes* pathogenesis; thus, the toxin represents a promising drug target for the treatment of *L. monocytogenes* infection.

Given the great importance of LLO in the pathogenesis of *L. monocytogenes*, we performed LLO-targeted biochemical screening and identified kaempferol, a type of flavanol present in many plants and some dietary sources, as an effective blocker of LLO. Further study revealed that kaempferol specifically inhibited LLO pore formation by engaging the residues Glu437, Ile468, and Tyr469 of LLO but did not exhibit a detectable effect on bacterial viability and toxin
production, indicating that the natural compound may be easily induce drug resistance compared to traditional antibiotics. LLO-mediated cytotoxicity and barrier dysfunction were both significantly repressed by kaempferol. Additionally, we found that kaempferol suppressed \textit{L. monocytogenes}-evoked inflammation mainly through inhibiting MyD88-dependent inflammation signaling. In a murine systemic infection model, kaempferol treatment effectively protected mice from \textit{L. monocytogenes} infection, with reduced bacterial burden and cytokine levels in the main target organs of liver and spleen, therefore alleviating pathological damage and prolonging survival time. These results characterize kaempferol as an effective LLO inhibitor and would render this natural small molecule an alternative lead compound to curb \textit{L. monocytogenes} infection.

RESULTS

Kaempferol exerted effective suppression of LLO pore formation. Acting like a Swiss army knife, LLO plays an indispensable role in \textit{L. monocytogenes} infection, and most contributions of LLO in \textit{L. monocytogenes} pathogenicity are dependent on its pore-forming activity. Based on this fact, we performed hemolysis-based screening to discover effective inhibitors of LLO pore formation. Kaempferol (Fig. 1A), a bioactive flavonoid broadly present in dietary sources and traditional Chinese herbs, exhibited a potent suppressive effect on the hemolytic activity of LLO naturally produced from \textit{L. monocytogenes}. Culture supernatants from wild-type \textit{L. monocytogenes} strain EGD, its hly-deficient mutant EGD\_hly, and the complementation strain EGD\_hly::hly were harvested and incubated with the indicated concentrations of kaempferol for the hemolysis assay. (E) The MIC values of kaempferol for \textit{L. monocytogenes} strains used in the study. (F) The growth curves of \textit{L. monocytogenes} EGD in the presence of the indicated concentrations of kaempferol. Similar results were obtained from three independent experiments. Data are presented as means ± SEM (n = 3); **, P < 0.01 compared to the vehicle-treated group.

FIG 1 Hemolysis-based screening identifies kaempferol as an effective inhibitor of LLO pore formation. (A) Chemical structure of kaempferol. (B) Dose response curve of kaempferol in the hemolysis assay. Recombinant LLO (0.1 μg/mL) pretreated with DMSO or indicated concentrations of kaempferol for 30 min was incubated with freshly washed sheep erythrocytes for 20 min. Hemoglobin release was measured on a Tecan microplate reader at 543 nm absorbance. The absorbance of samples treated with 0.1% Triton X-100 was regarded as the reference value for 100% lysis activity. Hemolysis was defined as the value of each sample relative to the Triton X-100-treated sample. (C) Negative-stain electron microscopy images of sheep erythrocyte ghosts that were incubated with LLO in the presence of DMSO vehicle or 32 μg/mL kaempferol. Arrows point to LLO pores. Scale bar = 100 nm. (D) Kaempferol suppressed the hemolytic activity of LLO naturally produced from \textit{L. monocytogenes}. Culture supernatants from wild-type \textit{L. monocytogenes} strain EGD, its hly-deficient mutant EGD\_hly, and the complementation strain EGD\_hly::hly were harvested and incubated with the indicated concentrations of kaempferol for the hemolysis assay. (E) The MIC values of kaempferol for \textit{L. monocytogenes} strains used in the study. (F) The growth curves of \textit{L. monocytogenes} EGD in the presence of the indicated concentrations of kaempferol. Similar results were obtained from three independent experiments. Data are presented as means ± SEM (n = 3); **, P < 0.01 compared to the vehicle-treated group.
Next, we evaluated the effect of kaempferol on the hemolytic activity of native LLO produced by *L. monocytogenes*. As expected, the hemolysis of the culture supernatants harvested from both wild-type *L. monocytogenes* EGD and the complementation strain EGDΔhly, was dose-dependently inhibited by kaempferol (Fig. 1D). However, kaempferol treatment did not affect the hemolytic activity of the culture supernatants from EGDΔhly, which hardly caused hemolysis (Fig. 1D), suggesting that kaempferol inhibited the pore-forming activity of native LLO derived from *L. monocytogenes*.

