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Vibrio parahaemolyticus flagellin induces cytokines expression via toll-like receptor 5 pathway in orange-spotted grouper, *Epinephelus coioides*

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ABSTRACT

Vibrio parahaemolyticus is the major pathogen of vibriosis in aquatic animals and causes inflammation that may be related to tissue damage. Here, we have established a *V. parahaemolyticus* flagellin stimulation model using grouper spleen (GS) cell line. Purified *V. parahaemolyticus* flagellin was used to stimulate GS cells. Our results showed that the mRNA levels of orange-spotted grouper (*Epinephelus coioides*) toll-like receptor 5M (*Ec*TLR5M), *Ec*TLR5S and downstream cytokines, such as interferon-γ2 (IFN-γ2), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), were all significantly increased after stimulation with *V. parahaemolyticus* flagellin in GS cells. Gene silencing of the *Ec*TLR5M and *Ec*TLR5S in GS cells by using small interfering RNA resulted in suppression of the *V. parahaemolyticus* flagellin-induced cytokines expression. We further demonstrated that activation of both mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) were required for cytokines expression. We observed that the phosphorylation of NF-κB inhibitor-α (IκBα), extracellular signal-regulated kinase (ERK) and p38 were induced following treatment with flagellin. Additionally, most of p65, a NF-κB subunit, was found to translocate to the nucleus after 60 min stimulation. Overall, our results suggest that *V. parahaemolyticus* flagellin influences cytokines expression, such as IFN-γ2, IL-6 and TNF-α, via *Ec*TLR5s recognition and MAPKs/NF-κB signaling pathway activation in GS cells.

1. Introduction

Pattern recognition receptors (PRRs), as a member of innate immune system, recognize pathogen-associated molecular patterns (PAMPs), and trigger the signaling pathways that initiate immune response to pathogen infection [1,2]. Toll-like receptors (TLRs), as an important part of PRRs, sense many different types of PAMPs to trigger myeloid differentiation primary-response protein 88 (MyD88)-dependent or independent signaling pathway to provide specificity immune response [3]. TLR5 specifically recognizes the certain conservative site of bacterial flagellin from both Gram-positive and Gram-negative bacteria, and passes signal through MyD88-dependent signaling pathway that leads to certain pro-inflammatory cytokines production in mammals [4]. There are two forms of TLR5, the membrane form of TLR5 (TLR5M) and the soluble form of TLR5 (TLR5S) in fish. TLR5S is a piscine-specific gene, and have been identified in teleost including rainbow trout (*Oncorhynchus mykiss*) [5], channel catfish (*Ictalurus punctatus*) [6], Japanese flounder (*Paralichthys olivaceus*) [7], gilthead sea bream (*Sparus aurata*) [8], orange-spotted grouper (*Epinephelus coioides*) [9] and darkbarbel catfish (*Pelteobagrus vachellii*) [10]. TLR5M contains the typical structure of mammalian TLRs, including leucine-rich repeats (LRRs) domains, transmembrane region and Toll/IL-1 receptor homology domain (TIR) domain, while TLR5S structure lacks the transmembrane region and TIR domain [2].

Flagellin is not only the major structural component of the flagellum that affects bacterial motility but also an important virulence factor of

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bacteria [11]. In mammals, flagellin can be recognized by the plasma membrane TLR5 and the cytoplasm of NOD-like receptor C4 (NLRC4) [12]. Similarly, membrane TLR5 and soluble TLR5 are involved in the recognition of flagellin in teleost. After stimulation with flagellin, rainbow trout TLR5M (rtTLR5M) activates Nuclear factor κB (NF-κB) to induce the expression of cytokines and rtTLR5S, and then inducible rtTLR5S amplifies the rtTLR5M-mediated immune responses [5]. *Vibrio parahaemolyticus*, as a halophilic Gram-negative bacterium, is one of the major pathogenic bacteria of Vibriosis which causes serious economic losses in aquaculture industry. *V. parahaemolyticus* infection causes higher mortality and histopathological changes, which were observed after *V. parahaemolyticus* injection in grouper (*E. coioides*) [13], zebrafish (*Danio rerio*) [14], Chinese amphioxus (*Branchiostoma belcheri tsingtaunese*) [15]. Interestingly, *V. parahaemolyticus* possesses two distinct types of flagellar systems that propel bacterial movement under different circumstances. The polar flagellum promotes bacterial movement in liquid environments and the lateral flagella moves the bacteria on surfaces or in viscous environments [16]. In previous study, *V. parahaemolyticus* induces the expression of pro-inflammatory cytokines interleukin (IL)-6, IL-8 and IL-12 in Pacific red snapper (*Lutjanus peru*) [17]. However, it is unclear how *V. parahaemolyticus* flagellin affects the fish immune system.

In fish, TLR5 signaling pathway and the molecular mechanism of the *V. parahaemolyticus* flagellin-induced innate immune response have not been well studied. In mammals, ligation of TLR5 recruits the adaptor protein MyD88, leading to IκBα phosphorylation and NF-κB translocation [18]. Concomitantly, mitogen-activated protein kinases (MAPKs) are activated, causes the translocation of *c-Jun* and *Fos*, two subunits of the activator protein-1 (AP-1) [19]. In teleost, the role of AP-1 in the induction of cytokines expression is poorly understood. In this study, we aim to determine whether *V. parahaemolyticus* flagellin induces cytokines expression via TLR5 pathway, and investigate whether the AP-1 and NF-κB are both required for cytokines expression. Our study sought to further explore the pathogenic mechanism of *V. parahaemolyticus*, and provide some theoretical basis for *V. parahaemolyticus* defense in marine aquaculture.

2. Materials and methods

2.1. Cell culture and stimulation

HEK 293T cell line was brought from Cell Bank, Chinese Academy of Sciences (Shanghai, China). HEK 293T cells were cultured in DMEM medium (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA), and incubated at 37 °C with 5% CO_2. For experiments, HEK 293T cells were seeded in 12-well plates, then transfected with specifically siRNA and recombinant plasmid.

The grouper spleen cell line (GS cell line) was a gift from Prof. Qiwei Qin, South China Agricultural of University. GS cells were cultured in L15 medium (Hyclone, USA) containing 10% FBS (Hyclone, USA), and incubated at 27 °C without CO_2. For stimulation experiments, GS cells were evenly seeded in 96-well or 12-well plates and then cultivated 36 h before stimulation.

