Beta-Arrestins negatively regulate the Toll pathway in shrimp by preventing Dorsal translocation and inhibiting Dorsal transcriptional activity.

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ABSTRACT

Toll signaling pathway plays an important role in innate immunity of Drosophila melanogaster and mammals. The activation and termination of Toll signaling are finely regulated in these animals. Although the primary components of Toll pathway were identified in shrimp, the functions and regulation of the pathway are seldom studied. We first demonstrated that Toll signaling pathway plays a central role in host defense against Staphylococcus aureus by regulating expression of antimicrobial peptides in shrimp. We then found that β-arrestins negatively regulate Toll signaling in two different ways. β-arrestins interact with the C-terminal PEST domain of Cactus through the arrestin-N domain, and Cactus interacts with the RHD domain of Dorsal via the ankyrin (ANK) repeats domain, forming a heterotrimeric complex of β-arrestin-Cactus-Dorsal, with Cactus as the bridge. This complex prevents Cactus phosphorylation and degradation, as well as Dorsal translocation into the nucleus, thus inhibiting activation of the Toll signaling pathway. β-arrestins also interact with non-phosphorylated ERK (extracellular regulated protein kinase) through the arrestin-C domain to inhibit ERK phosphorylation, which affects Dorsal translocation into the nucleus and phosphorylation of Dorsal at Serine 276 that impairs Dorsal transcriptional activity. Our study suggests that β-arrestins negatively regulate the Toll signaling pathway by preventing Dorsal translocation and inhibiting Dorsal phosphorylation and transcriptional activity.

The immune system is composed of humoral and cellular immunity. Toll and Toll like receptor pathways play important roles in humoral and cellular immune responses in Drosophila (1,2). Subsequent studies have revealed the central roles of mammalian Toll like receptors in innate immunity (TLRs) (3) Gram-positive bacterial or fungal infection activates the Toll pathway, which
Beta-Arrestins negatively regulate the Toll pathway in shrimp leads to production of several antimicrobial peptides (AMPs) that kill infective pathogens in *Drosophila melanogaster* (4). The Toll pathway is also involved in the hematopoiesis, encapsulation and killing of parasites (5). The core components of the Toll pathway in *Drosophila* include the cytokine-like ligand Spätzle, the receptor Toll, the intracellular adaptor MyD88, the kinases Tube and Pelle, and transcription factors, such as Dorsal and Dorsal-related immunity factor (Dif). After activation of the Toll pathway, Dorsal or Dif translocates into the nucleus, leading to activation of several AMP genes (2,6,7).

The Toll pathway is regulated by multiple factors at different levels. For example, Pellino, a Pellino-like IL-1R–associated kinase (IRAK) interacting protein, acts as a positive regulator of the Toll pathway (8). *Drosophila* Pellino mutants have impaired Drosomycin expression and reduced survival against Gram-positive bacteria. As all Pellino proteins contain a RING domain, it is speculated that *Drosophila* Pellino may ubiquitinate Pelle in a similar fashion to mammalian Pellinos’ polyubiquitination of IRAK1 (9). G protein-coupled receptor kinase 2 (Gprk2) was identified as a regulator of the Toll pathway (10). Gprk2 interacts with Cactus in S2 cells, but is not involved in Cactus degradation; the detailed mechanism remains to be investigated. A recent study showed that specific calcineurin isoforms are also involved in *Drosophila* Toll immune signaling as positive regulators (11).

The strength and duration of the activation of the Toll signaling pathway must be tightly controlled, because overactivation of Toll or Toll-like receptors (TLRs) can be dangerous to the host. The Toll signaling pathway is negatively regulated by different molecules that target each of the key molecules in Toll signaling through various mechanisms to prevent or terminate excessive immune responses. In *Drosophila*, the Cactus, a homolog of IkB in mammals, is the main cytoplasmic inhibitor of Dorsal (a homolog of mammalian NF-κB) (12). The activation of the Toll pathway is also negatively regulated by serpins (13). Kurtz, a β-arrestin in *Drosophila*, negatively controls Toll signaling and systemic inflammation at the level of sumoylation (14). A very recent report demonstrates that *Drosophila* Pellino functions as a negative regulator by targeting MyD88 for ubiquitination and degradation in Toll-mediated signaling (15).

The arrestin family in mammals comprises four members: two visual β-arrestins (βarrs), which are expressed in the rod and cone photoreceptor cells of the retina, respectively, and two ubiquitously expressed β-arrestins (βarrs 1 and 2) (16). As adapter proteins, βarrs are critical for mediating endocytosis of G protein-coupled receptors (GPCRs). In addition, βarrs function in the desensitization and endocytosis of different cell surface receptors (17). βArrs are also scaffold proteins, linking GPCRs to other signaling proteins, such as the src-family kinases and members of the mitogen-activated protein kinase (MAPK) cascade. βArrs are involved in the regulation of multiple signal pathways (18). Previous studies suggested that the role of βarrs in cell signaling is much broader and that βarrs regulate signaling molecules by modulating phosphorylation, ubiquitination, and/or subcellular distribution of their binding partners. βArrs appear to interact with TRAF6 and IκBα in the TLR signaling pathway and inhibit NF-κB activity (19-21). βArr1 functions as a positive regulator of CD4+ T cell survival and autoimmunity (22). βArrs also inhibit cell apoptosis by inhibiting pro-apoptotic extracellular regulated protein kinases (ERK1/2) and p38 MAPKs and anti-apoptotic Akt signaling pathways in mouse embryonic fibroblasts (23). In *Drosophila*, Kurtz inhibits MAPK and Toll signaling during
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Development (24). Another study connected Kurtz activity to sumoylation, and found that Kurtz negatively controls Toll signaling and systemic inflammation at the level of sumoylation in *Drosophila* although the mechanism was not clear (14). Therefore, mammalian β-arrs and *Drosophila* Kurtz both downregulate NF-κB signaling, but in different ways. It would be interesting to determine whether βarrs in other invertebrates regulate Toll/NF-κB signaling pathways like mammals or *Drosophila*.

In shrimp, the key molecules of the Toll pathway have been identified, such as Spätzle, Toll receptors, MyD88, Pelle, Cactus and Dorsal (25-31) and reviewed briefly for the signal pathway by Li and Xiang (32). Toll-interacting protein (Tollip) from *Litopenaeus vannamei* was reported to negatively regulate the shrimp antimicrobial peptide gene, penaeidin-4 (*PEN4*) (33). ERK was identified and involved in defense against White spot syndrome virus (WSSV) invasion in *Fenneropenaeus chinensis* (34). However, the function and regulation of the Toll signaling is seldom studied. In our work, we found that the bacterial clearance, shrimp survival rate, and expression of antimicrobial peptides in kuruma shrimp *Marsupenaeus japonicus*, were declined significantly after RNA interference (RNAi) of Dorsal, the transcription factor of the pathway and subsequently infected with *Staphylococcus aureus*. These results demonstrated that the Toll pathway plays a central role in host defense against *S. aureus* by regulating AMPs expression. Then the regulation of the signaling was analyzed. We found that two βarrs (designated as Mj-βarr1 and Mj-βarr2) interacted with Mj-Cactus and Mj-ERK. Knockdown of βarrs enhanced Dorsal translocation into the nucleus to induce expression of antimicrobial peptides (AMPs), while knockdown of ERK showed the opposite results. The possible mechanism was further studied. βArrs interacted with Cactus to form a βarr-Cactus-Dorsal complex, which retained Dorsal in the cytoplasm of cells. βArrs also interacted with non-phosphorylated ERK to inhibit its phosphorylation and affected Dorsal phosphorylation at Serine\(^276\). Therefore, βarrs negatively regulates the Toll signaling pathway by forming a βarr-Cactus-Dorsal complex to prevent Dorsal translocation and impair Dorsal phosphorylation by inhibiting ERK phosphorylation. To the best of our knowledge, this is the first work to demonstrate that Toll signaling plays central role in shrimp against gram-positive bacteria and βarrs regulate the Toll pathway in two different ways.