To investigate whether kaempferol exerted antimicrobial activity, the MICs and growth curves of *L. monocytogenes* were determined at the indicated concentrations. As shown in Fig. 1E, the MICs of kaempferol for *L. monocytogenes* strains used in this study were no less than 256 μg/mL. Moreover, no visible inhibitory effect on bacterial viability was observed at each time point under the concentrations required for hemolysis inhibition (Fig. 1F).

Collectively, these data demonstrated that the small molecule kaempferol represents a potential candidate for *L. monocytogenes* infection via effectively suppressing LLO pore formation rather than bacterial viability.

**Kaempferol attenuated *L. monocytogenes*-caused cytotoxicity via targeting LLO.**

The most essential function of LLO has been considered to mediate intracellular survival of *L. monocytogenes*, which is indeed critical for *L. monocytogenes* pathogenesis. However, accumulating evidence supports the idea that, as an extracellular pore-forming toxin, LLO also perforates the host cell membrane and thus causes appreciable cell death independent of host cell invasion (21–23). Using the J774A.1 cell line, we found that challenge of wild-type *L. monocytogenes* EGD caused obvious cytotoxicity, while kaempferol dose-dependently reduced the cytotoxicity (Fig. 2A). Further study revealed that the suppressive effect...
was dependent on LLO, because the lack of hly led to the invalidation of kaempferol, while the complementary of hly restored above observations (Fig. 2B). Consistently, LLO-induced cytotoxicity in Caco-2 cells was also remarkably reduced by 32 μg/mL kaempferol, even at an extremely high dose of LLO (50 μg/mL) (Fig. 2C). Live/Dead staining revealed that both wild-type *L. monocytogenes* EGD and the hly-complementary strain EGDΔhly::chly induced membrane-damaged cell death, where the dead cells were stained with membrane-impermeable red fluorescent dye. Conversely, no visible cell death was observed in cells infected with the hly-deficient strain EGDΔhly or without any stimulation or in the cells exposed to EGD with the addition of 32 μg/mL kaempferol (Fig. 2D). Taken together, these results suggested that kaempferol effectively protected cells from *L. monocytogenes*-caused cytotoxicity by targeting LLO pore formation.

**Kaempferol suppressed the *L. monocytogenes*-evoked inflammation response.** LLO also manipulates the host inflammation response by causing ion oscillations associated with membrane perforation (24). Studies of mouse peritoneal macrophages (MPMs) verified that the wild-type *L. monocytogenes* EGD infection caused pronounced activation of mitogen-activated protein kinase (MAPK) and NF-κB pathways (Fig. 3A to D), with a significant increase in the phosphorylation levels of p38, extracellular signal-regulated kinase (ERK), Jun N-terminal protein kinase (JNK), p65, and κB kinase (IKK), while 32 μg/mL kaempferol treatment significantly hindered the activation of these signaling. Although the activation of MAPK and NF-κB pathways was much lower in cells infected by EGDΔhly, the slight activation NF-κB p65 and IKK were both significantly blunted by kaempferol (Fig. 3A to D), indicating that the anti-inflammation activity of kaempferol upon *L. monocytogenes* was not entirely dependent on LLO inhibition but that an LLO-independent mechanism was also involved. Accordingly, the secretion and transcription of the inflammation cytokines tumor necrosis factor alpha (TNF-α) (Fig. 3E and H), interleukin-1β (IL-1β) (Fig. 3F and I), and IL-6 (Fig. 3G and J) were both suppressed by kaempferol in cells infected by EGD and EGDΔhly, confirming the LLO-independent anti-inflammation mechanism of kaempferol for *L. monocytogenes* infection.