2.2. Flagellin preparation

V. parahaemolyticus that was isolated from a diseased *Epinephelus coioides*, was grown overnight in thiosulfate citrate bile salts sucrose (TCBS) agar culture medium at 28 °C. We identified this strain by mass spectrometry as *V. parahaemolyticus*. Single colonies were selected and inoculated into a tube containing 1 mL 2216E liquid medium, then were grown at 28 °C with 150 rpm for 2 h before colony PCR analysis. Positive bacteria were then inoculated into a flask with 300 mL 2216E incubated at 28 °C with 150 rpm for 16 h. The procedure of *V. parahaemolyticus* flagellin extraction was modified based on methods previously described [20]. *V. parahaemolyticus* were harvested by centrifugation (5000 × *g*, 4 °C, 30 min) and resuspended in PBS (Hyclone, USA). The suspension was then adjusted to pH 2.0 with 1 M HCl and constantly stirred for 30 min at room temperature. Flagella, which were now divorced from bacterial, were separated by centrifugation (5000 × *g*, 4 °C, 30 min) and further centrifuged (13,000 × *g*, 4 °C, 30 min) to precipitate the pH 2.0-insoluble material. The pH of the supernatant was adjusted to 7.2 with 1 M NaOH and slowly added solid ammonium sulfate with stirred to achieve two-thirds saturation (2.67 M). The solution was held overnight at 4 °C and then was centrifuged (14,000 × *g*, 4 °C, 25 min). The pellet, which contained polymerized flagellin, was resuspended in 5 mL of PBS and transferred to ultrafiltration device, which had a molecular weight cutoff of 30,000 D (Millipore, UK), with centrifuge desalination. Flagellin was removed residual endotoxin using endotoxin removal kit (GenScript, USA) and measured endotoxin levels using Chromogenic LAL endotoxin assay kit (GenScript, USA). The flagellin solution was lyophilized and stored at −80 °C. The final concentration of the purified flagellin was measured using BCA protein assay kit (Thermo, USA). The extracted flagellin was observed through Jeol JEM-100CXII transmission electron microscopy (Japan), and confirmed the purified flagellin through SDS-PAGE and western blot experiments. Anti-*Vibrio parahaemolyticus* polar flagellin B/D antibody used in western blot experiment was purchased from Bioss (China) (Catalogue No. Bs-8876R).

2.3. Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR) analysis, extraction of total RNA and synthesis of cDNA were using SuperPrep Cell Lysis & RT Kit for qPCR Reagents (Toyobo, Japan), and performed on the ABI 7900 Real-time System using SYBR Green Real Time PCR Master Mix kit (Toyobo, Japan). Primer sequences used in qRT-PCR were listed in Table 1 and all samples were amplified in quadruplicate wells. The data were calculated using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method, and EF-1α served as an internal control.

2.4. Knockdowns of EcTLR5M and EcTLR5S

Small interfering RNAs (siRNAs) were designed and synthesized based on the sequences of *Ec*TLR5M and *Ec*TLR5S by GenePharma (Shanghai, China). In order to verify the siRNA knockdown efficacy, the full open reading frame (ORF) of *Ec*TLR5M and *Ec*TLR5S were amplified and inserted into the pcDNA-4.0 vector (Invitrogen, USA), respectively. Primers, which were used to amplify the ORF of *Ec*TLR5M and *Ec*TLR5S, were listed in Table 2.

The siRNA knockdown efficacy was analyzed by qRT-PCR and

Table 1
Sequences of primers used in quantitation.

Primer	Accession No.	Sequence (5′-3′)
IFN-γ1-RT-F		CGATTCGGTCATCAAGAGCAT
IFN-γ1-RT-R		CTCCGTCACGACCGACACCA
IFN-γ2-RT-F	JX013936.1	CAGCAATGGTGAGGTGGCA
IFN-γ2-RT-R		TTTGCTCTGGATGATAGGGTC
IL-6-RT-F	AFE62919.1	CAATCCCAGCACCTTCCAC
IL-6-RT-R		CCTGACAGCCAGACTTCCTCT
IL-12-F	AEM06414.1	GTGGATGCCAGCGGTCAA
IL-12-R		GGAAATGCTCCGTCGTCA
IL-1β-F	EF582837	GAACAGCGACATGGTGCGGTTTC
IL-1β-R		GTGGGTGTCTCCTCCTTGATTGC
TNF-α-RT-F	ACM46004.1	CCTGGTGATGTGGAGATG
TNF-α-RT-R		GTCCGACTTGATTAGTGCTT
*Ec*TLR5M-RT-F	KM282522.1	TAGCCACTCCAGACCCAAG
*Ec*TLR5M-RT-R		GAGACGGGCGTAAACAATC
*Ec*TLR5S-RT-F	KR005612.1	TGTTTCCCAAAACAATGTGA
*Ec*TLR5S-RT-R		CATGACCCAGAACACCAATG
EF-1α-RT-F	AEG78376.1	GGTCGTCACCTTCGCTCCAT
EF-1α-RT-R		TCCCTTGGGTGGGTCATTCT

Table 2
Sequences of primers used for plasmid constructions.

Primer	Accession No.	Sequence (5′-3′)
*Ec*TLR5M-4.0-*BamH*I-F	KM282522.1	CGCGGATCCATGCAGCACCACTACTGG
*Ec*TLR5M-4.0-*Xho*I-R		CCGCTCGAGCATGGCAATAGGAGCGATGG
*Ec*TLR5S-4.0-*BamH*I-F	KR005612.1	CGCGGATCCATGTGGACGCTGGGTCTTCA
*Ec*TLR5S-4.0-*Xho*I-R		CCGCTCGAGCTGCTGTGTGATGTCATCAG

western blot. GS cells, an endogenous grouper TLR5-expressing cell line, were seeded into 96-well plates and cultured overnight, then transfected with 0.72 μg/mL *Ec*TLR5M/*Ec*TLR5S siRNA or negative control siRNA (NC siRNA) using Lipofectamine 3000 (Invitrogen, USA). GS cells were collected at 36 h post transfection and processed by SuperPrep Cell Lysis & RT Kit for qPCR Reagents (Toyobo, Japan). Then the mRNA levels of *Ec*TLR5M and *Ec*TLR5S were detected to reflect the siRNA knockdown efficacy. Similarly, HEK 293T cells, a non-fish and no grouper TLR5-expressing cell line, were seeded into 12-well plates and culture 12 h, then co-transfected with 1500 ng/mL recombinant plasmids, which containing the ORF of grouper target genes, and 0.72 μg/mL siRNA by Lipofectamine 3000 (Invitrogen, USA). Cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, China) containing 1% protease inhibitor (Sigma, USA) at 24 h after transfection, and then the total proteins were detected by western blot. *Anti*-myc tag and anti-his tag antibody used in western blot experiments were obtained from Proteintech (USA).

