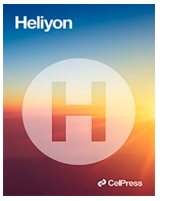
Heliyon 10 (2024) e23225

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



# Irisflorentin promotes bacterial phagocytosis and inhibits inflammatory responses in macrophages during bacterial infection

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ARTICLE INFO

*Keywords:*
Irisflorentin (IFL)
Pam3CSK4
Peptidoglycan
Methicillin-resistant *Staphylococcus aureus* (MRSA)
Inflammatory response
Phagocytosis

ABSTRACT

Bacterial infection remains a big concern in the patients of ICU, which is the main cause of life-threatening organ dysfunction, or even sepsis. The poor control of bacterial infection caused by antibiotic resistance, etc. or the overwhelming immune response are the most important patho genic factors in intensive care unit (ICU) patients. As main pathogens, antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), impose serious challenges during sepsis and require alternative therapeutic options. Irisflorentin (IFL) is one of the major bioactive compounds isolated from the roots of *Belamcanda chinensis* (Shegan). In this study, IFL could suppress inflammatory response induced by MRSA or a synthetic mimic of bacterial lipoprotein (Pam3CSK4). IFL treatment enhanced the ability of macrophages to phagocytose bacteria likely through up-regulating the expression of phagocytic receptors SR-A1 and FcγR2a. Furthermore, IFL inhibited Pam3CSK4-induced production of pro-inflammatory cytokines, including IL-6 and TNF-α in Raw 264.7 cells, mouse primary macrophages or dendritic cells. IFL treatment also inhibited heat-killed MRSA-induced secretion of IL-6 and TNF-α in mouse bone marrow-derived macrophages. Moreover, IFL attenuated M1 polarization of macrophages as indicated by the down-regulated expression of its polarization markers CD86 and iNOS. Mechanistically, IFL markedly decreased the Pam3CSK4-induced activation of ERK, JNK or p38 MAPK pathways in macrophages. Taken together, IFL may serve as a promising compound for the therapy of bacterial infection, particularly those caused by antibiotic-resistant bacteria, such as MRSA.

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https://doi.org/10.1016/j.heliyon.2023.e23225
Received 24 June 2023; Received in revised form 21 November 2023; Accepted 29 November 2023
Available online 9 December 2023

## 1. Introduction

Sepsis is a life-threatening multiple organ dysfunction caused by a dysregulated and overwhelming immune response to infection [1], which is associated with high morbidity and mortality. A recent epidemiological study has estimated that there are 31.5 million sepsis cases and 5.3 million deaths annually worldwide [2], which imposes a huge socioeconomic burden. Gram-positive bacteria *Staphylococcus aureus* is one of the most commonly isolated pathogens in patients with sepsis [3,4]. While timely intervention can be effective for the management of sepsis, no drug has been approved for treating sepsis [5]. Therefore, developing new and effective therapeutic options for sepsis is urgently required. With the increasing infection of antibiotic-resistant bacteria, the situation becomes tougher and new therapeutic drugs are eagerly needed.

Central to the pathogenesis of sepsis is sustained excessive inflammation characterized as overproduction of pro-inflammatory cytokines, well-known as cytokine storm, primarily by innate immune cells, including macrophages and dendritic cells, etc. During infections, pattern-recognition receptors (PRRs) on these cells, e.g., Toll-like receptors (TLRs), can specifically recognize distinct pathogen-associated molecular patterns (PAMPs), such as lipoteichoic acid, a cell wall component of Gram-positive bacteria, and its synthetic mimic Pam3CSK4. After ligation with its receptor TLR2, Pam3CSK4 can activate the downstream signaling pathways to initiate inflammatory responses. Upon TLR2 activation, adaptor protein MyD88 is recruited to the receptor and induces signaling cascades eventually activating MAP kinases (p38, JNK, and ERK1/2) and the famous transcription factor NF-κB for the expression of pro-inflammatory cytokines TNF-α IL-1β and IL-12. It has been demonstrated in animal models of sepsis that genetic depletion or pharmaceutical inhibition of these cytokines protects against acute infection[6], suggesting that the inhibition of excessive inflammation by down-regulating the overproduction of these cytokines may be a promising treatment for sepsis. Macrophages are the main innate immune cells secreting inflammatory cytokines during infection. According to the heterogenous character of macrophages, it can divide into 2 main subtypes, M1 or M2 macrophages. The polarization of M1 macrophages are closely related with the overexpression of inflammatory cytokines (cytokine storm) and poor outcome.

The phagocytosis of invading pathogens by professional phagocytes such as macrophages represents a critical component of the immune defense system, dysfunction of which is implicated in sepsis pathogenesis. The first step of phagocytosis is the specific recognition of pathogens by a various phagocytic receptor on the cell surface that can either directly or indirectly bind PAMPs of the pathogens. For instance, scavenger receptor A can detect lipopolysaccharide (LPS) on Gram-negative bacteria [6], and the Fc receptors (FcRs) are capable of binding opsonized pathogens [7]. In order for dynamic elimination of infection and maintenance of host homeostasis, cell-intrinsic mechanisms have evolved to regulate the expression levels of phagocytic receptors [7]. Enhanced phagocytosis of bacteria and the control of overwhelming inflammatory response in macrophages may have the potential therapeutic role to treat bacterial infectious diseases, or even sepsis.

Irisflorentin (IFL) is a bioactive isoflavone component in *Belamcandae Rhizoma* [8,9], an important herb that is used in traditional Chinese medicine (TCM) to treat inflammatory diseases [10]. The present study showed that IFL not only suppresses methicillin-resistant *Staphylococcus aureus* (MRSA) and Pam3CSK-induced production of proinflammatory cytokines in a macrophage cell line and primary macrophages by inhibiting MAPK signaling pathways but also promotes macrophage-mediated phagocytosis of bacterial pathogens likely through enhancing the expression levels of phagocytic receptors, exhibiting a potential to treat bacterial sepsis.

## 2. Materials and methods

### 2.1. Chemicals and agents

IFL was bought from Shanghai Yuanye Biotechnology Co. Ltd (Shanghai, China) (CAS：41743-73-1, HPLC≥98 %), dissolved in DMSO to 40 mM for storage at −20°**C**, and diluted with medium or sterile PBS for use. Pam3CSK4 and PGN were purchased from InvivoGen (San Diego, USA). LPS was bought from Sigma Chemical Co. St. Louis (MO, USA).