To interrogate the potential mechanism by which kaempferol suppressed *L. monocytogenes*-evoked inflammation independent of LLO, we further enumerated the effect of kaempferol on the inflammatory response induced by Toll-like receptor 4 (TLR4) and TLR2 ligands. The results revealed that kaempferol remarkably repressed MyD88-dependent activation of NF-κB and MAPK signaling responses to TLR4 ligand lipopolysaccharide (LPS) (Fig. 4A and B), which was consistent with previous studies (25, 26). Consequently, LPS-induced production of inflammatory cytokines was also significantly attenuated by kaempferol treatment (Fig. 4C and D). Importantly, similar suppression was also observed in the TLR2-mediated inflammation response, where the inflammation signaling and the burst of proinflammatory gene response to TLR2 ligand Pam3CSK4 were almost all blocked by 32 μg/mL kaempferol (Fig. 4E to H). These data further implicated a potential role of kaempferol in MyD88-dependent activation of inflammation signaling downstream of TLRs.

Taken together, these results confirmed that kaempferol could effectively suppress the *L. monocytogenes*-caused inflammation response through eliminating LLO perforation and suppressing the MyD88-dependent inflammation response upon recognition of *L. monocytogenes* by pattern recognition receptors.

**Kaempferol hindered LLO-induced barrier disruption of the Caco-2 monolayer.** As a foodborne pathogen, the natural route of infection of *L. monocytogenes* is through the gastrointestinal tract, followed by systemic dissemination. Functioning as a trigger for the disruption of tight junctions, the pore formation of extracellular LLO also promotes bacterial entry and spread via compromising barrier integrity. To examine the effect of kaempferol on LLO-induced barrier disruption, we evaluated the integrity of the Caco-2 monolayer, which mimicked the intestinal epithelium barrier *in vitro*. In line with a previous study (27), LLO treatment caused a dramatic decline in transepithelial electrical resistance (TEER) within the first 10 min (Fig. 5A), along with a significant increase in transcellular permeability to sodium fluorescein (Fig. 5B). Promisingly, 32 μg/mL kaempferol effectively prevented LLO-caused loss of epithelial barrier integrity. These data demonstrated that kaempferol could effectively restrain LLO-induced barrier disruption and suggested that kaempferol might prevent the translocation of *L. monocytogenes* from the intestines into the to the deep organs.
FIG 3 Kaempferol suppresses the *L. monocytogenes*-evoked inflammation response. (A) Western blot analysis of MAPK signaling cascades in MPMs infected with wildtype *L. monocytogenes* strain EGD and its hly-deficient mutant EGDDhly (MOI, 5) for 5 h in the presence of DMSO vehicle or 32 mg/mL kaempferol. (B) The levels of P-p38, P-ERK and P-JNK relative to β-actin determined using densitometry are shown. (C) Western blot analysis of NF-κB signaling cascades in MPMs infected with wild-type *L. monocytogenes* strain EGD and its hly-deficient mutant EGDDhly (MOI, 5) for 5 h in the presence of DMSO vehicle or 32 mg/mL kaempferol. (D) The levels of P-p65 and P-IKK relative to β-actin determined using densitometry are shown. (E to G) The levels of TNF-α (E), IL-1β (F), and IL-6 (G) in the coculture supernatants were detected using ELISA. (H to J) The transcriptions of TNF-α (H), IL-1β (I), and IL-6 (J) were determined by RT-qPCR. All the genes were normalized to the housekeeping gene GAPDH. Similar results were obtained from three independent experiments. Data are presented as means ± SEM (n = 3). **, *P* < 0.01 compared to the vehicle-treated group.
Kaempferol was protective against *L. monocytogenes* infection in mice. To investigate whether kaempferol conferred effective protection against *L. monocytogenes* infection *in vivo*, we established a systemic infection model in BALB/c mice. Following intraperitoneal inoculation with $5 \times 10^8$ EGD, approximately 92.31% of the mice died within 6 days, but 30.77% of those mice that received vehicle treatment survived ($P = 0.0343$) (Fig. 6A). Compared to the infected mice that received vehicle treatment, kaempferol-treated mice had a significantly lower bacterial load in the major target organs of spleen and liver (Fig. 6B and C), indicating limited bacterial dissemination in kaempferol-treated mice. Additionally, the good therapeutic efficiency of kaempferol was also validated by histological examination of spleen and liver using hematoxylin and eosin (H&E) staining. EGD infection caused significant histopathological injury in the spleens of vehicle-treated mice, manifested by visible lymphocyte depletion as well as obvious necrosis and congestion in the germinal center. Meanwhile, severe cell degeneration, apparent spotty necrosis, and significant inflammatory cell...
accumulation were observed in the liver. As expected, kaempferol-treated mice displayed slight pathological changes in these two target organs (Fig. 6D). Simultaneously, the levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 in the spleens of vehicle-treated mice were remarkably higher than those of kaempferol-treated mice (Fig. 6E). In contrast, kaempferol treatment did not significantly reduce the IL-1β level in the liver (Fig. 6F), but the amounts of TNF-α and IL-6 were significantly diminished. Taken together, our results demonstrated that kaempferol effectively protected mice from systemic L. monocytogenes infection.