2.5. SDS-PAGE and western blot analysis

Intracellular protein phosphorylation analysis of extracellular signal-regulated kinase (ERK), *c-Jun* N-terminal kinase (JNK), p38 and NF-κB inhibitor-α (IκBα) were detected after flagellin stimulation. After stimulation, GS cells were washed with ice-cold PBS and lysed with RIPA lysis buffer (Beyotime Ins. Bio., China) containing protease inhibitor (Sigma, USA) and phosphatase inhibitor (Sigma, USA). Total proteins were separated by 12% SDS-PAGE gels and elector-transferred onto PVDF membranes (Millipore, UK). Membranes were blocked for 2 h in TBST containing 4% Bovine Serum Albumin (BSA) and 0.05% Tween 20 (Beyotime Institute of Biotechnology, China). Anti-total and anti-phosphorylated ERK, JNK, p38 and IκBα rabbit antibodies were purchased from Cell Signaling Technology (USA) (Anti-total ERK: Catalogue No. 4695P, Anti-total JNK: Catalogue No. 9258P, Anti-total p38: Catalogue No. 8690P, Anti-total IκBα: Catalogue No. 9242S,Anti-phosphorylated ERK: Catalogue No. 9106S, Anti-phosphorylated JNK: Catalogue No. 4668T, Anti-phosphorylated p38: Catalogue No. 4511P, Anti-phosphorylated IκBα: Catalogue No. 2859S). Goat anti-mouse IgG and goat anti-rabbit IgG second antibody were obtained from Proteintech (USA). Membranes were probed overnight with indicated antibodies at 4 °C, washed five times with TBST (containing 0.05% Tween 20) and incubated for 1 h with the second antibodies (Proteintech, USA). Mouse *anti*-β-actin antibody (Proteintech, USA) was used as an endogenous and loading control. Chemiluminescence detection was carried out in an Alliance MINI HD9 system (UVITEC, USA).

2.6. Immunofluorescence staining

To investigate the cellular localization of NF-κB subunit p65, the full ORF of *Ec*NF-κB-p65 was inserted into the pEGFP-N3 vector (Clontech, USA). GS cells were seeded into the 8-well chamber slide system (Thermo, USA) in 250 μL culture medium. GS cells were transiently transfected with 300 ng of *Ec*NF-κB-p65 plasmids using Lipofectamine 3000 Reagent (Invitrogen, USA) at 24 h after incubation. The cells were stimulated with flagellin or PBS at 48 h after transfection, and then washed twice with HBSS prior to fixation by incubating in 4% paraformaldehyde in PBS for 15 min incubation at room temperature. The cells were washed with PBS for three times before using DAPI (Thermo, USA) to stained the nucleus, and then visualized by Zeiss LSM 880 Meta microscope (Zeiss, Germany).

2.7. Statistical analysis

Results are reported as means values ± SEM. All data were analyzed by GraphPad Prism 5.0 software, carried out by *t*-test, one-way ANOVA or two-way ANOVA analysis of variance.

3. Results

3.1. V. parahaemolyticus flagellin induces the expression of cytokines in GS cells

By transmission electron microscopy, we clearly observed the morphology of flagellum in the coarsely extracted *V. parahaemolyticus* flagellin. Then the purified *V. parahaemolyticus* flagellin was verified through SDS-PAGE and western blot experiments. SDS-PAGE result showed that the maximum molecular weight of purified *V. parahaemolyticus* flagellin was about 43 kda (Fig. S), which was consistent with previous studies [21,22]. In order to examine whether *V. parahaemolyticus* flagellin is capable of inducing inflammatory responses on GS cells, we quantified the cytokines expression, including interferon-γ1 (IFN-γ1), IFN-γ2, interleukin-6 (IL-6), IL-12, IL-1β and tumor necrosis factor-α (TNF-α) in GS cells after exposure to flagellin at 6 h. The transcriptional levels of IFN-γ2 ($P < 0.001$), IL-6 ($P < 0.001$) and TNF-α ($P < 0.001$) were significantly increased in the flagellin-incubated cells (Fig. 1), suggesting that *V. parahaemolyticus* flagellin was able to induce the downstream cytokine transcription.

3.2. The expression patterns of IFN-γ2, IL-6 and TNF-α in V. parahaemolyticus flagellin-stimulated GS cells

In order to understand the effect of different incubation concentration and time of flagellin-stimulation on cytokines expression, GS cells were stimulated with different doses or time of flagellin. As shown

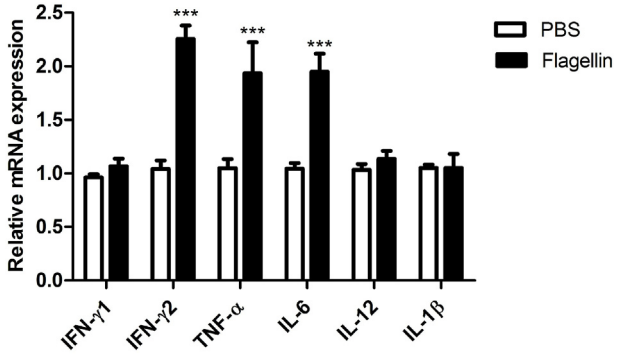

Fig. 1. *V. parahaemolyticus* flagellin induces the expression of IFN-γ2, IL-6 and TNF-α. GS cells were treated with PBS or 1 μg/mL V. *parahaemolyticus* flagellin for 6 h, and then collected and detected for cytokine expression. The mRNA expression levels of cytokines were measured by qRT-PCR using EF-1α served as an internal control. Data indicates the mean ± SEM (n = 4). ***$p < 0.001$.