**EXPERIMENTAL PROCEDURES**

**Bacterial challenge and sample collection**—Kuruma shrimp *Marsupenaeus japonicus* (6–8 g each) were purchased from a fish market in Jinan, Shandong Province, China, and cultured for one day in laboratory aquarium tanks with aerated seawater at 22°C for acclimation to the new environment. The 2 × 10\(^7\) CFU of *Staphylococcus aureus* (Shandong University Organism Culture Collection (SDMCC)) was injected into the abdomen of shrimp. The control group was injected with PBS (140 mM NaCl, 10 mM sodium phosphate, pH 7.4). Hemocytes, heart, hepatopancreas, gill, stomach, and intestine were collected from at least three shrimp. For the hemocytes collection, the hemolymph was extracted with a syringe preloaded with 1 ml anticoagulant buffer (0.45 M NaCl, 10 mM KCl, 10 mM EDTA and 10 mM HEPES, pH 7.45) and immediately centrifuged at 800 \( \times \) g for 8 min at 4°C and the hemocytes were suspended in PBS. The hemocytes and other tissues were used for RNA or protein extraction. Total RNA was...
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extracted using the Trizol reagent (Cwbio, Beijing, China).

**cDNA cloning and sequence analysis**—The full-length cDNA sequences of Mj-Dorsal, Mj-βarr1, Mj-βarr2 and Mj-ERK were obtained from hemocyte and intestine transcriptome sequencing of *M. japonicus*. The open reading frame of Mj-Cactus was also obtained by transcriptome sequencing. Similarity analysis was conducted using BLASTx ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). The corresponding cDNA was conceptually translated and the deduced proteins were predicted using ExPASy ([http://www.expasy.org/](http://www.expasy.org/)). The domain architecture prediction of the proteins was performed using SMART ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)). MEGA 5 was used for phylogenetic analysis.

**Tissue distribution and expression pattern analysis by reverse transcription-PCR (RT-PCR) and western blotting**—RT-PCR was used to assess the tissue distribution of Mj-βarr1, Mj-βarr2, Mj-Dorsal, Mj-ERK and Mj-Cactus using the primers shown in Table 1. Protein samples obtained from shrimp organs were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was blocked for 1–2 h with 3% non-fat milk in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and incubated with 1/200 diluted antiserum against the proteins of interest (Mj-βarrs, Mj-ERK, or Mj-Dorsal) in TBS with 3% non-fat milk for 3 h. After washing 3 times in TBS, Alkaline phosphatase-conjugated goat anti-rabbit IgG (1/10,000 diluted in TBS) was added. After incubation with the membrane for 3 h, unbound IgG was washed away. The membrane dipped into the reaction system was visualized by 4-chloro-1-naphthol oxidation in the dark for 5 min. Mj-βarr1, Mj-βarr2, Mj-ERK and Mj-Cactus antisera were prepared in our laboratory using recombinant proteins. Antibodies recognizing the phosphorylated forms of ERK were purchased from Abcam (USA) and NF-κB P65 (Serine²⁷⁶) antibody was purchased from ABGENT (San Diego, CA).

**Recombinant expression and antiserum preparation**—DNA encoding Mj-βarr1, Mj-βarr2 and Mj-Dorsal with His-Tag (pET32a (+) vector), Mj-βarr1, Mj-βarr2, Mj-ERK and Mj-Cactus with GST-Tag (pGEX4T-1) were expressed in *Escherichia coli* Rossata. *E. coli* with different plasmids were cultured until the OD₆₀₀ of the bacterial culture reached 0.5, and Isopropyl thiogalactoside (IPTG, 0.5 mM) was added to induce protein expression at 37°C for 4 h. The recombinant proteins were purified by affinity chromatography using His-Bind resin (Ni²⁺-resin; Novagen, Darmstadt, Germany) or GST-resin (GenScript, Nanjing, China). The antiserum preparation was performed as previously described (35).

**Quantitative real time PCR**—The cDNA templates were diluted 50-fold in nuclease-free water for qRT-PCR analysis. SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used in a real-time thermal cycler (Bio-Rad, USA) with a total volume of 10 μl containing 5 μl of 2× Premix Ex Taq, 1 μl of the 1:100 diluted cDNA, and 2 μl (1 μM) each of the forward primer and the reverse primer. qRT-PCR was performed with the following conditions: 94°C for 3 min, 40 cycles of 94°C for 10 s and 60°C for 1 min, and a final dissociation protocol from 65°C to 95°C. Three parallel experiments were conducted to increase the credibility of this study. We used the 2⁻^ΔΔCt method to calculate the mRNA relative expression (36). The obtained data were subjected to
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RNA interference assay—The cDNA fragments amplified by primers Fi and Ri (Table 1) linked to the T7 promoter (Table 1) were used as templates for the synthesis of dsRNA. The cDNA fragment of GFP used for dsGFP synthesis was amplified using primers GFP-Fi and GFP-Ri (Table 1).

The assay for dsRNA synthesis was performed in accordance with previous reports (35). The dsRNA (40 µg) of Mj-MEK, Mj-βarr1, Mj-βarr2, Mj-ERK, or Mj-Dorsal was injected into the abdominal segment of each shrimp. To enhance the RNAi effect, a second injection was performed 12 h after the first injection. The dsGFP was used as the control. The intestine was collected from the shrimp 24 h after the second injection, and total RNA was extracted and detected by RT-PCR using primers RT-F and RT-R (Table 1) to check the efficiency of RNAi. The experiments were repeated three times.

After setting up the RNAi assay, S. aureus was injected into the gene-silenced shrimp. The expression levels of AMPs and the tumor necrosis factor (TNF) superfamily (TNFSF) gene regulated by the Toll pathway (37) in hemocytes and intestine and gill, the bacterial clearance and the survival rate (see the following method) of the shrimp were analyzed for Mj-Dorsal RNAi shrimp; the translocation of Mj-Dorsal in hemocytes and intestine, the AMPs expression in hemocytes, intestine or gill, the bacterial clearance and survival rate were detected for Mj-βarr1 and Mj-ERK RNAi shrimp. The AMPs expression was analyzed by quantitative real-time reverse transcription PCR (qRT-PCR) with the AMPs primers (Table 1). And β-actin was used as the control. Data show the mean ± SD from three independent repeats. The p value was calculated by Student’s t test for paired samples, and significant or most significant differences were accepted when p < 0.05 or p < 0.01.

Nuclear and cytoplasmic protein extraction—To confirm Mj-Dorsal and Mj-ERK translocation into the nucleus, nuclear and cytoplasmic protein extraction assays were performed. Shrimp intestine and gill were cut into pieces with small scissors, washed with PBS and the centrifuged at 1000 × g for 7 min. The precipitate was suspended in 100µl buffer A (10 mM HEPES, PH 7.5, 10mMKCl, 0.2 mM EDTA, 3mM MgCl₂, 1mM DTT, 1mM PMSF, 1% inhibitor cocktail (Merck, Gemery), 1% NP-40) and incubated on ice for 10 min. The tissue solution was then centrifuged at 1000 × g for 10 min. The obtained supernatant contained cytoplasmic proteins, and the precipitate was resuspended in the buffer A and incubated for 10 min on ice to wash off the remaining cytoplasm, and then centrifuged at 1000 × g for 10 min again. Buffer B (100 µl) (20 mM HEPES, 400mM NaCl, 1 mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 1% cocktail) was added to the precipitate and vortexed for 30 min at 4°C. The solution was centrifuged at 14,000 × g for 10 min to obtain the nuclear proteins. Nuclear and cytoplasmic proteins were then subjected to western blotting.