Kaempferol engaged Glu437, Ile468, and Tyr469 on LLO to inactivate its biological activity. To further investigate the detailed molecular mechanism of kaempferol-driven LLO inhibition, we first analyzed the secondary structure of LLO in the presence or absence of kaempferol using circular dichroism (CD). As shown in Fig. 7A, kaempferol treatment caused significant changes in CD spectra, indicating that kaempferol might inactivate LLO via altering the secondary structure of LLO protein, which would depend on its interaction with certain sites in LLO.

Then, the key residues of LLO responsible for the binding with kaempferol were identified using molecular docking analysis. The root-mean-square fluctuations (RMSF) of the residues of the whole protein in the LLO-kaempferol complex and in the free LLO clearly depicted different flexibilities in the binding site of LLO in the presence and absence of the kaempferol (Fig. 7B). The majority of the residues in the LLO binding site for kaempferol showed lower flexibility, with an RMSF of less than 3 Å compared with the free LLO, indicating that these residues appeared to be the potential binding sites to kaempferol (Fig. 7B). To gain more information about the residues surrounding the binding site and their contribution to the system, the electrostatic, Van der Waals, solvation, and total contribution of the residues to the binding free energy were calculated. In the LLO-kaempferol complex, the residue Glu-437 had a prominent electrostatic (ΔΔEele) contribution, with a value of ≈10.5 kcal/mol (Fig. 7C). Detailed analysis showed that the residue Glu-437 was oriented to the hydroxyl group of the kaempferol and formed a strong hydrogen bond (bond length, 2.0 Å) interaction with kaempferol (Fig. 7D). Notably, the residue Tyr-469 had strong Van der Waals interactions with kaempferol because of the close proximity between each other (Fig. 7C). Regarding the other residues (i.e., Trp-435, Ile-468, Leu-470, Pro-471, and Ala-474), the majority of the decomposed energy interaction originated from Van der Waals interactions and apparent hydrophobic interactions. In addition, the total binding free energy calculated for the LLO-kaempferol complex revealed an ΔGbind (binding free energies) of approximately −13.3 kcal/mol, indicating that kaempferol could strongly bind to and interact with the predicted binding site of LLO (Fig. 7C).

In order to more definitively confirm the essential binding site based on the above-described results, we next introduced the mutations of LLO by replacing the predicted residues with Ala. By comparing the inhibition rate of kaempferol on the hemolytic activity of LLOwt and involved mutants, we determined that the residues Glu-437, Tyr-469, and Ile-468 critically contributed to the LLO inhibition by kaempferol, as evident by the
stronger inhibition of kaempferol on wild-type LLO (LLOWT) than those mutants (Fig. 7E). Consistently, the mutation of these essential binding sites also led to the invalidation of kaempferol in LLO-induced cytotoxicity (Fig. 7F), further confirming the interaction between kaempferol and LLO. Thus, these results verified that kaempferol blocked LLO pore formation mainly through engaging Glu-437, Tyr-469, and Ile-468 of LLO, which provided valuable information for further development of LLO inhibitors.

**DISCUSSION**

Although most *L. monocytogenes* are currently susceptible to the antibiotics commonly used in clinic, there is always a need to develop novel alternative approaches, because antibiotic resistance is considered to be a predictable result determined by the mechanism...
The pore-forming toxin LLO appears to be a promising therapy target due to its indispensable role in *L. monocytogenes* pathogenesis and intracellular life cycle.Indeed, LLO also acts as a predominant antigen for T cells; thus, an LLO-targeted vaccine is also being developed to prevent listeriosis (28). In addition to vaccines and antibodies,
Kaempferol was reported to stabilize the immunosuppressive function of FOXP3 regulatory T cells (Tregs) by inhibiting PIM1, a protein kinase specifically catalyzing the phosphorylation of the FOXP3 molecule (35). In this way, kaempferol may limit bacterial expansion during L. monocytogenes infection, because reduced Treg suppressive potency appears to eradicate infection via accelerating the expansion of protective antigen-specific CD8+ T cells in the case of acute L. monocytogenes infection (36). Last but not least, as opposed to the traditional broad-spectrum antibiotics that have a high risk of perturbing commensal bacteria in the gut, kaempferol was proved to rebalance the gut microbiota and microbial metabolism (37), which would preserve the first-line defense against numerous enteropathogenic bacterial infections, including L. monocytogenes. Here, we verified that kaempferol hardly influenced the viability of L. monocytogenes, suggesting a lower risk of inducing drug resistance than traditional antibiotics. The role of kaempferol in inhibiting LLO and inflammation provided new therapeutic indications for presenting this bioactive natural compound as an alternative approach to counteract L. monocytogenes infection.