### 2.2. Cell culture

Raw264.7 macrophage cell line was purchased from ATCC (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS) purchased from Biowest (Nuaill'e, France) [11]. The acquisition and induction of primary peritoneal macrophages and bone marrow-derived macrophages were performed as previously reported [11], plated according to the diameter of the culture plate, incubated overnight, and then stimulated. Bone marrow cells were obtained from the femurs and fibulas of 4-week-old male C57BL/6J mice, and then mouse bone marrow-derived macrophages (BMDMs) were induced by M-CSF for 5 consecutive days. After that, BMDMs were further differentiated into M1 or M2 macrophagesusing LPS (100 ng/mL) and IFN-γ (20 ng/mL) or IL-4 (20 ng/mL), respectively. Bone marrow cells were differentiated into immature DCs in the presence of mGM-CSF (10 ng/mL) and mIL-4 (1 ng/mL) for 5 constitutive days.

### 2.3. Preparation of heat-killed MRSA

The clinical isolates of Methicillin-resistant *Staphylococcus aureus* (MRSA, HS488) used in this study were provided by the Institute of Antibiotics, Huashan Hospital, Fudan University and Key Laboratory of Clinical Pharmacology of Antibiotics (Shanghai, China).

MRSA was grown in Luria-Bertani medium containing 1 % Tryptone, 0.5 % Yeast extract, and 1 % NaCl at 37 °C. Heat-killed MRSA was obtained by 90 °C water baths for 30 min and then washed twice with sterile PBS. The source of the bacterial strain, the culture conditions, and the preparation of heat inactivation were as previously reported [12].

### 2.4. Cell viability assay

Observing the cytotoxicity of IFL by detecting the cell viability of Raw264.7 cells at 24, 48 or 72 h using cell proliferation assay with cell counting kit-8 (CCK-8) (Beyotime Biotechnology, Shanghai, China). Raw264.7 cells were seeded in 96-well plates with a density of $1 \times 10^5$ cells/well and cultured overnight. The cell culture medium was replaced by complete medium containing different concentrations of IFL (0, 5, 10, 20, 40 or 70 μM) and cells were cultured for subsequent 24, 48 or 72 h. CCK-8 reagent was added into the medium (10 μL/well) 2 h before the detection and the OD values were measured at 450 nm using a Synergy 2 Microplate Reader (Bio-Tek, Vermont, USA).

### 2.5. Real-time PCR

Real-time PCR was performed as previously reported [12].The primer sequences for qRT-PCR amplification were listed in Table 1.

### 2.6. Detection of cytokine production

Enzyme-linked immunosorbent assay (ELISA) kits for murine interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), IL-12p70 and IL-10 were purchased from R&D Systems (Minnesota, USA). The supernatants of cell culture were collected to measure the secretion of cytokines according to the manufacturer's instructions.

### 2.7. Flow cytometric analysis

The phagocytosis of pHrodo™ Deep Red *E. coli* BioParticles™ Conjugate (Thermo Fisher, Californian, USA) was detected in Raw264.7 cells by Flow cytometry. Cells were stimulated by LPS with or without IFL for 24 h, and then were incubated with pHrodo-labeled *E. coli* (10 μL/100 μL) at 37 °C for 1 h. The phagocytosis of *E. coli* was investigated, and data were analyzed by Flowjo software (BD, San Jose, USA).

### 2.8. Western blot

Proteins were extracted from mouse primary peritoneal macrophage and detected by Western blot [11]. All the antibodies were bought from Cell Signaling Technology (Beverly, MA, USA).

### 2.9. Confocal microscopy

The phagocytosis of pHrodo-labeled *E. coli* (100 μL/1000 μL) in Raw264.7 cells was also detected by confocal microscopy. Cells

**Table 1**
The primer sequences for qRT-PCR amplification.

| | | |
|---|---|---|
| mβ-Actin | mβ-ActinF | 5′-AGTGTGACGTTGACATCCGT-3′ |
| | mβ-ActinR | 5′-GCAGCTCAGTAACAGTCCGC-3′ |
| mIL-6 | mIL-6F | 5′-TAGTCCTTCCTACCCCAATTTCC-3′ |
| | mIL-6R | 5′-TTGGTCCTTAGCCACTCCTTC-3′ |
| mTNF-α | mTNF-αF | 5′-AAGCCTGTAGCCCACGTCGTA-3′ |
| | mTNF-αR | 5′-GGCACCACTAGTTGGTTGTCTTTG-3′ |
| mMCP-1 | mMCP-1F | 5′- GCATCCACGTGTTGGCTCA -3′ |
| | mMCP-1R | 5′- CTCCAGCCTACTCATTGGGATCA -3′ |
| mIL-1β | mIL-1βF | 5′-GGTGTGTGACGTTCCCATTAGAC-3′ |
| | mIL-1βR | 5′-CATGGAGAATATCACTTGTTGGTTGA-3′ |
| mIL-10 | mIL-10F | 5′-GCTCTTACTGACTGGCATGAG-3′ |
| | mIL-10R | 5′-CGCAGCTCTAGGAGCATGTG-3′ |
| mCD206 | mCD206F | 5′-CTCTGTTCAGCTATTGGACGC-3′ |
| | mCD206R | 5′-TGGCACTCCCAAACATAATTTGA-3′ |
| mDectin-1 | mDectin-1F | 5′-AGGCATCCCAAACTACAGGAG-3′ |
| | mDectin-1R | 5′-GGAGCAGTGTCTCTTACTTCCAT-3′ |
| mSR-A1 | mSR-A1F | 5′-TTCACTGGATGCAATCTCCAAG-3′ |
| | mSR-A1R | 5′-CTGGACTTCTGCTGATACTTTGT-3′ |
| mMARCO | mMARCOF | 5′-GGGTCAAAAAGGCGAATCTTTC-3′ |
| | mMARCOR | 5′-CCCTCTGGAGTAACCGAGCA-3′ |
| mFcγR2a | mFcγR2aF | 5′-TGGACAGCCGTGCTAAATCTT-3′ |
| | mFcγR2aR | 5′-GGTCCCTTCGCATGTCAGTG-3′ |

were treated with IFL with or without LPS for 18 h, and then were incubated with pHrodo-labeled *E. coli* at 37 °C for 1 h. The dishes were gently rinsed with sterile PBS for three times, and Hoechst (33342) (2 μg/mL) were added into the dishes for 10 min. Cells were observed by Leica TCS SP2 confocal laser microscopy (Leica Microsystems, Wetzlar, Germany).

### 2.10. Statistic analysis

Data are presented as means ± standard deviation (SD). Student's *t*-test was used to evaluate the comparisons between the two groups. One-way ANOVA was used to obtain the differences between multiple groups. *P* values were considered as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

### 2.11. Animal ethics

All experiments involving the use of animal subjects were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (No. PZSHUTCM201231001).