Bacteria clearance and survival rate assays—The shrimp (6-8 g each) were divided into four groups, three groups for RNAi of target genes, and one group for GFP-RNAi control. After knockdown of Mj-Dorsal by RNAi, shrimp were injected with S. aureus (2×10⁷ CFU) at 24 h after the second dsRNA injection. At 6 h after S. aureus injection, S. aureus (2x10⁷ CFU) was injected into shrimp again and hemolymph was extracted at 1 h after second S. aureus injection. The hemolymph was then diluted and cultured on LB agar-plates overnight at 37°C. The number of bacterial colonies was counted. Same methods were performed for Mj-βarr1 and Mj-ERK RNAi and bacterial clearance.
For survival rate assay: After *Mj-Dorsal* was knocked down by RNAi, the shrimp were injected with *S. aureus* (2×10^7 CFU). The dead shrimp were monitored and counted every day. Data show the mean ± SD from three independent repeats. The *p* value was calculated by Student’s *t* test for paired samples, and significant or most significant differences were accepted when *p* < 0.05 or *p* < 0.01, respectively.

**Pull-down assay**—The recombinant proteins (30 µg) were added into 20 µl of charged Ni-NTA beads (for His-tagged proteins) or Glutathione resin (for GST-tagged proteins) and incubated at room temperature for 2 h with slight rotation. The mixture (resin and binding proteins) was washed three times by centrifugation at 500 × g for 3 min to remove the unbound proteins. The test protein, without a His-tag or GST-tag, was added into the mixture containing the Ni-NTA beads or Glutathione resin and the tagged protein, and gently rotated at room temperature for 2 h. After washed three times, the mixture was analyzed by SDS-PAGE. We also used recombinant proteins to pull down the natural proteins from shrimp. Shrimp gills were homogenized with lysis buffer (150 mM NaCl, 1.0% Nonident-P40, 0.1% SDS, 50 mM Tris containing protease inhibitor cocktail (Abcam USA) and then centrifuged at 14,000 × g for 12 min. The supernatant (1000 µl) was added into the GST resin with recombinant proteins, and incubated at room temperature for 2 h. The resin was washed three times by centrifugation at 500 × g for 3 min, and then analyzed using SDS-PAGE. The proteins in the gel were transferred onto a nitrocellulose membrane, followed by blocking with 3% non-fat milk dissolved in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated with 1/100 diluted antiserum in TBS with 3% non-fat milk for 2 h. Horseradish peroxidase goat anti-rabbit IgG (1/100,000 diluted in TBS) was then added. After incubation with the membrane for 2 h, unbound IgG was washed off. The membrane was dipped into the reaction system (9 ml of TBS, with 1 ml of 4-chlorine naphtol and 6 µl of H_2O_2) in the dark for 5 min to visualize the target protein.

**Immunocytochemical assays**—The hemolymph was collected from three shrimp using a syringe preloaded with 1 ml of anticoagulant and then fixed by adding 4% paraformaldehyde. The hemocytes were isolated by centrifugation (700 × g for 4 min at 4 °C), washed with PBS, incubated in 0.2% Triton X-100 at 37°C (5 min) twice. After blocking with 3% bovine serum albumin (BSA) for 30 min at 37°C, hemocytes were incubated overnight with anti-Mj-Dorsal serum (1:100 in blocking buffer) at 4°C. After washing with PBS, the hemocytes were then incubated with 3% BSA for 10 min, after which the second antibody, goat anti-rabbit-Alexa Fluor 488 (1:1,000 dilution in 3% BSA), was added. The reaction was kept in the dark for 1 h at 37°C, and then washed with PBS. Hemocytes were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI, AnaSpec Inc., San Jose, CA, USA) for 10 min at room temperature, and washed again.

Hemocytes were observed under a fluorescence microscope (Olympus BX51).

**Co-immunoprecipitation (Co-IP)**—Sixty milligrams of CNBr-activated Sepharose 4B (Amersham Biosciences) was swollen in 500 µl of HCl (1 mM) and 100 µl of medium, and then washed twice with 1 mM HCl. The antibodies of interest were incubated with CNBr-activated Sepharose 4B with rotation at 25°C for 1 h. The resin was washed five times with coupling buffer and then equilibrated in 0.1 M Tris-HCl (pH 8.0) at 25°C for 2 h. Then, after four rounds of alternate washing with acetic acid buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0) and Tris buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0), the
resin was equilibrated in Tris buffer. The supernatant (1000 µl) from normal and challenged shrimp hemocytes, intestine or gill was prepared and then mixed with 100 µl of resin and gently rotated overnight at 4°C. After five washes with Tris buffer, the native protein was eluted using elution buffer (0.1 M glycine, pH=2.5) and neutralized with neutralization buffer (1 M Tris-HCl, pH=8.0). The purified proteins were confirmed by western blotting.

*PD98059 inhibitor assay in shrimp*-PD98059, a mitogen-activated protein kinase kinase MEK1/2 inhibitor, was purchased from Cell Signaling Technology (Danvers, MA, USA). PD98059 (4 µM) was injected into shrimp (8-10 g), and the same volume of DMSO injection was used as the control. *S. aureus* was injected after 1 h of inhibitor injection. Subsequently, shrimp intestine were collected 1 h after bacterial injection for western blotting. Antibodies against Mj-ERK and Mj-Dorsal were used in the analysis. The total RNAs from intestine were extracted from shrimps 6 h after *S. aureus* injection to detect AMPs expression.

**RESULTS**

*The Toll pathway is activated by *S. aureus* challenge and plays a central role in defense against *S. aureus* in shrimp*-To study the function and regulation of the Toll pathway in shrimp during bacterial infection, we firstly ascertained activation of the Toll pathway by translocation of Mj-Dorsal (GenBan accession no. KU160503) and expression of AMPs after bacterial infection. The Gram-positive bacterium *S. aureus* was used to activate the Toll pathway. After bacterial activation of the Toll pathway in shrimp, the protein level of Mj-Dorsal increased in hemocytes, intestine and gill (Fig. 1A-C). Mj-Dorsal was translocated from the cytoplasm into the nucleus in hemocytes, intestine and gill at 1 h after *S. aureus* challenge (Fig. 1D and F). The expression of antimicrobial peptides (AMPs), ALF-C2 (GenBank accession no. KU160498), Cru-I (KU160502) and another effector TNFSF (KU160505) but not ALF-D1 (KU160499) regulated by the Toll pathway also was increased significantly (Fig. 1G, J and M). After knockdown of Mj-Dorsal by RNA interference and subsequent challenge by *S. aureus*, the expression of the effectors ALF-C2, Cru-I and TNFSF in hemocytes (Fig. 1H), intestine (Fig. 1K) and gill (Fig. 1N) were significantly decreased compared with the control (Fig. 1I, L and O). These results indicated that expression and translocation of Mj-Dorsal and expression of the Toll pathway effectors (AMP genes) were significantly increased in hemocytes, intestine and gill after *S. aureus* infection, and suggest that systemic immune responses occurred also in the intestine and gill in addition to hemocytes. Therefore, we mainly used intestine or gill in the following studies for the convenience of sample collection.

To evaluate whether the Toll pathway plays a central role in host defense against *S. aureus*, the bacterial clearance and survival of shrimp were conducted after shrimp were injected with dsRNA of Mj-Dorsal and subsequently infected with *S. aureus*. dsGFP injection followed by *S. aureus* infection was used as the control. The results showed that after RNAi of Mj-Dorsal, *S. aureus* clearance was significantly reduced compared with the control group (Fig. 1P), and the survival rate of Mj-Dorsal-RNAi shrimp infected with *S. aureus* was significantly lower than that of the control from day 3 to day 5 post-infection (Fig. 1Q). These data indicated that the Toll signaling may play a central role in defense against Gram-positive bacteria in shrimp.