In conclusion, our study discovered that, acting as a potent inhibitor of LLO pore formation and MyD88-dependent inflammation response, kaempferol effectively suppressed L. monocytogenes virulence and L. monocytogenes-evoked inflammation and thus exhibited good therapeutic efficiency against L. monocytogenes infection in vivo.
These findings presented kaempferol as a promising therapeutic candidate for *L. monocytogenes* infection, which paved the load for the development of antibacterial infection strategies.

**MATERIALS AND METHODS**

**Reagents.** Kaempferol (≥98%) was purchased from Herbpurify (Chengdu, China) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). A cytotoxicity detection kit (Roche, Switzerland) and Live/Dead cell imaging kit (Invitrogen, USA) were used for the analysis of cytotoxicity. T-PER tissue protein extraction reagents (Thermo Scientific, USA) and a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai) were used to prepare biological protein samples. Fluorescein sodium salt used for the evaluation of barrier permeability was purchased from Sigma-Aldrich. Total RNAiso reagent and a MutanBEST kit were from TaKaRa (Tokyo, Japan). FastStart Universal SYBR green master mix was purchased from Roche.

**Bacterial strains.** *Escherichia coli* DH5α and *E. coli* BL21 used for protein expression and purification were purchased from TransGen (Beijing, China) and cultured in LB medium. Wild-type *L. monocytogenes* strain EGD and its hly-deficient mutant EGDΔhly and the complementation strain EGDΔhly::hly were kind gifts from Pascale Cossart (Institut Pasteur, Paris, France) and were grown in Trypticase soy broth (TSB) medium with shaking at 37°C.

**Expression and purification of recombinant protein.** Recombinant LLO<sub>wt</sub> and its mutants without signal peptide (amino acids 1 to 24) were heterologously overexpressed in *E. coli* BL21 based on the pET21a vector. In short, the overnight cultures were diluted 1:100 in the fresh LB medium containing 100 μg/mL ampicillin, and then protein expression was induced with 300 μM IPTG (isopropyl-β-D-thiogalactopyranoside) while reaching an optical density at 600 nm (OD<sub>600</sub>) of 0.6 to 0.8; at the same time, the culture temperature was turned down to 16°C. After 18 h, the bacterial pellets were obtained and sonicated for the collection of soluble protein fractions. The His-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) columns. After the concentrations were determined, the protein fractions were stored at −80°C.

**Hemolysis assay.** Erythrocyte leakage was determined by the release of hemoglobin that could be spectrophotometrically detected at 543 nm (38). Recombinant LLO proteins dispensed in the 96-well plates were incubated with the indicated concentrations of kaempferol at 37°C for 30 min before the addition of freshly washed sheep erythrocytes. The released hemoglobin was quantified after a 20-min incubation. The absorbance of samples treated with 0.1% Triton X-100 was regarded as the reference value for 100% lysis activity. Hemolysis was defined as the value of each sample relative to that of the 0.1% Triton X-100-treated sample.

**Specimen preparation and EM.** The specimen for electron microscopy (EM) was prepared as described previously (30). Briefly, the resealed ghosts were treated with 2 μg/mL LLO protein in the presence or absence of 32 μg/mL kaempferol and then transferred to EM grids, followed by negative staining with 1% uranyl acetate. Then, the specimens were analyzed with a Hitachi H-7650 transmission EM (Tokyo, Japan) operating at 80 kV.