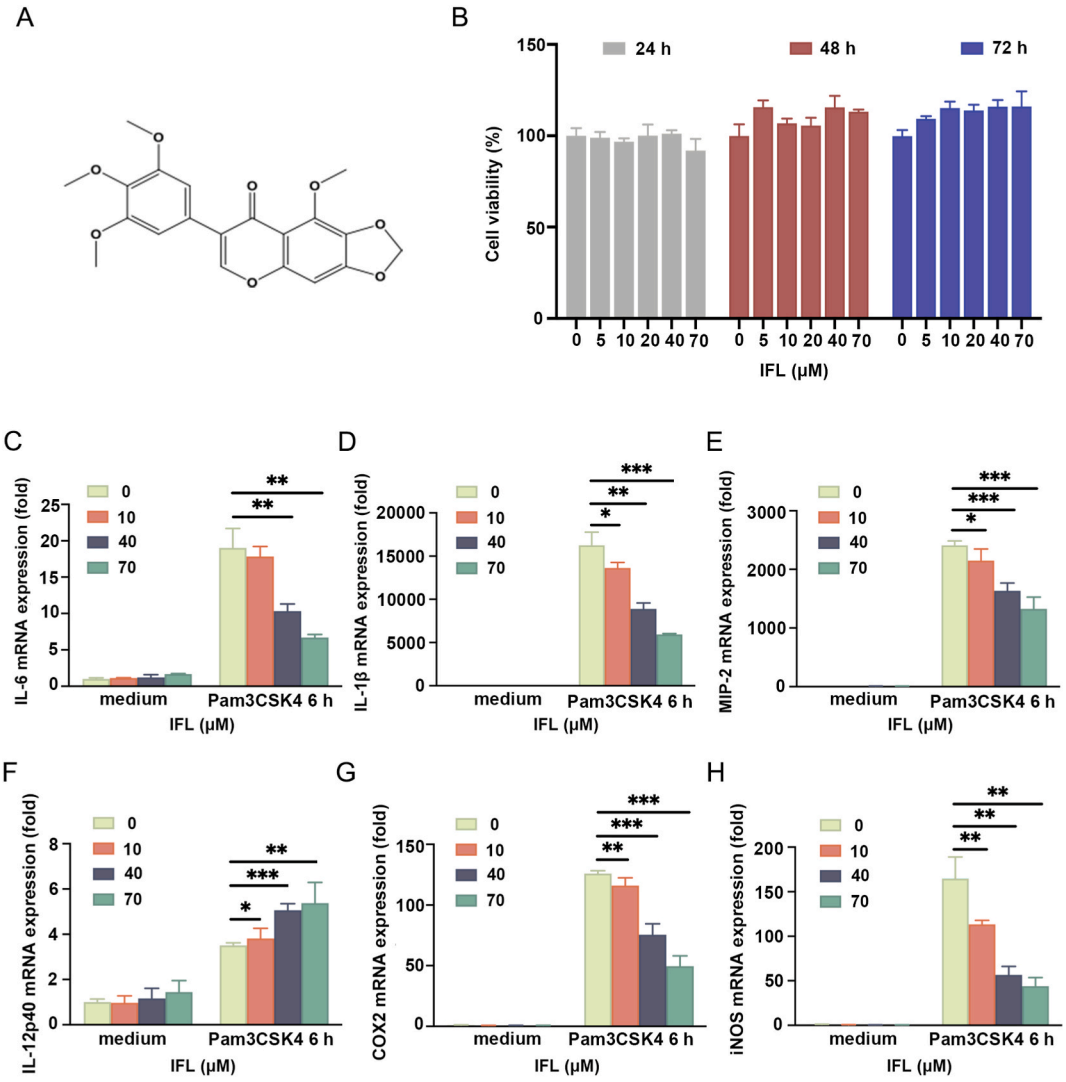


**Fig. 1.** The inflammatory effects of irisflorentin on Pam3CSK4-induced inflammatory response in Raw264.7. (A) The chemical structure of irisflorentin; (B) Cytotoxicity of irisflorentin was examined by CCK8 assay in Raw264.7. Data were presented as mean ± SD (n = 4). Raw 264.7 cells were seeded in 24-well plates ($4 \times 10^5$ cells/500 μL) overnight and were then treated with Pam3CSK4 (100 ng/mL) and IFL (0–70 μM) for 6 h. Total RNA samples were extracted and the mRNA expression of IL-6 (C), IL-β (D), MIP-2 (E), IL-12p40 (F), COX2 (G) and iNOS (H) was examined by qRT-PCR. Data were presented as mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

## 3. Results

### 3.1. IFL inhibited TLR2-activated production of inflammatory cytokines and mediators in Raw264.7 cells

The chemical structure of IFL is displayed in Fig. 1A. To test the possible toxicity of IFL to macrophage cells, different concentrations (0–70 μM) of IFL were used to treat Raw264.7 cells for 24–72 h and CCK-8 assay was performed to measure cell viability. As shown in Fig. 1B, none of the IFL concentrations tested caused cytotoxicity. Pam3CSK4 is a synthetic triacyl lipopeptide which mimics the acylated amino terminus of bacteria and can stimulate inflammatory response through Toll-like receptor 2 (TLR2). When assessing the effect of IFL on Pam3CSK4-induced expression of pro-inflammatory cytokines and mediators by real-time PCR, we found that IFL reduced the mRNA levels of IL-6, IL-1β, MIP-2, IL-12p40, COX2 and iNOS in a dose-dependent manner in Raw264.7 cells (Fig. 1C–H). This inhibitory effect is not stimulus-specific, IFL also inhibited the expression of pro-inflammatory cytokines induced by another TLR-2 agonist peptidoglycan (PGN), a main component of the bacterial cell wall (Fig. S1).

### 3.2. IFL reduced the secretion of IL-6, TNF-α, IL-12p70, IL-10 and IL-1β in Pam3CSK4-induced mouse primary macrophages and inhibited the polarization of M1 macrophages

In addition to Raw264.7 cells, we also used mouse primary macrophages to assess the effect of IFL on Pam3CSK4-induced gene expression. Similar to what was observed in Raw264.7 cells, IFL decreased the transcription of IL-6, TNF-α, IL-1β, MIP-2 and COX2 in Pam3CSK4-induced bone marrow-derived macrophages (BMDMs) (Fig. 2A–E). Consistently, ELISA results indicated that Pam3CSK4-induced secretion of inflammatory cytokines IL-6, TNF-α and IL-12p70 was also reduced by IFL in mouse primary peritoneal