*Mj-βarr1, Mj-βarr2 and Mj-ERK participate in regulation of the Toll pathway*-The full-length cDNA sequences of Mj-βarr1 (GenBank
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accession no. KU160500), Mj-βarr2 (KU160501) and Mj-ERK (KU160504) were obtained by transcriptome sequencing of *M. japonicus*. To analyze whether Mj-βarr1, Mj-βarr2 and Mj-ERK participate in regulation of the Toll signaling pathway, translocation of Mj-Dorsal and expression of the Toll pathway effectors were detected after RNAi knockdown of βarrs and ERK, followed by bacterial challenge. The results showed that in the βarr-silenced shrimp, most of Mj-Dorsal was detected in the nucleus of intestine cells by Western blotting (Fig. 2 A and A1) and the expression of effector transcripts, such as ALF-C2, CruI-1 and TNFSF mRNAs, were significantly upregulated (Fig. 2A2). The Mj-Dorsal translocation was also detected in hemocytes in βarr-silenced shrimp following bacterial challenge (Fig. 2A3-A4). The results showed that in the normal (untreated) shrimp, Mj-Dorsal could be observed in the cytoplasm and nucleus of hemocytes, but most of the Mj-Dorsal was located in the cytoplasm (Fig. 2A4). In the dsGFP-injected shrimp, most of Mj-Dorsal was translocated into the nucleus and only a small amount of Mj-Dorsal could be detected in the cytoplasm after *S. aureus* challenge (Fig. 2A4). After βarrs knockdown and bacterial challenge, Mj-Dorsal was only detected in the nuclei of hemocytes (Fig. 2A4). These results suggested that βarrs may inhibit the expression of the Toll pathway effector genes by preventing Mj-Dorsal from translocation into the nucleus. On the contrary, Most Mj-Dorsal was detected in the cytoplasm but not in the nuclei of intestine cells (Fig. 2B1) after Mj-ERK was knocked down (Fig. 2B) followed by *S. aureus* challenge. This result indicated that Mj-Dorsal could not be translocated into the nucleus after bacterial challenge in the Mj-ERK-knockdown shrimp and the expression of effectors was also significantly decreased (Fig. 2B1-B2) at 6 h after bacterial injection. In the hemocyte immunocytochemical assay, almost no Mj-Dorsal could be detected in the nucleus after Mj-ERK knockdown (Fig. 2B3-B4). These results suggested that Mj-ERK promoted the nuclear translocation of Mj-Dorsal and increased the expression of the AMPs. Taken together, our results suggested that Mj-βarrs negatively regulate the Toll pathway and Mj-ERK positively regulates the pathway.

To further confirm that Mj-βarrs and Mj-ERK participate in regulating the Toll pathway, bacteria clearance and survival rate of Mj-βarr1-RNAi or Mj-ERK-RNAi shrimp were also analyzed. In the Mj-βarr1-RNAi shrimp injected with *S. aureus*, bacteria clearance was enhanced while in the Mj-ERK-RNAi shrimp bacteria clearance was reduced compared with the control group (Fig. 2A5 and B5). The survival rate of the Mj-βarr1-RNAi shrimp injected with *S. aureus* was about 55% at day 5 post-infection, which was significantly higher than that (20%) of the GFP-RNAi control group (Fig. 2A6). However, the survival rate of Mj-ERK-RNAi shrimp injected with *S. aureus* was about 40% at day 3 post-infection, which was significantly lower than that (75%) of dsGFP shrimp (Fig. 2B6). These data indicated that Mj-βarr1 and Mj-ERK play important roles in antibacterial immunity of shrimp.

*Mj-βarrs interact with Mj-Cactus to form a βarr-Cactus-Dorsal complex*—To understand the regulatory mechanism of Mj-βarrs in the Toll pathway, the interactions of Mj-βarrs with Mj-Cactus and Mj-Dorsal were analyzed by Co-IP assays. We firstly determined the amount of Mj-Cactus after *S. aureus* challenge, and the results showed that the amount of Mj-Cactus was decreased from 10 to 60 min in hemocytes, intestine and gill of the shrimp, and only few amount of Mj-Cactus was detected in hemocytes at 60 min post bacterial challenge (Fig. 3A).
These results suggested that Mj-Cactus was degraded after activation of Toll pathway. The antibody against Mj-βarr1 was coupled to CNBr-activated Sepharose 4B for binding of natural Mj-Cactus and Mj-Dorsal from unchallenged and bacteria-challenged shrimp. The results showed that in the unchallenged shrimp, Mj-Cactus and Mj-Dorsal could be co-immunoprecipitated with Mj-βarr1, but in the bacteria-challenged shrimp, no Mj-Cactus and Mj-Dorsal were co-immunoprecipitated (Fig. 3B and B1). It is known that after activation of the Toll pathway by *S. aureus*, Cactus is degraded to release Dorsal, which then translocates into the nucleus. Therefore, the above results suggest that Mj-βarrs may interact with Mj-Cactus and/or Mj-Dorsal. When Mj-Cactus antibody was used for the Co-IP assay, Mj-βarr1, Mj-βarr2 and Mj-Dorsal were co-precipitated in the unchallenged shrimp, but only a little Mj-Dorsal was co-precipitated in the bacteria-challenged shrimp and almost no Mj-βarrs were detected in gill (Fig. 3C-C1). The results suggested that Mj-Cactus was not completely degraded in gill of shrimp challenged by bacteria. From Fig. 3A, we can see that most of Mj-Cactus was degraded at 60 min post bacterial challenge, we used hemocytes to do the co-immunoprecipitation assay, and the results showed that Mj-βarr1, Mj-βarr2 and Mj-Dorsal were not co-immunoprecipitated in the bacterial challenged shrimp (Fig. 3D-D1). These results suggested that Mj-Cactus interacted with Mj-Dorsal and Mj-βarrs, almost no interaction of Mj-Cactus and Mj-βarrs was detected at the early stage of bacterial infection in *S. aureus* challenged shrimp, probably because the upstream signal pathway was activated, and the Mj-Cactus would be degraded to lead to the dissociation of Mj-βarrs with Mj-Cactus. The Mj-Dorsal antibody was also used for Co-IP assays, and the results showed that Mj-βarrs and Mj-Cactus were detected in the unchallenged shrimp, and no Mj-βarrs and Mj-Cactus were detected in the challenged shrimp (Fig. 3E-E1). Taken together, our results suggested that Mj-βarrs, Mj-Cactus and Mj-Dorsal could form a heterotrimeric complex and the complex would disassociate followed by Mj-Cactus degradation.

To further confirm the possibility of forming heterotrimeric complex in vivo, the tissue distribution of Mj-βarr1, Mj-βarr2, Mj-Dorsal and Mj-Cactus were detected at the mRNA and protein levels. The results showed that the four mRNAs and proteins were distributed in all tissues tested, including hemocytes, heart, hepatopancreas, gill, stomach and intestine (Fig. 3F), suggesting that heterotrimeric complex of Mj-βarr-Cactus-Dorsal could be formed in shrimp.

The N-terminal domain of Mj-βarrs interacts with the PEST domain of Mj-Cactus-
Pull-down assays were performed to confirm the above-mentioned interactions using recombinant proteins of Mj-βarrs and Mj-Cactus. Mj-βarr contains arrestin-N and arrestin-C domains, while Mj-Cactus contains an N-terminal domain (1-168), ANK repeats domain (169-395), and a PEST domain (396-452) at the C-terminus (Fig. 4A). The full-length βarrs and their two individual domains were expressed and purified. The full-length Mj-Cactus protein, the N-terminal domain-ANK repeats domain (1-395), N-terminal domain (1-168), ANK repeats domain (169-395) and PEST domain (396-452) were also expressed and purified. Two different pull down assays with GST-binding resin and His-binding resin were performed.

The recombinant Mj-βarrs and Mj-Cactus were used for GST- and His-pull-down assays. As shown in Fig. 4B and C, Mj-βarr1 or Mj-βarr2 interacted with Mj-Cactus. To understand which domain of Mj-βarrs was responsible for the interaction with Mj-Cactus, the N and C terminal
domains of \( \beta \) arrs (His-Mj-\( \beta \) arr1-N, His-Mj-\( \beta \) arr1-C; and His-Mj-\( \beta \) arr2-N, His-Mj-\( \beta \) arr2-C) were used in GST- and His-pull down assays. The results showed that Mj-\( \beta \) arrs-N, but not Mj-\( \beta \) arrs-C, interacted with Mj-Cactus (Fig. 4D, E, F and G). To further study which domain of Mj-Cactus interacted with Mj-\( \beta \) arrs-N. The GST-tagged N-ANK and PEST domains of Mj-Cactus were used for pull-down assays. The results showed that The Mj-Cactus-N-ANK (1-395) could not interact with Mj-\( \beta \) arrs-N (Fig. 4H-I); however, Mj-Cactus-PEST (Fig. 4F and G) interacted with Mj-\( \beta \) arrs-N (Fig. 4J-K). Thus, the results suggested that the N-terminal domain of Mj-\( \beta \) arrs interacted with the C-terminal PEST domain of Mj-Cactus.