**MIC determination and growth curve assay.** The MICs of kaempferol for the involved *L. monocytogenes* strains were determined by broth microdilution according to the guidance of the Clinical and Laboratory Standards Institute (CLSI) (39). To assess the effect of kaempferol on the viability of *L. monocytogenes* strain EGD, the growth curve assay was performed as previously described (40).

**Cell culture and treatments.** The human colon carcinoma cell line Caco-2 and mouse macrophage-like cell line J774A.1 were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin G plus 100 μg/mL streptomycin sulfate. Mouse peritoneal macrophages (MPMs) derived from C57BL/6 mice were obtained as previously reported and cultured in RPMI medium with 10% FBS (41). All the cells were grown at 37°C in a 5% CO₂ incubator. To investigate the effect of kaempferol on *L. monocytogenes*-caused inflammation response, MPMs grown overnight in the 6-well plate at 5 × 10⁶ cells/well were challenged with *L. monocytogenes* at a multiplicity of infection (MOI) of 5 for 5 h in the presence of dimethyl sulfoxide (DMSO) vehicle or 32 μg/mL kaempferol. To clarify the effect of kaempferol on the MyD88-dependent inflammation response downstream of TLRs, MPMs grown overnight in the 6-well plate at 5 × 10⁶ cells/well were stimulated with TL1R2 ligand Pam3CSK4 (1 μg/mL) and TL4 ligand LPS (1 μg/mL).

**Cytotoxicity assays.** Cell death was evaluated by the lactate dehydrogenase (LDH) release assay using a cytotoxicity detection kit (Roche). For the determination *L. monocytogenes*-caused cytotoxicity, J774 A.1 cells grown overnight in the 96-well plate at 1 × 10⁵ cells/well were challenged with *L. monocytogenes* at an MOI of 50. Meanwhile, the indicated concentrations of kaempferol were added into the coculture system. After a 6-h incubation at 37°C, cytotoxicity was determined by detecting the amount of LDH released in the supernatant according to the manufacturer’s instructions. Next, cells at the bottom were stained using a Live/Dead cell imaging kit, where the live cells and membrane-damaged dead cell were labeled with green and red fluorescence, respectively. Cells were visualized by an inverted fluorescence microscope (Olympus, Tokyo). To assess the effect of kaempferol on LLO-induced cytotoxicity, Caco-2 cells grown overnight in the 96-well plate at 2 × 10⁵ cells/well were stimulated with serially diluted LLO protein in the presence of DMSO vehicle or 32 μg/mL kaempferol. After a 3-h incubation at 37°C in 5% CO₂, the cytotoxicity directly resulted from LLO stimulation was determined as described above.

**Immunoblot analysis.** Cells were washed with cold phosphate-buffered saline (PBS) and then lysed with T-PER mammalian protein extraction reagent. Cell debris was removed by centrifugation at 10,000 × g for 8 min (4°C). Then, the total protein concentrations of the supernatant fractions were determined using the BCA protein assay kit according to the manufacturer’s instructions, followed by the addition of 5 × SDS loading buffer and heat denaturation at 100°C for 8 min. The protein samples were resolved in 5 to 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). The target proteins on the membranes were probed with the specific primary antibodies as follows: rabbit polyclonal anti-JNK (Proteintech, catalog
TABLE 1  Sequence of primers used for RT-qPCR

| Gene       | Primer     | Sequence (5'→3')               |
|------------|------------|--------------------------------|
| Mouse GAPDH | Sense      | AGGTCGGTGTGACGAGTTTG            |
|            | Antisense  | GGGTGCGTGTATGGCAACA             |
| Mouse IL-1β | Sense      | ACGTTGTCCTTCCGTTG               |
|            | Antisense  | TCATCTGGAGGCTGTAATG              |
| Mouse IL-6  | Sense      | CACCTTCAAAGTGGAGGCCTTA          |
|            | Antisense  | GCAAGTGATCATAGTGTTGATAC          |
| Mouse TNF-α | Sense      | CCTATGTCCTAGGCTTCTCAT           |
|            | Antisense  | CACTTGGTGTTTGGCTACGA            |