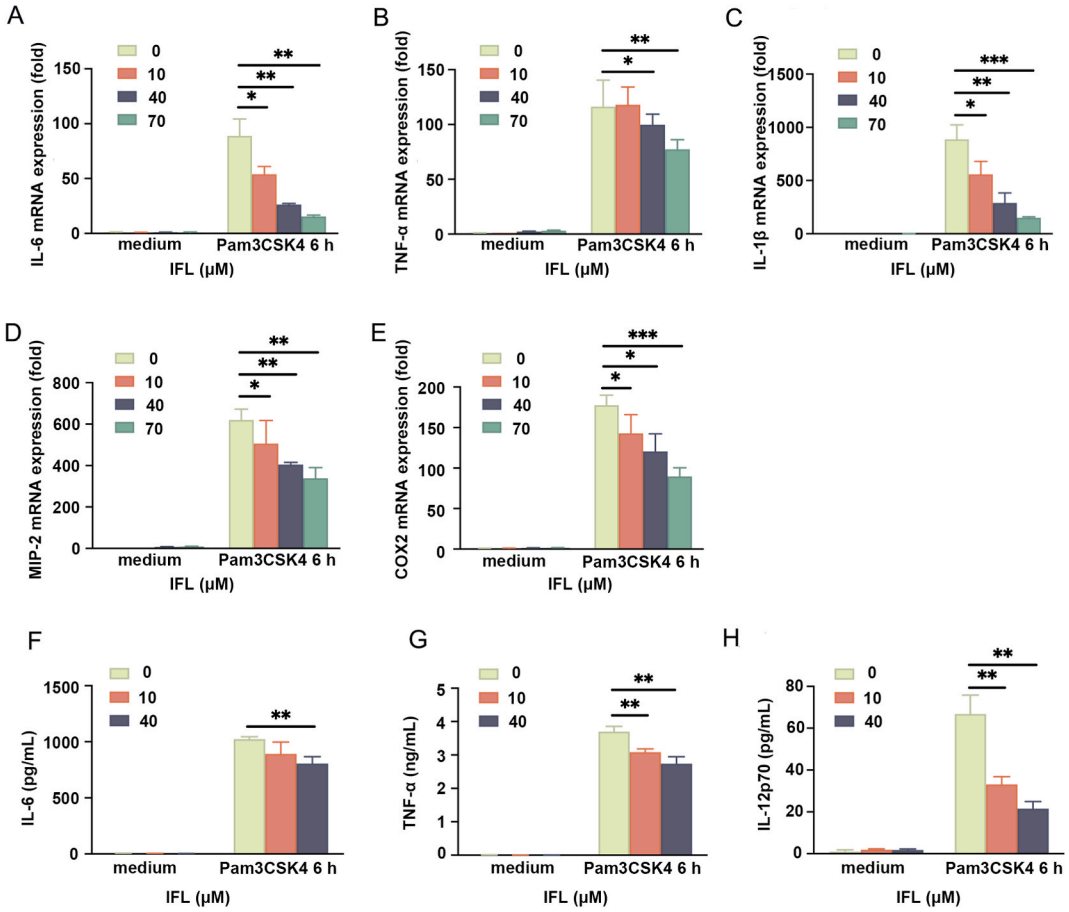


**Fig. 2.** IFL downregulated the production of Pam3CSK4-induced inflammatory cytokines in both bone marrow-derived macrophages (BMDMs) and mouse primary macrophages. BMDMs were plated overnight on Day 5 and stimulated with Pam3CSK4 (100 ng/mL) and IFL (0–70 μM) for 6 h. The mRNA levels of IL-6 (A), TNF-α (B), IL-1β (C), MIP-2 (D) and COX2 (E) were examined by qRT-PCR. Mouse primary peritoneal macrophages were seeded into 24-well plates ($3.5 \times 10^5$ cells/well) and incubated overnight. Cells were treated with Pam3CSK4 (100 ng/mL) and IFL (0–40 μM) for 6 h. The concentrations of IL-6 (F), TNF-α (G) and IL-12p70 (H) in the supernatants were measured by ELISA. Data were shown as mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

macrophages (Fig. 2F–H).

The overwhelmed inflammatory response and imbalanced polarization of M1 macrophage are usually related to cytokine storm and poor prognosis in infectious diseases. To investigate whether IFL can influence the polarization of macrophages, we induced M1 and M2 macrophages as illustrated, in the presence or absence of IFL (Fig. 3A). The expression of classical M1 markers, IL-6, CD86 and iNOS were analyzed by qRT-PCR. IFL decreased the expression of CD86 and iNOS significantly, suggesting that IFL could inhibit the polarization of M1 macrophages, accounting for the anti-inflammatory role of IFL (Fig. 3B–D).

### 3.3. IFL reduced the secretion of IL-6, IL-12p70 and IL-10 in Pam3CSK4-stimulated dendritic cells (DCs)

During bacterial infections, DCs not only produce inflammatory factors but also serve as professional antigen-presenting cells to initiate adaptive immunity. To investigate the effect of IFL on DC-mediated inflammatory responses, bone marrow-derived dendritic cells (BMDCs) were stimulated by Pam3CSK4 for two time points in the presence or absence of IFL and the cytokine production in culture supernatants was examined by ELISA. As shown in Fig. 4, all doses of IFL significantly decreased the expression of IL-6, IL-12p70 and IL-10.

### 3.4. IFL decreased the secretion of IL-6, TNF-α and IL-10 in HK-MRSA-stimulated macrophages

Heat inactivation of MRSA (HK-MRSA) was performed to eliminate viability but maintain the structure of bacteria and stimulate the biological activity of PAMPs. BMDMs were then provoked by HK-MRSA to elicit an inflammatory response, and the regulatory effect of IFL on inflammatory cytokines secretion was measured by ELISA. Results showed that IFL suppressed the production of IL-6, TNF-α and IL-10 in a dose-dependent manner in primary macrophages (Fig. 5A–C).

### 3.5. IFL enhanced the phagocytosis of E. coli by macrophages

During the defense against bacterial infections, the elimination of invading pathogens by macrophage-mediated phagocytosis plays a vital role. To investigate whether IFL regulates this process, Raw264.7 cells were pretreated with LPS in the presence or absence of IFL for 24 h and then co-cultured with *E. coli* labeled with a pH-sensitive dye, pHrodo. Flow cytometry and confocal microscopy were used to analyze the amount and status of engulfed *E. coli*. Flow cytometry results indicated that a substantial number of Raw264.7 cells were able to phagocytize *E. coli,* but IFL treatment significantly enhanced the number of cells that phagocytosed *E. coli* (Fig. 6A–D). When we closely examined the amount of engulfed *E. coli* in individual macrophage cells under a confocal microscope, we found that IFL treatment also increased the intensity of fluorescence from pHrodo-*E. coli* in each phagocyte (Fig. 6E), indicating that IFL enhances