The ANK domain of Mj-Cactus interacts with the RHD domain of Mj-Dorsal and \( \beta \) arrs, Cactus and Dorsal form a complex-To detect which domain of Mj-Cactus is responsible for the interaction with Mj-Dorsal, the recombinant proteins of Cactus and its individual domains with GST-tags (Mj-Cactus, Mj-Cactus-N, Mj-Cactus-ANK, Mj-Cactus-N-ANK, and Mj-Cactus-PEST) (Fig. 5A) and Mj-Dorsal and its different domains with His-tags (Mj-Dorsal, Mj-Dorsal-RHD, Mj-Dorsal-IPT) (Fig. 5B) were used for pull down assays. As shown in Fig. 5C, Mj-Cactus could interact with Mj-Dorsal, further study showed that Mj-Cactus interacted with the RHD domain, but not the IPT domain of Mj-Dorsal (Fig. 5D). Recombinant Mj-Cactus-N-ANK, Mj-Cactus-N, Mj-Cactus-ANK and Mj-Cactus-PEST were used for pull-down assays to analyze the interaction with RHD domain of Mj-Dorsal (Fig. 5E). The results showed that only the ANK repeats domain of Mj-Cactus interacted with the RHD domain of Mj-Dorsal.

In summary, the arrestin-N domain of Mj-\( \beta \) arrs interacts with the PEST domain of Mj-Cactus. The ANK repeat domain of Mj-Cactus interacts with RHD domain of Mj-Dorsal. The \( \beta \) arrs-Dorsal complex is formed using Mj-Cactus as a bridge. To confirm the result, we used recombinant Mj-\( \beta \) arr1 or Mj-\( \beta \) arr2, Mj-Cactus, and Mj-Dorsal in GST-pulldown assay and the result showed that Mj-\( \beta \) arrs, Mj-Cactus and Mj-Dorsal could form a complex (Fig. 5F) but Mj-\( \beta \) arrs could not interact with Mj-Dorsal (Fig. 5H).

**Mj-\( \beta \) arrs interacts with non-phosphorylated Mj-ERK to decrease phosphorylation of Mj-ERK**

In Figs. 2B-B4, we noticed that ERK positively regulated Dorsal translocation into the nucleus and expression of AMPs. The possible mechanism was further studied. The interactions of Mj-\( \beta \) arrs with Mj-ERK were firstly studied by pull-down (Fig. 6A) and Co-IP (Fig. 6B) assays. Here, we used recombinant proteins to pull down the native proteins from shrimp. The results showed that Mj-ERK interacted with native Mj-\( \beta \) arr1 or Mj-\( \beta \) arr2 (Fig. 6A), and that Mj-\( \beta \) arrs interacted with native non-phosphorylated Mj-ERK, but little native phosphorylated Mj-ERK was co-precipitated (Fig. 6B). To further confirm if native phosphorylated Mj-ERK could interact with Mj-\( \beta \) arr1 or Mj-\( \beta \) arr2, phosphorylated Mj-ERK antibody was used to do the Co-IP assay. The results showed almost no native Mj-\( \beta \) arr1 or Mj-\( \beta \) arr2 was co-precipitated (Fig. 6C). Therefore, the phosphorylated Mj-ERK could not interact with Mj-\( \beta \) arr1 or Mj-\( \beta \) arr2.

After knockdown of Mj-\( \beta \) arr1 or Mj-\( \beta \) arr2 (Fig. 6D), the phosphorylation level of Mj-ERK was detected by western blotting. The results showed that the phosphorylation level of Mj-ERK was increased in Mj-\( \beta \) arr1 and Mj-\( \beta \) arr2 knockdown shrimp challenged with S. aureus for 1 h (Fig. 6E). These results suggested that Mj-\( \beta \) arrs inhibited ERK phosphorylation by interacting with non-phosphorylated Mj-ERK.
The C-terminus of Mj-βarrs interacts with Mj-ERK-To detect which domain of Mj-βarrs was responsible for the interaction with Mj-ERK, recombinant Mj-βarrs and their domains (His-Mj-βarr1, His-Mj-βarr1-N, His-Mj-βarr1-C, His-Mj-βarr2, His-Mj-βarr2-N, His-Mj-βarr2-C) (Fig. 7A and B) and Mj-ERK (GST-Mj-ERK) were used for pull-down assays. The results showed that Mj-βarr1 interacted with Mj-ERK (Fig. 7A1). The C-terminal domain of Mj-βarr1 (Mj-βarr1-C) (Fig. 7A2), but not the N-terminal domain of Mj-βarr1 (Fig. 7A3), was interacted with Mi-ERK. The same results were obtained for the interaction of Mj-βarr2 with Mj-ERK (Fig. 7B-B3).

Mj-ERK affected the phosphorylation of Mj-Dorsal in the nucleus-To further study the possible mechanism of ERK in regulation of the Toll pathway, we analyzed the phosphorylation of Mj-ERK and Mj-Dorsal using antibodies to phosphorylated ERK (Abcam, USA) or phosphorylated Mj-Dorsal. To determine whether commercial phosphorylated Dorsal antibody could recognize the phosphorylated Mj-Dorsal from shrimp, Mj-Dorsal was knocked down in shrimp and then challenged with S. aureus, and western blot was performed to detect the Dorsal. As shown in Fig. 8A, phosphorylated Mj-Dorsal could be detected by the antibody to phosphorylated Dorsal. Then phosphorylation of Mj-Dorsal and Mj-ERK was analyzed by western blotting. The results showed that S. aureus challenge enhanced phosphorylation of Mj-ERK and Mj-Dorsal. The phosphorylation of Mj-ERK increased rapidly at 15 min and phosphorylation at Serine^276 of Mj-Dorsal increased at 30-60 min (Fig. 8B). After knockdown of Mj-ERK and challenged with S. aureus in shrimp, the phosphorylation of Mj-Dorsal decreased obviously (Fig. 8C). To confirm the result, Mj-MEK, which affect the phosphorylation of Mj-Dorsal and Mj-ERK was detected. The results showed phosphorylation of Mj-Dorsal and Mj-ERK was detected in Mj-MEK-silenced shrimp (Fig. 8E). PD98059, a MEK1/2 inhibitor, was also used to inhibit ERK phosphorylation, and similar result was obtained (Fig. 8F). We further detected the subcellular distribution of phosphorylated Mj-Dorsal by western blotting. The distribution and phosphorylation levels of Mj-Dorsal in the nucleus decreased after PD98059 injection and challenge with S. aureus for 1 h (Fig. 8G). The expressions of effectors (MjALF-C2, CruI-1 and TNFSF) regulated by the Toll pathway in the intestine were significantly decreased after PD98059 injection and challenge with S. aureus at 6 h (Fig. 8H). Taken together, the results showed that phosphorylation of Mj-ERK induced Mj-Dorsal phosphorylation and the expression of effectors regulated by the Toll pathway. However, the interaction of Mj-βarr with Mj-ERK inhibited the phosphorylation of Mj-ERK (Fig. 6), and subsequently reduced Mj-Dorsal phosphorylation, which inhibited Toll signaling and reduced the expression of the effectors.