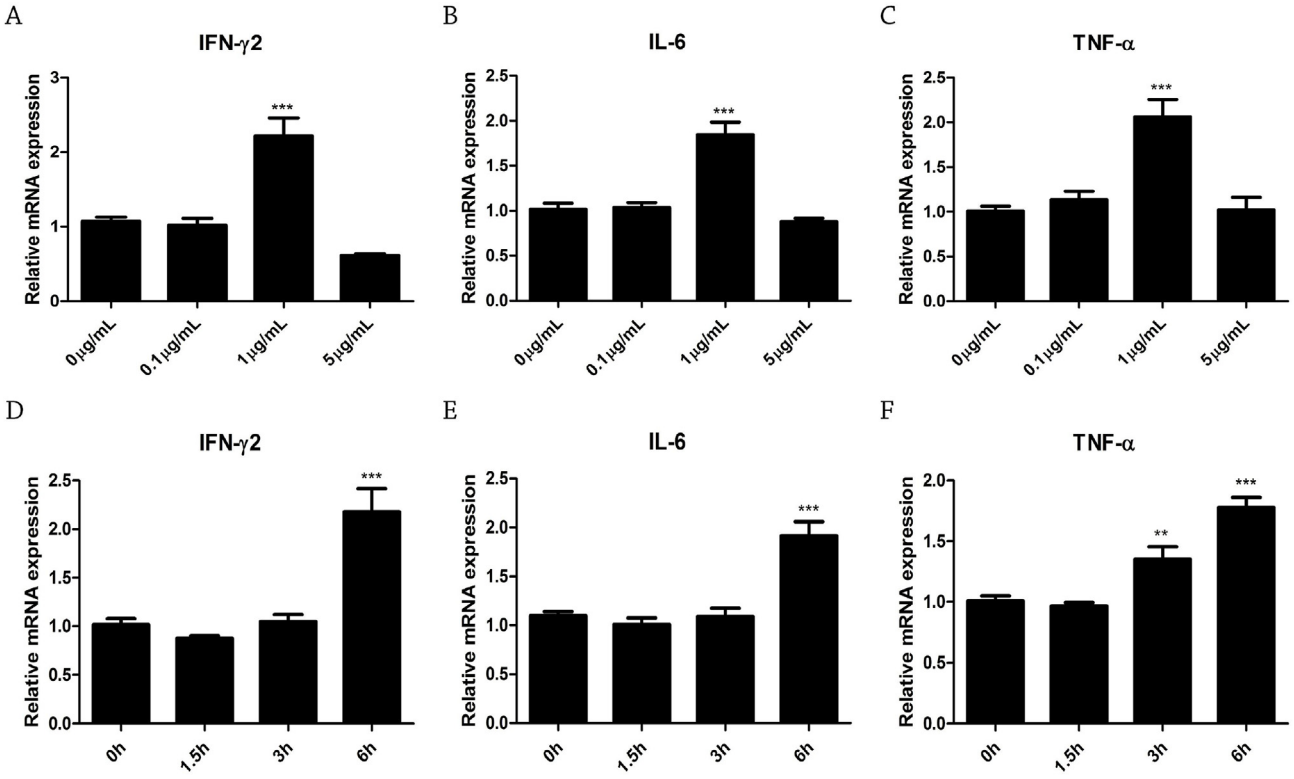

Fig. 2. *V. parahaemolyticus* flagellin-induced cytokine expression is affected by incubation dose and time. **(A–C)** GS cells were stimulated with 0, 0.1, 1 or 5 μg/mL of *V. parahaemolyticus* flagellin for 6 h. The mRNA expression levels of IFN-γ2 (A), IL-6 (B) and TNF-α (C) were analyzed by qRT-PCR. Data indicates the mean ± SEM (n = 4). $^{***}p < 0.001$. **(D–F)** GS cells were stimulated with 1 μg/mL flagellin for 0, 1.5, 3, or 6 h. The mRNA expression levels of IFN-γ2 (D), IL-6 (E) and TNF-α (F) were analyzed by qRT-PCR using EF-1α served as the internal control. Data indicates the mean ± SEM (n = 4). These dates were carried out by *t*-test analysis of variance. $^{**}p < 0.01$, $^{***}p < 0.001$.