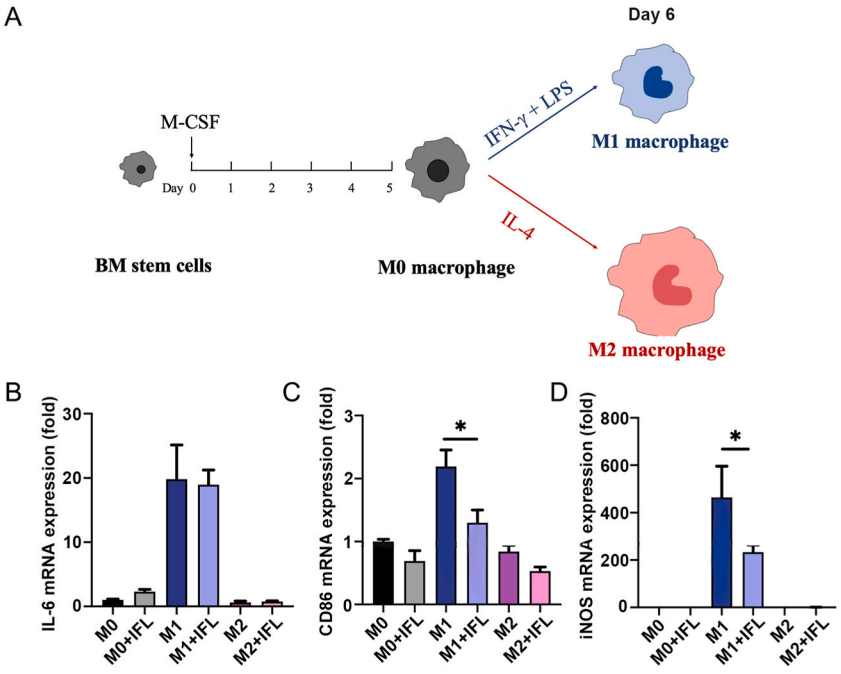


**Fig. 3.** IFL inhibits the polarization of M1 macrophages. Mouse bone marrow stem cells were induced into M0 macrophages and differentiated into M1 or M2-polarized macrophages (A). BMDMs were seeded in 24-well plates ($3.5 \times 10^5$ cells/well) overnight and treated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) along with IFL (70 μM) for 24 h, and the expression of IL-6 (B), CD86 (C) and iNOS (D) were analyzed. Data were presented as mean ± SD (n = 3). *, $P < 0.05$.

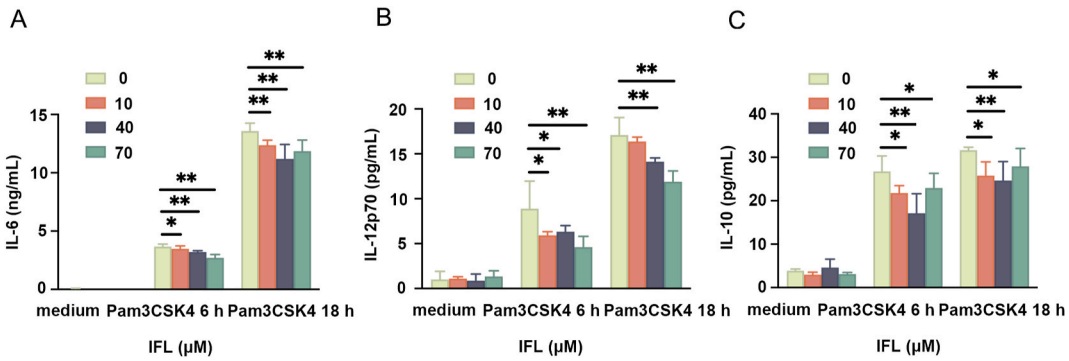


**Fig. 4.** IFL reduced the secretion of IL-6, IL-12p70 and IL-10 in Pam3CSK4-stimulated dendritic cells (DCs). Immature DCs were seeded in 24-well plates ($5 \times 10^5$ cells/well) and then stimulated with Pam3CSK4 (100 ng/mL) and IFL (0–70 μM) for 6 or 18 h. The concentrations of IL-6 (A), IL-12p70 (B) and IL-10 (C) in cell supernatants were determined by ELISA. Data were presented as mean ± SD (n = 3). *, $P < 0.05$ and **, $P < 0.01$.

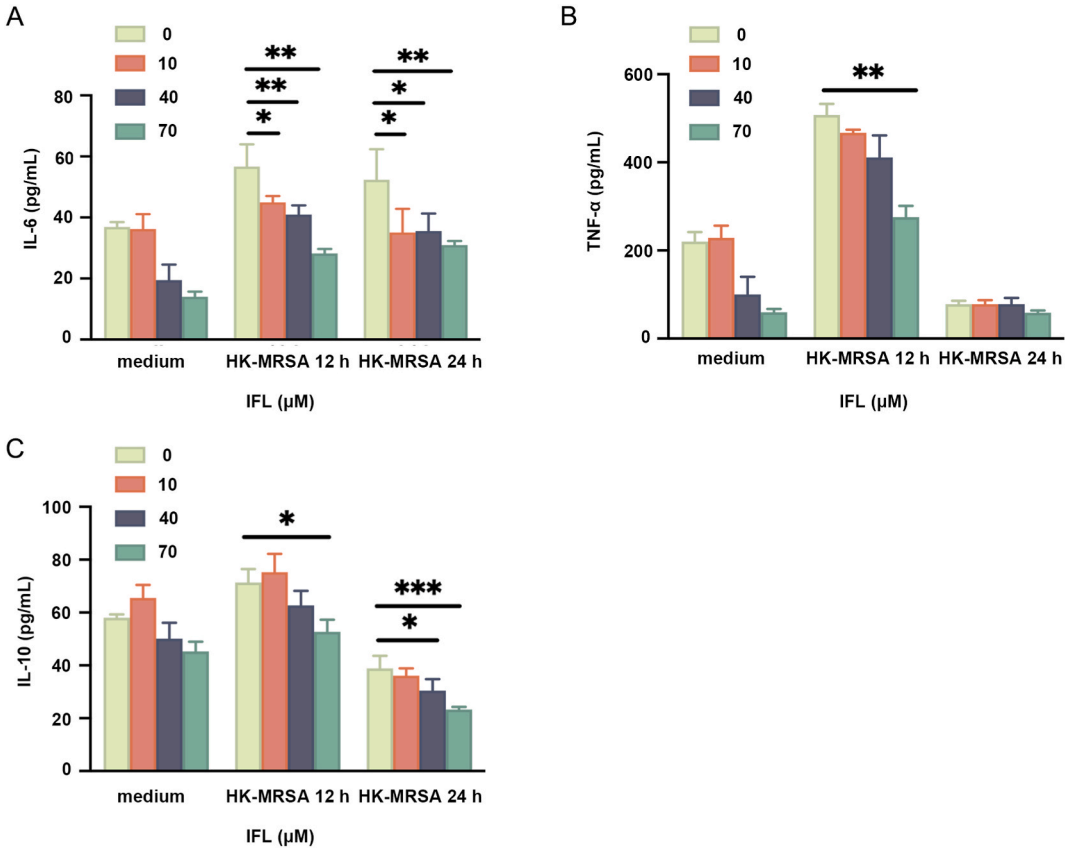


**Fig. 5.** IFL reduced the secretion of IL-6, TNF-α and IL-10 in heat-killed MRSA (HK-MRSA)-stimulated macrophages. Bone-marrow cells were differentiated into macrophages and seeded in 24-well plates ($3.5 \times 10^5$ cells/well). After stimulation with HK-MRSA (MOI = 10) and treatment with IFL (0–70 μM) for 12 or 24 h, the culture supernatants were collected for ELISA to measure the levels of IL-6 (A), TNF-α (B), and IL-10 (C). Data were presented as mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

the ability of macrophages to phagocytose pathogens.