DISCUSSION
The Toll signaling pathway plays an important role in innate immunity in invertebrates (38,39). Dorsal is a transcription factor that modulates the transcriptional activity of the downstream targets. In Drosophila, the Toll signal pathway is activated by fungi and Gram-positive bacteria to regulate expression of AMPs to defend against pathogen invasion (40,41). In shrimp, the Toll pathway plays an important role in regulating AMPs expression (42,43). In this report, we find that the bacterial clearance and survival rate of Mj-Dorsal-silenced shrimp infected with S. aureus were declined significantly, suggesting that the Toll pathway plays a central role in host defense.
against *S. aureus* in shrimp. Toll signaling is activated by *S. aureus*, resulting in Mj-Dorsal nuclear translocation and an increase in its phosphorylation level. The expression of the effectors (MjALF-C2, Cru-1 and TNFSF) is regulated by the Toll pathway. Mj-βarrs interacted with Mj-Cactus and Mj-ERK to negatively regulate the Toll pathway.

Systemic immune response in *Drosophila* is induced synthesis of several families of AMPs by cells in the fat body, but AMP induction in epidermis and epithelia of gut is regulated by the immune deficiency (IMD) pathway for local immune challenges (44). In our study, we found that *S. aureus* challenge could induce expressions of same kinds of AMPs in hemocytes, intestine and gill. AMPs expression was not induced in these tissues of Mj-Dorsal RNAi shrimp. These results indicated that *S. aureus* challenge could induce the systemic immune responses in intestine and gill.

β-Arrestins were initially identified as mediators of GPCR desensitization and endocytosis (45). Nowadays, they are acknowledged to have signaling functions in a wide variety of signaling pathways and modes of regulation, including the Hedgehog, Wnt, Notch, and TGFβ pathways (46). As negative regulators, β-arrestins regulate the TLR-IL-1R signal pathway in mammals by interacting with TRAF6 and IKBα (19,20). β-Arrestins also regulate the MAPK signaling pathway by interacting with inactive ERK to inhibit its phosphorylation (47,48). In *Drosophila*, Kurtz inhibits MAPK and Toll signaling during development (24). Anjum et al. demonstrated that Kurtz and SUMO protease Ulp1 work synergistically to regulate the Toll immune pathway in *Drosophila* (14). In shrimp, we found that the arrestin-N domain of Mj-βarrs interacted with the C-terminal PEST domain of Mj-Cactus, the ANK repeats domain of Mj-Cactus interacted with the RHD domain of Mj-Dorsal, and the βarr-Cactus-Dorsal complex was formed using Mj-Cactus as a bridge. The complex prevented phosphorylation and degradation of Mj-Cactus and Mj-Dorsal translocation from the cytoplasm to the nucleus. The C-terminal domain of Mj-βarrs also interacted with non-phosphorylated Mj-ERK and inhibited its phosphorylation. Phosphorylation of Mj-ERK could promote Mj-Dorsal translocation into the nucleus and the phosphorylation of Serine276 of Mj-Dorsal (47). The interaction of Mj-βarrs and Mj-ERK inhibited Mj-ERK phosphorylation and prevented Mj-Dorsal translocation and phosphorylation.

MAPK signaling has been implicated in stress and immunity in evolutionarily diverse species (45). The relationship between MAPK and Toll signaling still needs to be studied in invertebrates. ERK, as one of the three MAPKs, plays a role in regulating transcription activity of P65 in mammals (47,48). In our study, Mj-βarrs bound to non-phosphorylated Mj-ERK and inhibited its phosphorylation by MEK. Mj-ERK phosphorylation regulated Mj-Dorsal nuclear translocation and phosphorylation of Serine276 of Mj-Dorsal, and then regulated Mj-Dorsal transcriptional activity and DNA binding activity. Therefore, the interaction of Mj-βarrs with Mj-ERK indirectly inhibited Mj-Dorsal translocation and phosphorylation, and limited Toll signaling activity. Uncontrolled immune responses have detrimental outcomes in organisms; therefore, the intensity and duration of immune signaling must be finely regulated. As described above, activation of the Toll pathway is under multiple layers of control, which would limit the damage caused an inappropriate immune response.

Anti-microbial responses in insects are mainly consisted of a two-stage process:
Constitutive defense, such as engulfment and melanization, acts immediately and more than 99% of bacteria in the hemocoel can be cleared in less than an hour. The second stage is inducible defense, that is the production of antimicrobial peptides, which eliminate or suppress the remaining microbes (49). Similar immune responses occurred in shrimp. On the other hand, some pre-synthetized AMPs, such as penaeidins, stored in hemocytes could be release upon immune stimulation (50). Therefore, we performed an initial challenge and then made a second challenge against *S. aureus* in the bacterial clearance assay (see Materials and Methods).

In mammals, β-arrests appear to interact with TRAF6 and IκBα in the TLR signaling pathway and inhibit NF-κB activity (19-21). In *Drosophila*, Kurtz negatively controls Toll signaling and systemic inflammation at the level of sumoylation (14). In our study, we found that β-arrestins could interact with Cactus and inhibit Toll signaling activity. The function of β-arrestins in shrimp is similar to that of mammals.

In summary, our study revealed that in shrimp, bacterial challenge could activate Toll signaling pathway and the pathway played a central role in antibacterial immunity. We also showed that that Mj-β-arrestins could block or limit Toll signaling by forming a heterotrimeric complex of β-arrestin-Cactus-Dorsal to prevent Mj-Cactus phosphorylation and degradation. Mj-β-arrestins also inhibited phosphorylation of Mj-ERK, which affected Mj-Dorsal translocation and phosphorylation. Therefore, Mj-β-arrestins negatively regulate the Toll pathway by limiting the translocation and phosphorylation of Mj-Dorsal (Fig. 9). These findings for the first time demonstrate that Mj-β-arrestins negatively regulate the Toll signaling pathway in two different ways in shrimp.

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**Conflict of interest**-The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions**-Conceived and designed the experiments: JXW, JJS, XFZ. Performed the experiments: JJS, JFL, XZS, MCY, GJN, DD. Analyzed the data: JJS, JXW. Wrote the paper: JJS, JXW, XQY.
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**FOOTNOTES**

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1The abbreviations used are: Mj-βarr, *Marsupenaeus japonicas* β-arrestin; Mj-ERK, *Marsupenaeus japonicus* extracellular regulated protein kinase; AMPs, antimicrobial peptides; Cru, Crustin; Mj-ALF, *Marsupenaeus japonicus* antilipopolysaccharide factor; Mj-TNFSF, *Marsupenaeus japonicus* tumor necrosis factor superfamily; *S. aureus*, *Staphylococcus aureus*; Co-IP, Co-immunoprecipitation; DMSO, dimethylsulfoxide; dsRNA, double-stranded RNA;
FIGURE LEGENDS

FIGURE 1. Toll pathway plays a central role in host defense against *S. aureus* in shrimp. (A-C) The upper panels show Dorsal expressions in the hemocytes (A), intestine (B) and gill (C) of shrimp challenged with *S. aureus*, as analyzed by western blotting, the bottom panels are the statistical analysis of Mj-Dorsal expression in shrimp for the upper panels. (D) Translocation of Mj-Dorsal induced by bacterial challenge in shrimp hemocytes, as analyzed by immunocytochemistry. Green fluorescence signal indicates the distribution of Mj-Dorsal; blue indicates the nucleus of hemocytes stained with DAPI. More than 90% of cells exhibits the representative image (Mj-Dorsal was translocated from the cytoplasm into the nucleus) in three repeated experiments. (E-F) The subcellular distributions of Mj-Dorsal in shrimp intestine and gill. Nuclear and cytoplasmic proteins were extracted from shrimp intestine and gill after *S. aureus* challenge at 1 h and analyzed by western blotting. (G, J and M) qRT-PCR was used to detect the expression of AMPs (MjALF-D1, MjALF-C2, CruI-1) and another effector (TNFSF) in the hemocytes (G), intestine (J) or gill (M) of shrimp challenged with *S. aureus* at 6 h. (H, K and N) The efficiency of Mj-Dorsal RNAi in hemocytes (H), intestine (K) or gill (N), as analyzed by western blotting (upper) and qRT-PCR (bottom). (I, L and O) The expression of AMPs and other effectors were detected in the hemocytes (I), intestine (L) or gill (O) of Mj-Dorsal-silenced shrimp challenged with *S. aureus* at 6 h. (P) The bacterial clearance of the Mj-Dorsal-silenced shrimp. The dsGFP injection was used as the control. (Q) The survival rate of the dsMj-Dorsal-silenced shrimp challenged by *S. aureus*. The shrimp were injected with dsMj-Dorsal and then with *S. aureus*. The shrimp were monitored daily for mortality, and the survival rate was calculated. The dsGFP injection was used as the control. The experiments were repeated three times. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