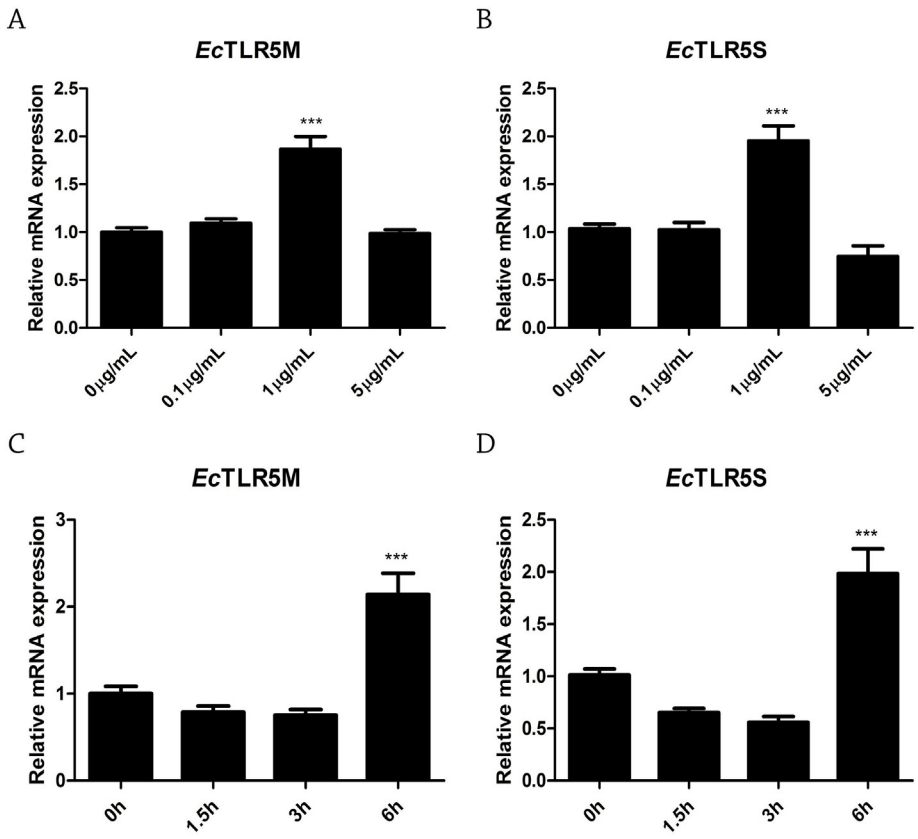

Fig. 3. *V. parahaemolyticus* flagellin induces the mRNA expression of *Ec*TLR5M and *Ec*TLR5S. **(A–B)** GS cells were stimulated with 0, 0.1, 1 or 5 μg/mL of *V. parahaemolyticus* flagellin for 6 h. The mRNA transcription levels of *Ec*TLR5M (A) and *Ec*TLR5S (B) were analyzed by qRT-PCR. Data indicates the mean ± SEM (n = 4). $^{***}p < 0.001$. **(C–D)** GS cells were stimulated with 1 μg/mL V. *parahaemolyticus* flagellin for 0, 1.5, 3, or 6 h. The mRNA expression levels of *Ec*TLR5M (C) and *Ec*TLR5S (D) were analyzed by qRT-PCR using EF-1α served as an internal control. Data indicates the mean ± SEM (n = 4). $^{***}p < 0.001$.

Table 3
Sequences of siRNA.

Named	Accession No.	Sense (5′-3′)	Antisense (5′-3′)
*Ec*TLR5M-siRNA-1	KM282522.1	GCGUGACCAUAGCAAGAAATT	UUUCUUGCUAUGGUCACGCTT
*Ec*TLR5M-siRNA-2	KM282522.1	GCAGCUCGCUCAGAAGAUATT	UAUCUUCUGAGCGAGCUGCTT
*Ec*TLR5S-siRNA-1	KR005612.1	CCAGCCUUCAAUGUUCUUUTT	AAAGAACAUUGAAGGCUGGTT
*Ec*TLR5S-siRNA-2	KR005612.1	CCUUCUGUUGAGUGACAAUTT	AUUGUCACUCAACAGAAGGTT

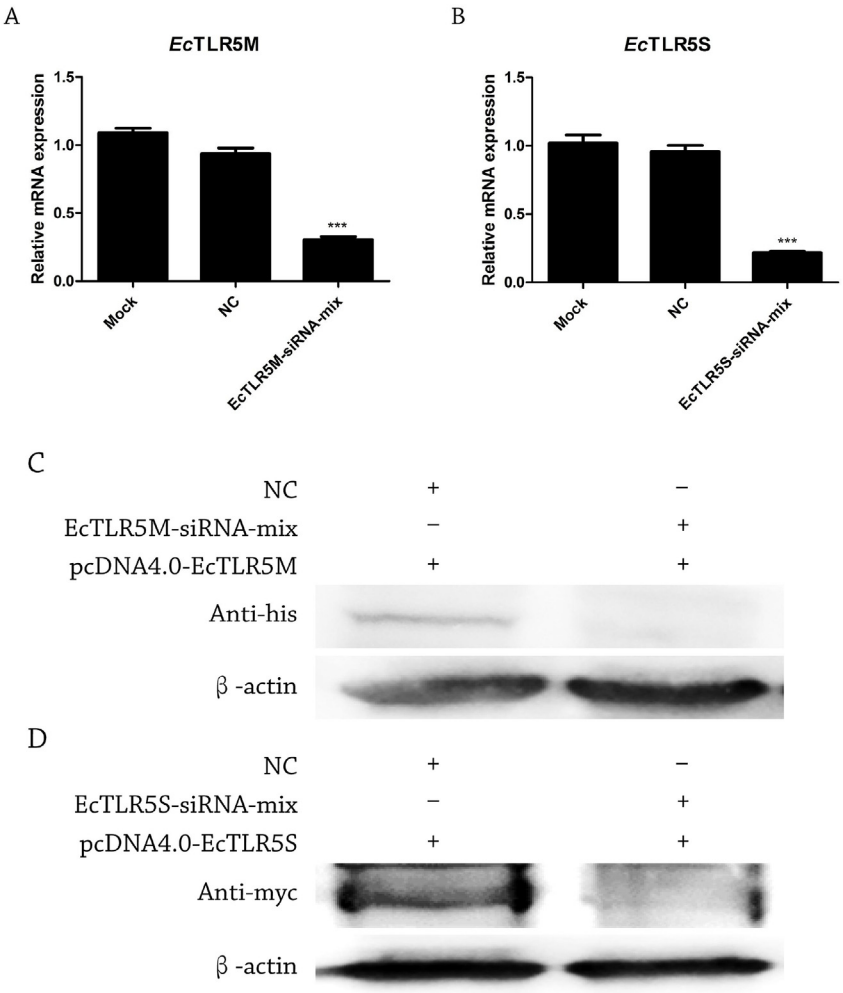

Fig. 4. The siRNA-mix knockdown verification of *Ec*TLR5M and *Ec*TLR5S. Two specific siRNAs were designed and screened for *Ec*TLR5M and *Ec*TLR5S, respectively. The two siRNA were transfected into cells at a ratio of 1:1. **(A–B)** *Ec*TLR5M and *Ec*TLR5S siRNA-mix knockdown efficacy were evaluated by qRT-PCR. GS cells were transfected with *Ec*TLR5M/*Ec*TLR5S-siRNA-mix or NC siRNA. The relative expression levels of *Ec*TLR5M and *Ec*TLR5S were normalized by Mock groups. Data indicates the mean ± SEM (n = 3). ***$p < 0.001$. These dates were carried out by *t*-test analysis of variance. **(C–D)** *Ec*TLR5M and *Ec*TLR5S siRNA-mix knockdown efficacy by western blot. HEK 293T cells were transfected with 1500 ng/mL *Ec*TLR5M/*Ec*TLR5S plasmids and 0.72 μg/mL siRNA-Mix or NC siRNA, then the total protein was extracted from cells at 48 h post transfection. β-actin served as internal control.

in Fig. 2A–C, the highest levels of IFN-γ2 ($P < 0.001$), IL-6 ($P < 0.001$) and TNF-α ($P < 0.001$) mRNA expression were observed when the cells were treated with 1 μg/mL flagellin. In flagellin-stimulated GS cells experiments at multiple time points (0, 1.5, 3 or 6 h), a significantly increase and peak of IFN-γ2 ($P < 0.001$), IL-6 ($P < 0.001$) and TNF-α ($P < 0.001$) mRNA expression were observed at 1 μg/mL flagellin treatment for 6 h (Fig. 2D–F). These conditions were used for subsequent experiments.