### *3.6. IFL increased the expression of FcγR2a and SR-A1 in macrophages*

Macrophage-mediated phagocytosis requires the recognition of pathogens by various phagocytic receptors. To determine the mechanism by which IFL regulates bacterial phagocytosis by macrophages, we explored the effect of IFL on the expression of

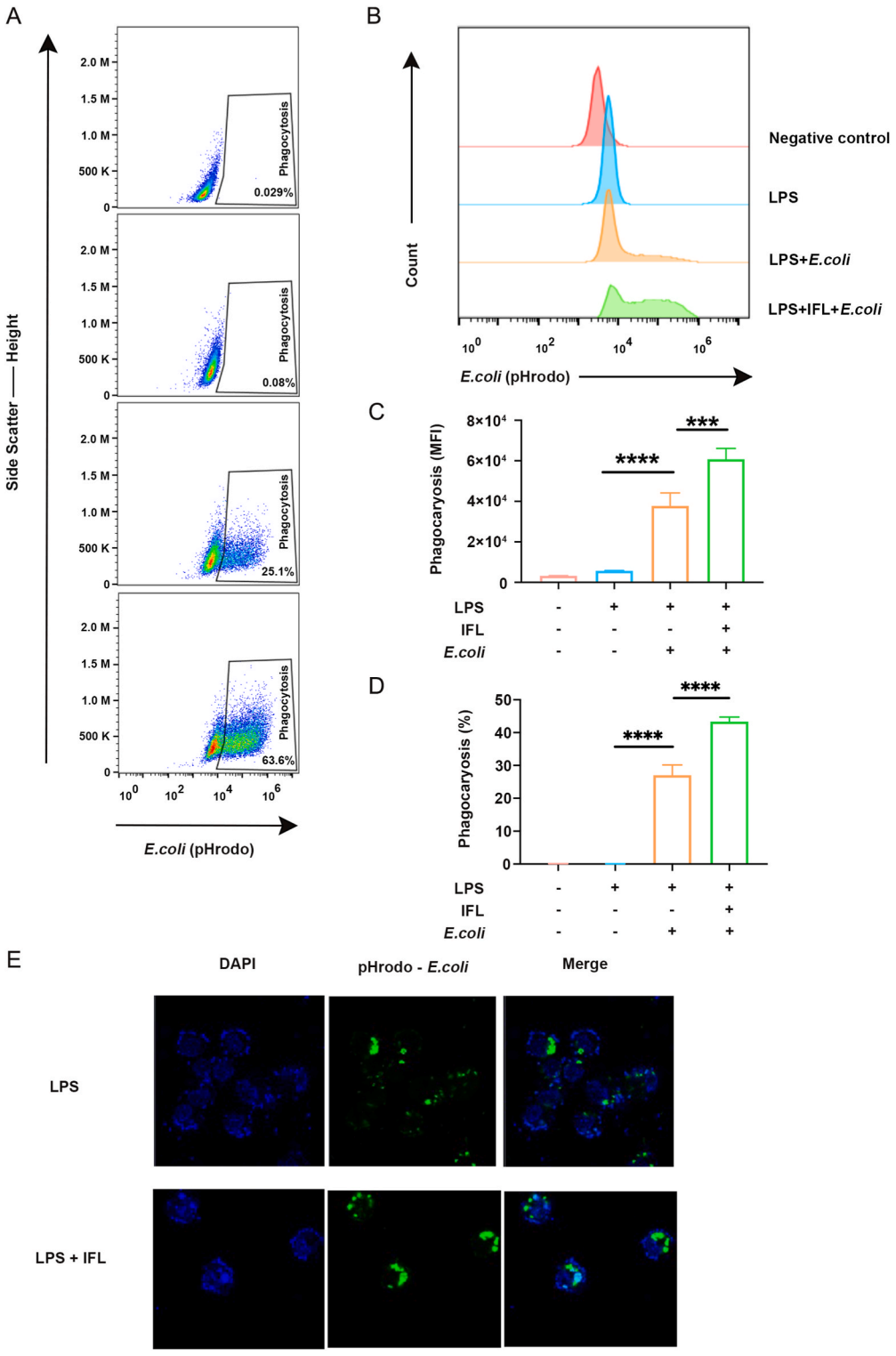


**Fig. 6.** IFL enhanced the phagocytosis of pHrodo-labeled *E. coli* by Raw264.7 cells. Raw264.7 cells were seeded ($1 \times 10^5$ cells/well) in 96-well plates and incubated overnight. Cells were unstimulated or stimulated with LPS (100 ng/mL) and IFL (40 μM) for 24 h followed by incubation with pHrodo-labeled *E. coli* for 1 h. Extracellular bacteria were washed away with sterile PBS and the phagocytosis of pHrodo-labeled *E. coli* was quantified by Flow cytometry. The scatter plot was shown (A). The mean fluorescence intensity (MFI) of pHrodo-labeled *E. coli* phagocytized by Raw264.7 cells were analyzed (B, C). The proportion of macrophages that phagocytized pHrodo-labeled *E. coli* were shown (D). Raw264.7 cells were seeded in 35 mm dishes ($1 \times 10^5$ cells) and incubated overnight. Cells were treated with LPS (100 ng/mL) and IFL (40 μM) for 18 h. Before the

examination, cells were incubated with pHrodo-labeled *E. coli* for 1 h. The bacteria outside of cells were washed twice with sterile PBS and then the nuclei were stained by Hochest 33342. The phagocytosis of pHrodo-labeled *E. coli* by macrophages was evaluated by confocal microscopy (E). Data were presented as mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

phagocytic receptors by RT-PCR. Consistent with previous findings, among the five receptors tested, expression levels of SR-A1 and FCγR2a were markedly up-regulated by Pam3CSK4 and HK-MRSA stimulations; IFL treatment further increased their expression levels. However, IFL had little or no effect on the expression of other phagocytic receptors tested, including CD206, Dectin-1 and CD169. Intriguingly, treatment of cells with IFL alone dramatically increased the expression of SR-A1 (Fig. 7A–F). Taken together, these results suggested that IFL is capable of explicitly promoting the expression of FCγR2a and SR-A1 in the presence or absence of stimulations, respectively.

### 3.7. IFL suppressed the activation of ERK and p38 MAPKs in Pam3CSK4-induced macrophages

As previously described, IFL exerted an anti-inflammatory effect in response to stimulations by TLR2 ligands Pam3CSK4, PGN, or HK-MRSA. Hence, we dissected the underlying molecular mechanism by examining the effect of IFL on TLR2-mediated activation of ERK and p38 MAPK and NF-κB signaling pathways. As shown in Fig. 8, IFL greatly reduced the phosphorylation of ERK, p38 in Pam3CSK4-stimulated mouse peritoneal macrophages, and moderately inhibited the phosphorylation of IKKα/β, leading to a slight delay of IκBα degradation under the same condition. Collectively, IFL suppressed the activation of MAPKs and NF-κB in response to Pam3CSK4 stimulation of macrophages, which may account for the anti-inflammatory role of IFL.