FIGURE 2. Knockdown of Mj-βarrs or Mj-ERK affects Toll signaling in shrimp. (A) The efficiency of RNAi for Mj-βarr1 and Mj-βarr2 in shrimp intestine analyzed at protein (upper) and RNA (lower) levels. DsGFP injection was used as the control. (A1) Mj-Dorsal was detected in the cytoplasm and nucleus of the intestine in Mj-βarrs-RNAi shrimp after bacterial challenge. (A2) The expression of effectors (MjALF-D1, MjALF-C2, CruI-1 and TNFSF) regulated by the Toll pathway in Mj-βarrs-RNAi shrimp challenged by bacteria. (B) The effects of Mj-ERK RNAi in shrimp intestine. (B1) Mj-Dorsal was detected in the cytoplasm and nucleus of intestine from Mj-ERK-silenced shrimp challenged by *S. aureus*. (B2) The expression of effectors (MjALF-D1, MjALF-C2, CruI-1 and TNFSF) regulated by the Toll pathway in the intestine of Mj-ERK-silenced shrimp. (A3) The effects of RNAi of Mj-βarr1 and Mj-βarr2 in shrimp hemocytes. (A4) Mj-Dorsal translocation in hemocytes of Mj-βarrs-RNAi-shrimp challenged with *S. aureus* at 1 h. Green fluorescence signal indicates the distribution of Mj-Dorsal in hemocytes, blue shows the nucleus of hemocytes stained with DAPI. More than 90% of cells exhibits the representative image (Mj-Dorsal was almost completely translocated from the cytoplasm into the nucleus) in three repeated experiments. (B3) The effect of Mj-ERK RNAi in shrimp hemocytes. (B4) Mj-Dorsal translocation in hemocytes of Mj-ERK-silenced shrimp at 1 h after *S. aureus* challenge. Green fluorescence signal indicates the distribution of Mj-Dorsal in hemocytes, blue shows the nucleus of hemocytes stained with DAPI. More than 90% of cells exhibits the representative image (Mj-Dorsal could not be translocated from the cytoplasm into the nucleus) in three repeated experiments. (A5) The bacterial clearance of Mj-βarr1-silenced shrimp. Shrimp were injected with dsMj-βarr1 and then with *S. aureus*.
After 6 h of the first bacterial injection, same number of *S. aureus* was injected again and the hemolymph was drawn out and cultured on the LB plate for bacterial count. The *dsGFP* injection was used as the control. (A6) The survival rate of the *Mj-βarr1*-silenced shrimp challenged by *S. aureus*. Shrimp were injected with *dsMj-βarr1* and then with *S. aureus*. The shrimp were monitored daily for mortality, and the survival rate was calculated. The *dsGFP* injection was used as the control. (B5) The bacterial clearance of *Mj-ERK*-silenced shrimp. (B6) The survival rate of the *Mj-ERK*-silenced shrimp challenged by *S. aureus*. The experiments were repeated three times. Asterisks indicate significant differences (*p* < 0.05, **p** < 0.01, ***p** < 0.001).

**FIGURE 3. Mj-βarrs interacts with Mj-Cactus and Mj-Dorsal, forming a βarr-Cactus-Dorsal complex.** (A) The shrimp was injected with *S. aureus* and the proteins from different tissues (hemocytes, intestine and gill) were extracted at 10, 30 and 60 min post injection and analyzed by western blotting using Mj-Cactus antibody. (B) The interaction of Mj-βarr1 with Mj-Cactus and Mj-Dorsal in gill was analyzed by a co-immunoprecipitation assay using an anti-Mj-βarr1 antibody. The antibody was coupled with CNBr-activated Sepharose 4B. The gill tissues of untreated shrimp or *S. aureus* challenged shrimp were collected at 1 h after bacterial injection. The tissue extraction solution was incubated with CNBr-activated Sepharose 4B to bind the target proteins. The eluate was assayed by western blotting. The Mj-Cactus and Mj-Dorsal could be detected in the unchallenged shrimp gill tissues, but not in the bacteria-challenged shrimp. (B1) The interaction of Mj-βarr2 with Mj-Cactus and Mj-Dorsal in gill was analyzed by co-immunoprecipitation using an anti-Mj-βarr2 antibody. The antibodies were coupled with CNBr-activated Sepharose 4B to bind natural Mj-Cactus and Mj-Dorsal. (C-C1) The interaction of Mj-Cactus with Mj-βarrs and Mj-Dorsal in gill was analyzed by co-immunoprecipitation using an Mj-Cactus antibody. The antibodies were coupled with CNBr-activated Sepharose 4B to bind natural Mj-βarrs and Mj-Dorsal. (D-D1) The interaction of Mj-Dorsal with Mj-βarrs and Mj-Cactus was analyzed by co-immunoprecipitation using an anti-Mj-βarr antibody. The antibody was coupled with CNBr-activated Sepharose 4B to bind natural Mj-βarrs and Mj-Dorsal. (E-E1) The interaction of Mj-Cactus with Mj-βarrs and Mj-Dorsal in hemocytes was analyzed by co-immunoprecipitation using an Mj-Cactus antibody. The antibody was coupled with CNBr-activated Sepharose 4B to bind Mj-βarrs and Mj-Dorsal. (F) The distribution of Mj-βarr1, Mj-βarr2, Mj-ERK, Mj-Cactus and Mj-Dorsal in mRNA and proteins was examined by semi-quantitative RT-PCR and western blotting. The experiments were repeated three times.

**FIGURE 4. N-terminus of Mj-βarrs interacts with the PEST domain of Mj-Cactus.** (A) Domain architectures of Mj-βarr1 and Mj-βarr2 (left and middle panels) and Mj-Cactus (right panel). (B) Interaction of Mj-Cactus with Mj-βarr1, as analyzed by GST- and His-pull-down. Left panel, GST-Pull-down: GST-Mj-Cactus was first bound to GST resin, and could pull down His-Mj-βarr1. Right panel, His-Pull-down: His-Mj-βarr1 was first bound to His resin, and could pull down GST-Mj-Cactus. (C) Interaction of Mj-Cactus with Mj-βarr2, as analyzed by GST- and His-pull-down. (D-E) Mj-Cactus interacted with the Mj-βarr1-N domain and Mj-βarr2-N domain. Left panel: GST-Mj-Cactus was used to pull down of the Mj-βarr1-N domain and Mj-βarr2-N domain (His-Mj-βarr1-N and His-Mj-βarr2-N). Right panel: The His-Mj-βarr1-N domain and His-Mj-βarr2-N domain were used to pull down
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GST-Mj-Cactus. (F-G) GST- and His-pull-down with Mj-Cactus and the Mj-βarr1-C domain and Mj-βarr2-C domain. Left panel: GST-Mj-Cactus was used to pull down the Mj-βarr1-C domain and Mj-βarr2-C domain (His-Mj-βarr1-C and His-Mj-βarr2-C). Right panel: The His-Mj-βarr1-C domain and His-Mj-βarr2-C domain were used to pull down GST-Mj-Cactus. (H-I) GST- and His-pull-down using Mj-Cactus-N-ANK and Mj-βarrs-N. Left panel: GST-Mj-Cactus-N-ANK was used to pull down His-Mj-βarr1-N and His-Mj-βarr2-N. Right panel: His-Mj-βarr1-N and His-Mj-βarr2-N were used to pull down GST-Mj-Cactus-N-ANK. (J-K) GST- and His-pull-down using Mj-Cactus-PEST and Mj-βarr1-N and Mj-βarr2-N. Left panel: GST-Mj-Cactus-PEST was used to pull down His-Mj-βarr1-N and His-Mj-βarr2-N. Right panel: His-Mj-βarr1-N and His-Mj-βarr2-N were used to pull down GST-Mj-Cactus-PEST. The experiments were repeated three times.