3.3. *EcTLR5M and EcTLR5S are involved in cytokines expression in V. parahaemolyticus flagellin-stimulated GS cells*

In previous study, membrane-bound and soluble forms of TLR5 have been identified in *Epinephelus coioides* [9]. To determine whether *V. parahaemolyticus* flagellin induce the expression of cytokines through the activation of *Ec*TLR5s, the expressions of *Ec*TLR5M and *Ec*TLR5S mRNA were quantified in GS cells in response to flagellin stimulation. In multiple concentrations flagellin-stimulated GS cells, a significant increase in *Ec*TLR5M ($P < 0.001$) and *Ec*TLR5S ($P < 0.001$) gene expressions were observed at 1 μg/mL flagellin stimulation (Fig. 3A–B). In flagellin-stimulated GS cells experiments at multiple time points, moderate increases in *Ec*TLR5M ($P < 0.001$) and *Ec*TLR5S ($P < 0.001$) mRNA expressions were detected at 6 h (Fig. 3C–D). These data suggested that *Ec*TLR5M and *Ec*TLR5S are likely to be involved in the recognition of *V. parahaemolyticus* flagellin in GS cells.

3.4. *Gene silencing of the EcTLR5M and EcTLR5S suppresses cytokines expression in response to V. parahaemolyticus flagellin in GS cells*

To further investigate whether *Ec*TLR5M and *Ec*TLR5S recognize flagellin and induce cytokines expression, siRNA was used to suppress *Ec*TLR5M and *Ec*TLR5S expressions in GS cells. According to the results of pre-experiment, two specific siRNAs with higher efficiency for gene silencing of *Ec*TLR5M and *Ec*TLR5S were screened, respectively (data not shown). These siRNAs were named *Ec*TLR5M siRNA-1, *Ec*TLR5M-siRNA-2, *Ec*TLR5S-siRNA-1 and *Ec*TLR5S-siRNA-2. The siRNAs sequences were all listed in Table 3.

We mixed *Ec*TLR5M-siRNA-1 and *Ec*TLR5M-siRNA-2 at a 1:1 ratio,

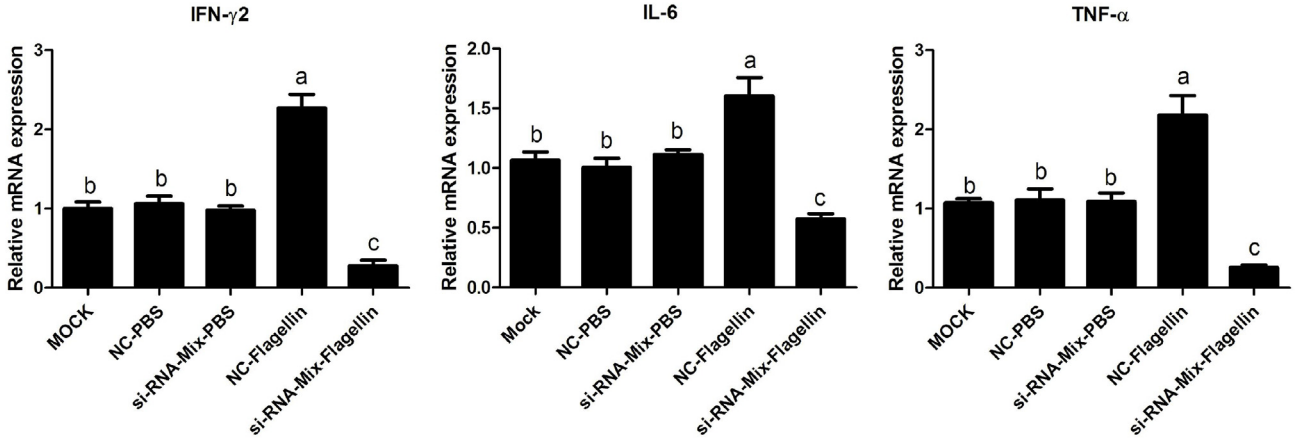

Fig. 5. *Ec*TLR5M and *Ec*TLR5S are required for the expression of *V. parahaemolyticus* flagellin-induced cytokines. *Ec*TLR5M and *Ec*TLR5S siRNA-Mix were equivalent mixed and transfected into GS cells. Then cells were challenged with 1 μg/mL V. *parahaemolyticus* flagellin for 6 h. The relative expression levels of cytokines were normalized by Mock groups. Data indicates the mean ± SEM (n = 3). These data were analyzed by one-way ANOVA. Different letters denote statistically significant differences ($p < 0.05$).

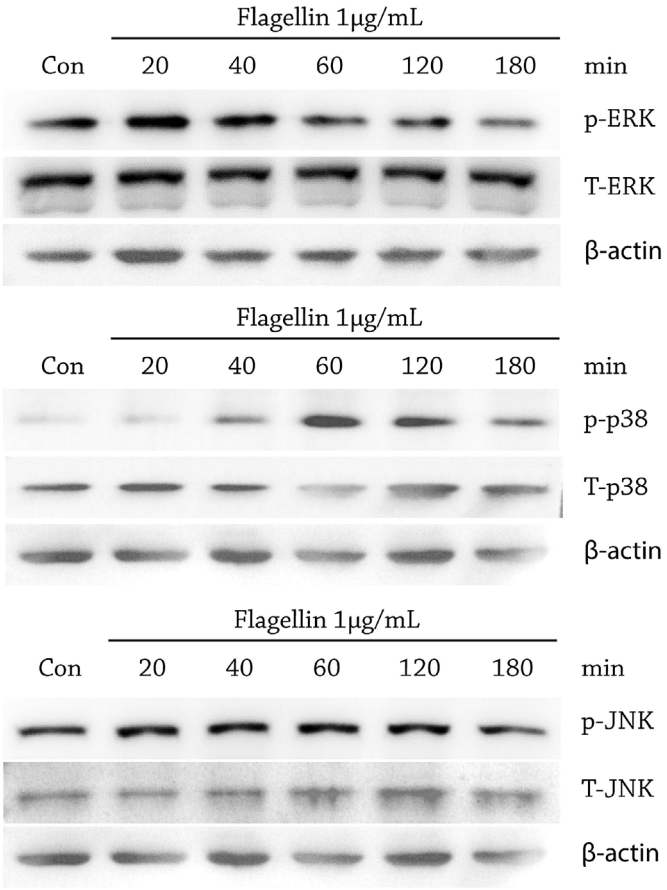

Fig. 6. *V. parahaemolyticus* flagellin induces MAPKs activation. GS cells were stimulated with 1 μg/mL V. *parahaemolyticus* flagellin for different time periods, and then the total protein was extracted. Total and phosphorylated forms of ERK, p38, and JNK were analyzed by Western Blot. P- and T- stands for phosphorylated and total target protein, respectively. β-actin served as internal control.

transfected into cells to detect the knockdown efficacy of *Ec*TLR5M expression using *Ec*TLR5M-siRNA-Mix by both qRT-PCR and western blot. Similarly, the knockdown efficacy of *Ec*TLR5S-siRNA-Mix was verified (Fig. 4A–D).