## 4. Discussion

Sepsis caused by bacterial infections is a very serious condition. Severe antibiotic resistance, exemplified by the high isolation rate of methicillin-resistant *Staphylococcus aureus* in clinical isolates, has become a major challenge to antibiotic therapy of sepsis. Therefore, new therapeutic strategies are urgently needed. One complementary strategy is the emerging host-directed therapy (HDT), which bypasses antibiotic problems by directly regulating host immune response during infection [13]. It is of great value to discover or develop HDT drugs targeting the innate immunity of the host that is responsible for the immediate responses to invading pathogens, initiation of adaptive immunity, and eventual involvement in the pathogenesis of sepsis.

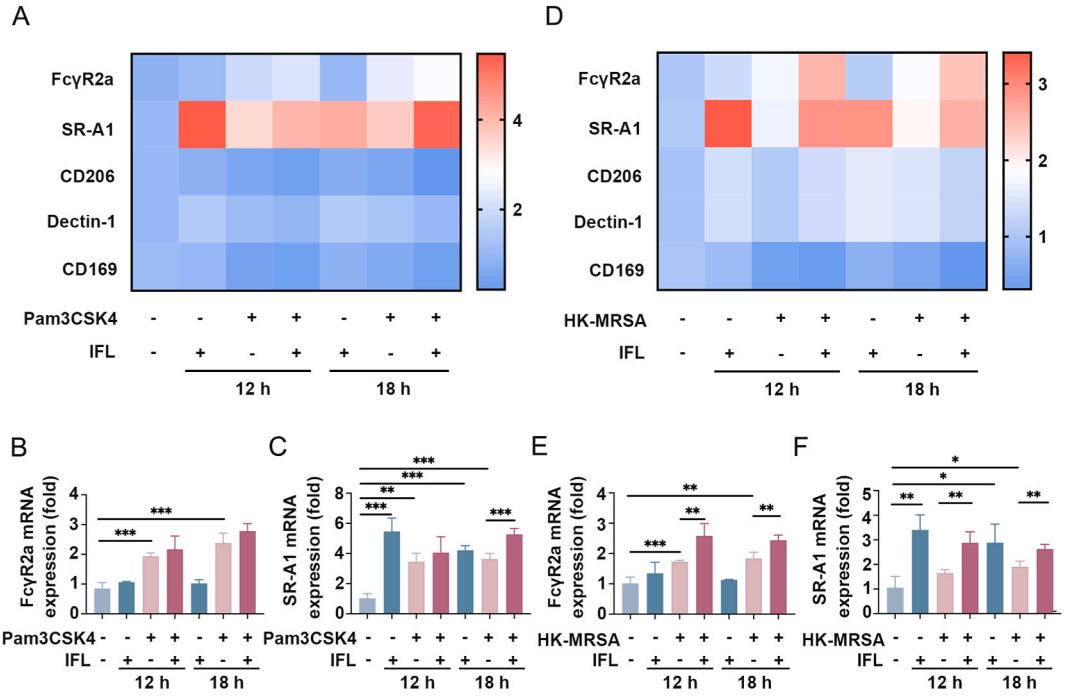


**Fig. 7.** IFL increased the expression of phagocytic receptors FcγR2a and SR-A1 in HK-MRSA and Pam3CSK4-induced mouse primary macrophage. Mouse primary peritoneal macrophages were seeded in 24-well plates ($3.5 \times 10^5$ cells/well) and incubated overnight. Cells were treated with Pam3CSK4 (100 ng/mL) or HK-MRSA (MOI = 10) separately along with IFL (40 μM) for 12 h or 18 h. Total RNAs were extracted and the expression of FcγR2a (A, B, E), SR-A1 (A, B, F), CD206 (A, D), Dectin-1 (A, D) and CD169 (A, D) were detected by qRT-PCR. Heat map was used for a global comparison of expression levels of all genes tested (A, D), and bar graphs were used to show the expression levels of FcγR2a (B, E) and SR-A1 (C, F). Data were shown as mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