[cat.] no. 51151-1-AP, rabbit polyclonal anti-INK phosho (Thr183 [221] plus Thr185 223) (Arigo, cat. no. ARGS1807), rabbit monoclonal anti-p38 MAPK (CST, cat. no. 8690), rabbit monoclonal anti-p38 MAPK phospho (Thr180/Tyr182) (CST, cat. no. 4511S), rabbit polyclonal anti-ERK1/2 (Proteintech, cat. no. 16443-1-AP), rabbit polyclonal anti-ERK1/2 phosho (Thr202/Tyr204) (Arigo, cat. no. ARG52277), rabbit polyclonal anti-ALKα (Arigo, cat. no. ARG65746), rabbit polyclonal anti-ALKα phospho (Thr23) (Arigo, cat. no. ARG51630), rabbit monoclonal anti-ALKα phosho (Thr23) (CST, cat. no. 30335), mouse polyclonal anti-β-actin (Proteintech, cat. no. 66009-1-lg), and horseradish peroxidase (HRP)-conjugated anti-rabbit (Proteintech, cat. no. SA00001-2) and anti-mouse IgG (Proteintech, cat. no. SA00001-1) secondary antibodies. All the primary antibodies and secondary antibodies were diluted 1:1,000 and 1:4,000, respectively, in Tris-buffered saline with Tween 20 (TBST). The immunoblots were developed with the enhanced chemiluminescence ECL kit (Biosharp) and visualized using an ECL Plus Western blotting detection system (Tanon). The Western blot bands were semiquantitated using Image-Pro software.

Measurement of cytokines. The levels of cytokines TNF-α, IL-6, and IL-1β in the culture supernatants as well as liver and spleen homogenates were measured using the mouse enzyme-linked immuno-sorbent (ELISA) MAX Deluxe set (BioLegend) according to the manufacturer’s instructions.

RNA isolation and RT-qPCR. Total RNA was isolated using RNAiso reagent and then reverse-transcribed into cDNAs using a Prime Script reverse transcription (RT) reagent kit (Tokyo, Japan). Reverse transcription-quantitative PCR (RT-qPCR) was performed using FastStart Universal SYBR green master mix, and the gene-specific primer pairs are listed in Table 1. Samples were loaded onto 96-well reaction plates and run on an Applied Bioscience 7500 thermocycler. Data were analyzed by the ΔΔCT method and normalized to GAPDH abundance. The data shown in the graph represent the fold induction relative to untreated cells.

Measurements of transepithelial electrical resistance (TEER) and permeability of the Caco-2 monolayer. Caco-2 cells were grown in Corning Transwell permeable supports (0.4 μM) to mimic the intestinal epithelial barrier in vitro (42). After 14 days of differentiation, the monolayer resistance was documented every 3 days using an epithelial voltohmmeter (Millicell ERS-2) until approximately 600 to 800 Ω was reached. On the day of experiment, the monolayer was washed with warm PBS and the culture medium with replaced with serum-free DMEM. Then, sodium fluorescein was added to the insert compartment at a final concentration of 0.01 mg/mL, followed by the addition of LLO protein (1 μg/mL) treated with DMSO vehicle or 32 μg/mL kaempferol. TEER was measured for the first 10 min and then at intervals of 30 min, and at the same time, 100 μL of medium in the basolateral chamber was moved to the 96-well plate to quantify the paracellular fluorescein by measuring the fluorescence intensity at 520 nm with an excitation of 460 nm. The TEER changes were presented as the mean percentage of the initial TEER value.

Drug administration and mouse infection. C57BL/6 female mice (18 to 20 g) and BALB/c female mice aged 6 to 8 weeks (20 to 25 g) were purchased from Liaoning Changsheng Biotechnology Co. Ltd. (Ben Xi, China) and maintained in individually ventilated cages (IVCs) under specific-pathogen-free and standard housing conditions (25 ± 1°C, 55% humidity, 12-h light/dark cycle) with free access to standard pellet food and sterilized water. All the in vivo experiments were carried out with the approval of the Jilin University Institutional Animal Care Committee and strictly conducted in accordance with the guidelines.

For animal injection, kaempferol stock initially dissolved in DMSO (100 mg/mL) was diluted to 40 mg/mL with sterilized water containing 5% polyethylene glycol (PEG) 400 and 5% Tween 80. To perform the survival rate analysis, mice were randomly divided into a vehicle group and a kaempferol group (n = 13 mice each group). Mice in the vehicle group were intraperitoneally inoculated with 5 × 10⁷ CFU of wild-type L. monocytogenes EGD and subcutaneously administered with 50 μL vehicle (sterilized water containing 5% PEG 400 and 5% Tween 80). Mice in the kaempferol group were challenged with the same amount of EGD and treated with 100 mg/kg kaempferol. Following infection, drug delivery was maintained at intervals of 8 h for 4 days, and the survival rate of the mice was recorded for 6 days. Finally, the mice that survived were anaesthetized with 5% isoflurane by inhalation and then killed by rapid cervical dislocation.