GS cells were seeded onto 96-well plates for 24 h before the transfection. We mixed *Ec*TLR5M-siRNA-1, *Ec*TLR5M-siRNA-2, *Ec*TLR5S-siRNA-1 and *Ec*TLR5S-siRNA-2 at a 1:1:1:1 ratio. Cells were transfection with 0.72 μg/mL siRNA-Mix or negative control siRNA (NC siRNA). At 36 h post transfection, the cells were stimulated with *V. parahaemolyticus* flagellin for 6 h. With silencing of both *Ec*TLR5M and *Ec*TLR5S, IFN-γ2, IL-6 and TNF-α mRNA expressions were significantly downregulated in GS cells stimulated with 1 μg/mL flagellin as compared to that of NC siRNA (Fig. 5). It further implied that *Ec*TLR5M and *Ec*TLR5S played an important role in mediating cytokines expression in response to *V. parahaemolyticus* flagellin.

3.5. *V. parahaemolyticus flagellin induces MAPKs pathway activation in GS cells*

In mammals, MAPKs pathways are involved in the production of pro-inflammatory cytokines after TLR5-flagellin recognition [23]. To confirm whether *V. parahaemolyticus* flagellin could activate MAPKs pathway in GS cells, we examined the phosphorylated and non-phosphorylated forms of ERK, p38 and JNK with Western Blot following treatment of GS cells with 1 μg/mL flagellin for 20, 40, 60, 120 or 180 min. The ERK phosphorylation was increased significantly at 20 min flagellin-treated group, and p38 phosphorylation was increased significantly at 60 min treated with flagellin compared to medium alone, whereas augmented JNK phosphorylation have not been observed flagellin treatment (Fig. 6).

3.6. *V. parahaemolyticus flagellin induces NF-κB pathway activation in GS cells*

Mammalian NF-κB signaling also participates in the regulation of cytokines production [24]. Flagellin was involved in the activation of NF-κB pathway via the plasma membrane TLR5. NF-κB subunit p65 translocated to the nucleus following IκBα degradation, which enhanced the transcription activity of NF-κB to induce cytokines production. We examined the phosphorylated and non-phosphorylated forms of IκBα through Western Blot to confirm whether *V. parahaemolyticus* flagellin could trigger NF-κB signaling. As shown in Fig. 7A, a significant degradation of IκBα occurred at 60 min stimulation with flagellin. To further confirm whether *V. parahaemolyticus* flagellin could activate NF-κB pathway in GS cells, we used an immunofluorescence method to detect NF-κB subunit p65 nuclear translocation. We observed that the most p65 were located in nucleus after flagellin-stimulation for 60 min (Fig. 7B). It suggested that NF-κB signaling is involved in *V. parahaemolyticus* flagellin-induced cytokines

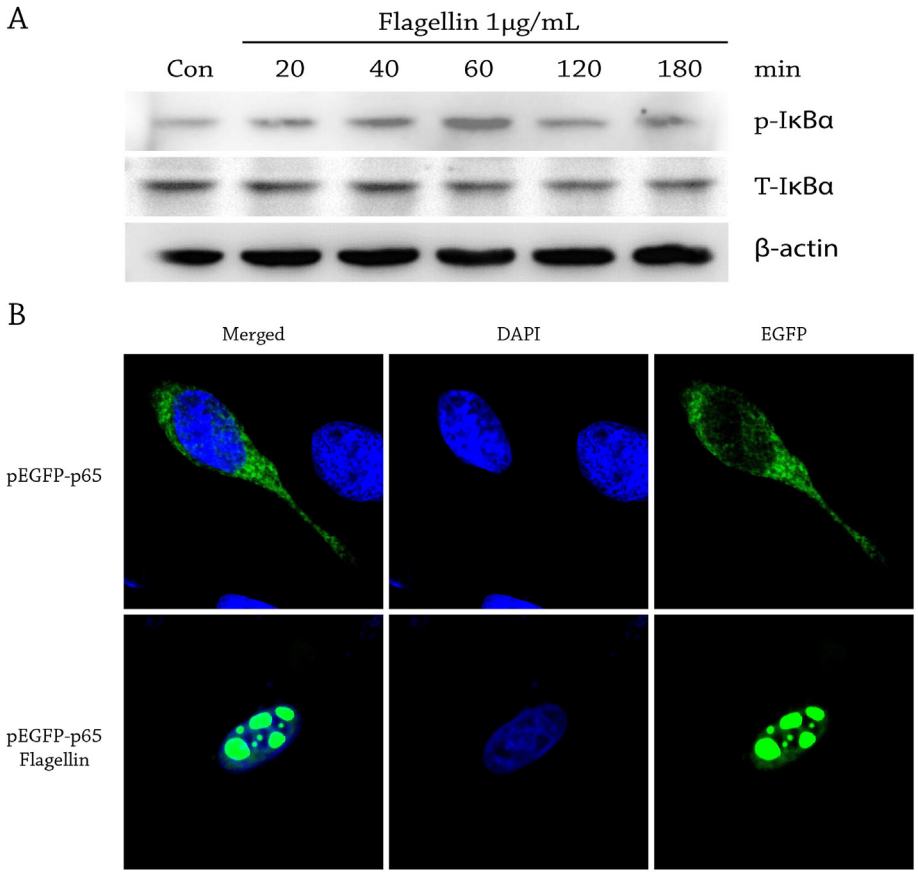

Fig. 7. *V. parahaemolyticus* flagellin induces NF-κB activation. **(A)** GS cells were stimulated with 1 μg/mL V. *parahaemolyticus* flagellin for different time periods, and then the total protein was extracted. Total and phosphorylated forms of IκBα were analyzed by Western Blot. β-actin served as internal control. **(B)** GS cells were transiently transfected with pEGFP-N3-NF-κB p65 plasmid. After 36 h, the cells were treated with 1 μg/mL V. *parahaemolyticus* flagellin for 30 min, then washed twice with HBSS, followed by stained with DAPI at 37 °C for 10 min. Images were obtained by a confocal microscope.

expression.