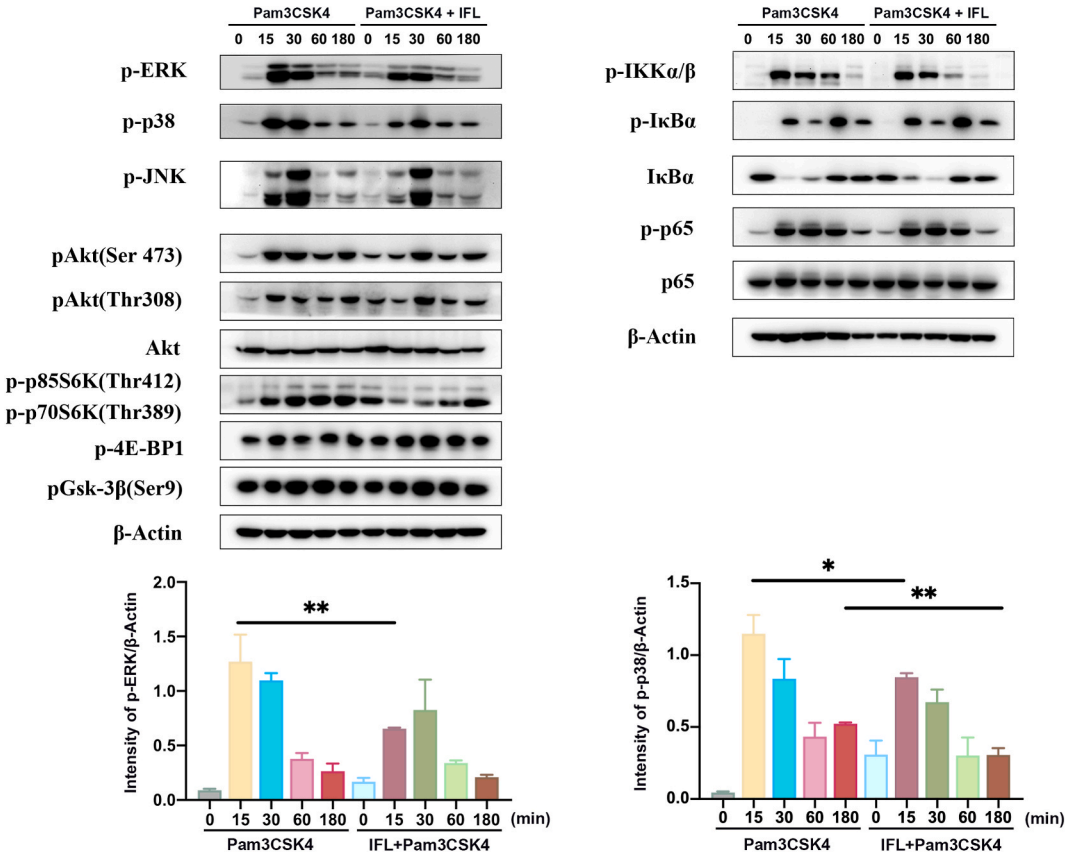


**Fig. 8.** IFL suppressed the phosphorylation of ERK, p38 and JNK MAPKs in Pam3CSK4-induced mouse primary macrophage. Mouse primary peritoneal macrophages were seeded in 6-well plates ($1 \times 10^6$ cells/well) and incubated overnight. Cells were treated with Pam3CSK4 (100 ng/mL) alone or together with IFL (40 μM) for 0–3 h. The phosphorylation of important signaling molecules in MAPK, NF-κB and PI3K/Akt pathways were assessed by Western blot.

Natural products are the most promising source of bioactive substances and innovative drugs, studies of which have led to the discovery, design and synthesis of many new drugs [14]. For example, by the end of 2014, 49 % of the 175 small molecule anti-cancer drugs and >50 % (82/112) of the 112 small molecule anti-bacterial drugs approved were natural products or derived directly from natural products [15]. The utilization of natural products and/or their novel structures for drug discovery and development remains a research hotspot [15]. TCM has a long history. While the therapeutic mechanisms of TCM remain largely undefined, it represents a treasure of medicines and deserves to be explored in depth for novel drug discovery. As a natural product that serves as an important gradient in anti-infective and anti-inflammatory TCM, IFL has been reported to suppress the secretion of LPS-induced inflammatory mediators in Raw264.7 and dendritic cells, but the underlying mechanism has not been characterized [16,17]. In the present study, we demonstrated that IFL could remarkably inhibit bacterial mimics, Pam3CSK4 or PGN -stimulated production of proinflammatory cytokines, such as IL-6, TNF-α, IL-12p70, IL-10 and IL-1β, not only in Raw264.7 cells but also in mouse primary macrophages (BMDMs or peritoneal macrophages) and dendritic cells through suppressing the activation of ERK and p38 MAPK pathways. More importantly, our results defined a previously uncharacterized role for IFL in modulating macrophage-mediated phagocytosis of bacteria. Given that IFL can promote the phagocytosis of a bacterial pathogen and suppress TLR2/4-dependent cytokine production, it is tempting to speculate that IFL has the potential to be a HDT drug candidate to treat bacterial infectious disease.

Macrophages are the primary cellular component in the innate immune system and play critical regulatory functions in the inflammatory response, pathogen phagocytosis, and tissue remodeling via distinct signaling pathways [18]. MAPK pathways are important signaling pathways controlling the induction, strength, and duration of bacterial infection-elicited inflammatory responses. Upon encountering bacteria or their components, TLR2/4 signal through a set of adaptor or scaffold proteins to activate TAK1 kinase, which subsequently phosphorylates and activates MAPKs for the activation of transcription factors (AP-1, ATF, etc.) and transcription of the pro-inflammatory cytokines. Our findings showed that IFL inhibited the activation of ERK and p38 MAPKs in Pam3CSK4-treated macrophages, accounting for its anti-inflammatory immunoregulatory role. Determination of the specific target(s) of IFL needs further investigation.

As professional phagocytes for clearance of infections, macrophages specifically recognize invading pathogens by various phagocytic receptors that can be categorized into four families, mannose receptors, scavenger receptors (SRs), complement receptors

and the Fcγ receptors (FcgRs) [19]. The expression of many phagocytic receptors is dynamically regulated to adapt to infections [20]. To determine whether IFL regulates the expression of these phagocytic receptors in macrophages, we selected a panel of representative members from these families for RT-PCR examination. The results revealed that in Raw264.7 cells, IFL dramatically promoted the expression of SR-A1, a member of SRs. Interestingly, in combined treatments, IFL further enhanced Pam3CSK4 or HK-MRSA-induced expression of SR-A1, suggesting that IFL may up-regulate SR-A1 expression during bacterial infection, at least including MRSA infection. Since SR-A1 can bind LPS, LTA and bacterial CpG DNA to mediate phagocytosis of both Gram-negative (e.g., *E. coli*) and Gram-positive bacteria (e.g., *Staphylococcus aureus*) [21], it is reasoned that IFL may broadly augment macrophage-mediated phagocytosis of many other bacterial pathogens, which is of potential significance for the treatment of bacterial infection in the clinic.

However, the bioavailability of IFL is predicted to be low by TCMSP [22], which may hamper in vivo study of IFL. To this end, the molecular structure of IFL should be modified to improve its solubility but maintain its bioactivity in the future. In a word, IFL is a potential anti-inflammatory and immunoregulatory compound, which has dual effects on promoting pathogen clearance and limiting the inflammatory response and immune damage during bacterial infection.

## 5. Conclusions

This study showed that IFL can down-regulate Pam3CSK4- and HK-MRSA-induced inflammatory response in macrophages and dendritic cells via suppressing the activation of ERK, p38 and JNK MAPK signaling pathways. IFL promotes the ability of macrophages to phagocytize bacterial pathogens by specifically up-regulating the expression of phagocytic receptor SR-A1. Taken together, IFL is a potential leading compound of host-directed therapy for bacterial infection.

## Data availability statement

Data associated with our study has not been deposited into a publicly available repository, and data will be made available on request.

## Summary sentence

Irisflorentin is a promising immunomodulatory drug candidate to maintain the appropriate level of immune response during MRSA infection.

## Ethics Declarations

This study was reviewed and approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine, with the approval number: No. PZSHUTCM201231001.

## CRediT authorship contribution statement

**Tiannan Xiang:** Data curation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Yingxiang Zou:** Conceptualization, Data curation, Investigation, Validation, Visualization, Writing - original draft. **Xinru Jiang:** Formal analysis, Software. **Lirong Xu:** Software. **Lu Zhang:** Methodology. **Chunxian Zhou:** Software. **You Hu:** Software. **Xiaolan Ye:** Software. **Xiao-Dong Yang:** Supervision, Writing - review & editing. **Xin Jiang:** Writing - review & editing. **Yuejuan Zheng:** Formal analysis, Supervision, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This research was funded by the National Key Research and Development Program of China (2021YFE0200900, 2022YFC3502000), National Natural Science Foundation of China (82073901, 82141203), Shanghai Municipal Science and Technology Major Project (ZD2021CY001, ZXS001-F4R4Z3), Three-year Action Plan for Shanghai TCM Development and Inheritance Program [ZY(2021–2023)-0401, ZY(2021–2023)-0103], and Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (ZYYCXTDD-202004).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23225.

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