To further assess bacterial burden and cytokine levels in livers and spleens, as well as the histopathology of the two target organs, a negative group was created (n = 7 mice in each group). In the experiment, mice in the negative group received only vehicle, mice in the vehicle group were intraperitoneally injected with 5 × 10⁷ CFU of wild-type L. monocytogenes EGD and received 50 μL vehicle, and mice in the kaempferol group were challenged with the same amount of EGD and administered with 100 mg/kg kaempferol at intervals of 8 h following infection. Then, 48 h after infection, mice were anaesthetized with 5% isoflurane and then killed by rapid cervical dislocation. The liver and spleen of each mouse were dissociated and homogenized in sterilized PBS (10% [wt/vol]). Bacterial burden was determined by microbiological plating, and cytokine levels in the supernatants of those tissue homogenates were measured using a mouse ELISA kit according to the manufacturer’s instructions. For histopathological analysis, the spleens...
and livers were fixed with 10% formalin and stained with hematoxylin and eosin (H&E). The pathological
sores of liver and spleen were evaluated as previously described (43).

**Molecular docking and dynamics analysis.** The kaempferol molecule docking with the three-dimensional
(3D) structure of the LLO (PDB ID: 4CDB) was performed using AutoDock Vina 1.1.2 and ChemBioDraw Ultra
14.0. Then, the molecular dynamics was performed on a Dell Precision T5500 workstation, and the Amber 14
and AmberTools 15 programs were used for molecular dynamics simulations of the selected docked pose. To
further explore the key protein residues responsible for the binding mode, the binding free energy was decom-
posed on a per-residue basis.

**Site-directed mutagenesis.** Site-directed mutants of LLO were all generated using the MutanBEST
kit based on the DNA template of a wild-type construct. The primer pairs used for mutations are shown
in Table 2. Finally, the sequences of all mutant constructs were verified by nucleotide sequencing.

**Statistical analysis.** The numeric data are shown as the mean ± the standard error of the mean
(SEM), and the statistical analyses were performed using GraphPad Prism 5.0 software. The comparison between
more than two groups were performed by one-way analysis of variance (ANOVA) with Bonferroni's cor-
rection. Mouse survival curves and the statistics were presented and analyzed using the Mantel-Cox log-rank test.
P<0.05; **P<0.01; **P<0.001 was considered to be statistically significant.

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J.W. and X.D. conceived the study. T.W. and J.W. designed the experiments. T.W. performed
the recombinant LLO proteins

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**TABLE 2** Sequence of primers used for site-directed mutagenesis

| Gene   | Primer              | Sequence (5′→3′)                  |
|--------|---------------------|----------------------------------|
| LLO933A Sense | CCGCCAAGAAAGGTACGCGAGTGGAATATAATTTG |
| LLO933A Antisense | CAATATATCATTCCCATTCCGCTAATTCGCG |
| LLO425A Sense | GTGTGCTCAATCCACATTCTCGGAGAAGTAAATGATCC |
| LLO425A Antisense | GGACATATTCTATCTCCGCAAGAATGTTGAAATTTG |
| LLO437A Sense | CATTCTTCGTTGACGTAATTTGATCC |
| LLO437A Antisense | GGACATATTCTATCTCCGCAAGAATGTTGAAATTTG |
| LLO468A Sense | CATTCTTCGTTGACGTAATTTGATCC |
| LLO468A Antisense | GGACATATTCTATCTCCGCAAGAATGTTGAAATTTG |
| LLO469A Sense | CATCTCAGGCGAGGCAGATGTGAAATG |
| LLO469A Antisense | GGACATATTCTATCTCCGCAAGAATGTTGAAATTTG |
| LLO470A Sense | CATCTCAGGCGAGGCAGATGTGAAATG |
| LLO470A Antisense | GGACATATTCTATCTCCGCAAGAATGTTGAAATTTG |
| LLO471A Sense | CATCTCAGGCGAGGCAGATGTGAAATG |
| LLO471A Antisense | GGACATATTCTATCTCCGCAAGAATGTTGAAATTTG |
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