4. Discussion

V. parahaemolyticus, a rod-shaped and Gram-negative bacterium, has a strong pathogenicity to *Branchiostoma lanceolatum* [15], *Danio rerio* [14] and *Tetraodon nigroviridis* [25], which can cause serious inflammatory responses and severe tissue damage in host. *V. parahaemolyticus* is even risky for humans by eating raw or undercooked seafood [26]. When exposed to viscous or surfaces environments, *V. parahaemolyticus* would develop two flagellar systems, which are multiple lateral flagella in addition to the single polar flagellum [27,28]. By transmission electron microscopy, we clearly visualized that the rod-shape appearance of *V. parahaemolyticus* with a single polar flagellum, since it was cultured in a liquid medium. Flagellin, as the important extracellular virulence factor of bacteria, can elicit immune response in teleost and mammals [29,30]. The effect of flagellin immunostimulatory on the cytokines expression and immune system activation were studied, like *Salmonella* [31], *Pseudomonas aeruginosa* [32] *and Vibrio anguillarum* [5]. However, the pathogenic molecular mechanism of *V. parahaemolyticus* flagellin is still poorly understood compared to other bacterial flagellin. In our study, *V. parahaemolyticus* flagellin is capable of up-regulating IFN-γ2, IL-6 and TNF-α expressions in GS cells.

TLR5 only responds to a monomeric form of flagellin but not filamentous flagellin and the recognition site is on the D1 domain of flagellin [33]. Yoon et al. suggested that the flagellin D1 domain bound to TLR5 forming a 1:1 heterodimer, and then two heterodimers oligomerize to a 2:2 complex of secondary dimerization [34]. Afterward, Yoon et al. reported that the first 10 leucine-rich repeat (LRR) regions of TLR5 is involved in combination with flagellin, and the LRR9 loop plays a major role in mediating the interactions [35]. Flagellin-binding activated TLR5, then the cytoplasmic Toll/interleukin-1 receptor (TIR) domain of TLR5 recruits MyD88 and transduces signal. In fish, *Ec*TLR5M/S transcripts were significantly up-regulated in spleen with *C. irritans* infection [9], similar to our results, suggesting that *Ec*TLR5M/S might be involved in fish immune response. TLR5M and MyD88 were responsible for NF-κB activating in *Oplegnathus fasciatus* [36]. Bacterial infection and flagellin exposure significantly induced the expressions of TLR5, MyD88, TRAF6 and downstream cytokines in *Cirrhinus mrigala* [37]. Previous studies reported that rtTLR5S can physically bind *V. anguillarum* flagellin and amplifies TLR5M-mediated NF-κB activity in *Oncorhynchus mykiss* [38]. Our results showed that the expressions of *Ec*TLR5M and *Ec*TLR5S genes increased significantly following *V. parahaemolyticus* flagellin stimulation in GS cells. *V. parahaemolyticus* flagellin may contain some amino acid sites that can be recognized by *Ec*TLR5M and *Ec*TLR5S. Further study should aim to identify the amino acid sites of flagellin recognized by *Ec*TLR5s, whether *Ec*TLR5M and *Ec*TLR5S recognition sites are difference.

In our study, *V. parahaemolyticus* flagellin induces cytokines expression through *Ec*TLR5s-mediated MAPKs and NF-κB activation. It was determined that both of MAPKs and NF-κB were required for immune response, and played a crucial role in regulating the effectors and cell signaling. ERK, p38, and IκBα phosphorylation were observed in stimulated GS cells. In mammals, Caco-2 cells were infected with *V. parahaemolyticus*, then activated ERK and p38 signal pathway leading to pro-inflammatory cytokines secretion [39]. Consistently, human monocytes were stimulated with *Treponema pallidum* recombinant flagellin, then the significantly augmented phosphorylation of ERK and p38 (but not JNK) were observed at 60 min [40]. Similarly, the rat intestinal epithelial cells were treated with *Salmonella* flagellin to examine the effects on MAPKs activations, the significant phosphorylation of ERK and p38 (but not JNK) were also seen at 60 min [41]. Canonical NF-κB signaling pathway has been also associated with the production of pro-inflammatory cytokines [24]. IκBα is the important member of IκBs, as an inhibitor of NF-κB function [42]. The p50/p65 heterodimer

bound to IκBα in steady state, and stimulation signal induced the phosphorylation degradation of IκBα is essential for the nuclear translocation of p50/p65 [43,44]. Afterward, NF-κB binding DNA drives pro-inflammatory cytokines expression, such as IL-6 and TNF-α [45], and induces IκBα expression generating a negative feedback loop [46]. So, IκBα degradation was examined, and p65 nuclear import proved the activation of NF-κB. Previous study showed that TLR5 activation leading to p65 translocation to nucleus which import and export at 30- and 100-mins post flagellin stimulation in MCF-7 cells, respectively [47]. Similar to our results, *V. cholerae* recombinant flagellin was recognized by TLR5 leading to the activation of MAPKs and NF-κB and the induction of pro-inflammatory cytokines expression in T84 intestinal epithelial cells [48]. We demonstrated that the dissociation of IκBα and the nuclear translocation of p65 happened at *V. parahaemolyticus* flagellin-stimulated in GS cells.

In summary, we have established a *V. parahaemolyticus* flagellin stimulation model using GS cells, suggesting the hypothesis that *V. parahaemolyticus* flagellin might have a direct role in the induction of IFN-γ2, IL-6 and TNF-α expressions in GS cells through the phosphorylation of ERK, p38 and degradation of IκBα following the activation of *Ec*TLR5M and *Ec*TLR5S. Our results provide an insight into understanding the physiological process of fish resistance to bacterial infections, especially vibrio invasion.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.054.

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