FIGURE 5. Mj-Cactus interacts with Mj-Dorsal to form a βarr-Cactus-dorsal complex. (A-B) Schematic representations of the full-length protein, and the RHD and IPT domains of Mj-Dorsal (A); full-length protein, N-terminus, N-ANK, ANK and PEST domains of Mj-Cactus (B) for the recombinant expression. (C) Mj-Cactus interacted with Mj-Dorsal. Left panel, GST-Pull-down: GST-Mj-Cactus was first bound to GST resin, and could pull down His-Mj-Dorsal. Right panel, His-Pull-down: His-Mj-Dorsal was first bound to His resin, and could pull down GST-Mj-Cactus. (D) Mj-Cactus interacted with RHD but not the IPT domain of Mj-Dorsal. Left panel: GST-Mj-Cactus was used to pull down the RHD domain of Mj-Dorsal (His-Mj-Dorsal-RHD). Right panel, GST-Mj-Cactus was used to pull down the IPT domain of Mj-Dorsal (His-Mj-Dorsal-IPT). (E) Different domains of Mj-Cactus (GST-Mj-Cactus-N-ANK, Mj-Cactus-N, Mj-Cactus-ANK and Mj-Cactus-PEST) were used in pull-down assays to analyze their interactions with Mj-Dorsal-RHD. (F) Heterotrimeric complex analysis of βarr-Cactus-Dorsal with recombinant proteins. Left panel: GST-Mj-Cactus interacted with His-Mj-βarr1 and His-Mj-Dorsal. Right panel, GST-Mj-Cactus interacted with His-Mj-βarr2 and His-Mj-Dorsal. (G) GST was used as the control for (F). (H) GST-pulldown: GST-Mj-βarr1 and GST-Mj-βarr1 were used to pull down His-Mj-Dorsal. The experiments were repeated three times.

FIGURE 6. Mj-βarrs interacts with non-phosphorylated Mj-ERK and phosphorylation of Mj-ERK was increased in the Mj-βarrs-knockdown shrimp challenged by S. aureus at 1 h. (A) GST pull-down assay demonstrated the interaction of GST-Mj-ERK with native Mj-βarrs. The extract solution of gill tissues from normal shrimp was incubated with GST-resin bound to Mj-ERK. The eluate was assayed by western blotting. The recombinant GST protein was used as control. (B) Co-immunoprecipitation assay using anti-Mj-βarr1 or Mj-βarr2 antibodies. Shrimp intestine tissues were homogenized and centrifuged. The Mj-βarr1 or Mj-βarr2 antibodies were incubated with the supernatant, and then isolated using CNBr-activated Sepharose 4B. (C) Co-immunoprecipitation assay using Mj-ERK or Mj-ERK-P antibodies. Shrimp intestine was homogenized and centrifuged. These antibodies were coupled with CNBr-activated Sepharose 4B to bind natural Mj-βarrs. (D) Mj-βarr1 and Mj-βarr2 expression at the mRNA level in intestine after injection of dsMj-βarr1 or dsMj-βarr2. (E) The distribution of Mj-ERK in the nucleus and cytoplasm was detected in intestine after knockdown of Mj-βarr1 or Mj-βarr2 and then challenged by S. aureus for 1 h. Bound proteins were eluted and detected by western blotting with the antibody of interest. (F) The left panels are the statistical analysis of Mj-ERK-P expression in shrimp for
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the right panels. The experiments were repeated three times. Asterisks indicate significant differences (*p < 0.05, **p < 0.01).

FIGURE 7. The arrestin-C domain of Mj-βarrs interacts with Mj-ERK. (A-B) Schematic representation of the full-length protein, and the arrestin-N and arrestin-C domains of Mj-βarr1 (A) and Mj-βarr2 (B). (A1-B1) Interaction of Mj-ERK with Mj-βarr1 or Mj-βarr2. Left panel, GST-Pull-down: GST-Mj-ERK was first bound to GST resin, and could pull down His-Mj-βarr1 and His-Mj-βarr2. Right panel, His-Pull-down: His-Mj-βarr1 and His-Mj-βarr2 was first bound to His resin, and could both pull down GST-Mj-ERK. (A2-B2) Mj-ERK interacted with the Mj-βarr1-C domain (A2) and Mj-βarr2-C domain (B2). Left panel: GST-Mj-ERK was used to pull down the Mj-βarr1-C or Mj-βarr2-C domain (His-Mj-βarr1-C or His-Mj-βarr2-C). Right panel: His-Mj-βarr1-C or His-Mj-βarr2-C domains were used to pull down GST-Mj-ERK. (A3-B3) Interaction of Mj-ERK with the Mj-βarr1-N domain or Mj-βarr2-N domain. Left panel: GST-Mj-ERK was used to pull down the Mj-βarr1-N domain or Mj-βarr2-N domain (His-Mj-βarr1-N and His-Mj-βarr2-N). Right panel: His-Mj-βarr1-N or His-Mj-βarr2-N domains were used to pull down GST-Mj-ERK. The experiments were repeated three times.

FIGURE 8. The inhibitor of Mj-ERK or knockdown of Mj-ERK decreased the phosphorylation of Mj-Dorsal in nucleus. (A) Western blot detected reduced the Serine276 phosphorylated Mj-Dorsal in the Mj-Dorsal knockdown shrimp challenged by S. aureus at 1 h. The lower panel is statistical analysis of Mj-Dorsal-P expression after digitizing by software Quantity One of BIO-RAD. (B) The intestine proteins from shrimp challenged with S. aureus were collected at different times and the phosphorylations of Mj-ERK and Mj-Dorsal were analyzed by western blotting. The lower panel is statistical analysis of Mj-ERK-P and Mj-Dorsal-P expression after digitizing by Quantity One. (C) The phosphorylation of Mj-Dorsal was detected by western blotting in the Mj-ERK knockdown shrimp challenged by S. aureus at 1 h. The lower panel is statistical analysis of Mj-Dorsal-P expression after digitizing by Quantity One. (D) Mj-MEK expression at the mRNA level in intestine after injection of dsMj-MEK. (E) The phosphorylations of Mj-ERK and Mj-Dorsal were analyzed by western blotting in the Mj-MEK knockdown shrimp challenged by S. aureus at 1 h. The lower panel is statistical analysis of Mj-ERK-P and Mj-Dorsal-P expression after digitizing by Quantity One. (F) Western blot detected reduced phosphorylation of Mj-ERK and Mj-Dorsal after injection of PD98059 and challenge with S. aureus at 1 h. The low panel is statistical analysis of Mj-ERK-P and Mj-Dorsal-P expression after digitizing by Quantity One. (G) The Mj-Dorsal phosphorylation in the nucleus and cytoplasm of cells was detected by western blotting after PD98059 injection and challenge with S. aureus at 1 h. (H) The expression of effectors (MjALF-D1, MjALF-C2, CruI-1 and TNFSF) that are regulated by the Toll pathway in the intestine were detected after PD98059 injection and challenge with S. aureus at 6 h. The experiments were repeated three times. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

FIGURE 9. A model for βarrs’ involvement in the Toll signaling pathway in shrimp. In shrimp, the Toll signaling pathway is activated by bacterial challenge. The transcription factor Dorsal is translocated into the nucleus and ERK is phosphorylated. Several effector molecules (ALF-C2, CruI-1 and TNFSF) were then expressed. βArRs interacts with Cactus to form the
βArr-Cactus-Dorsal complex, which inhibits Cactus phosphorylation and degradation, thereby preventing Dorsal from translocating into nucleus, resulting in inhibition of the activation of the Toll pathway. βArrs also interacts with inactive ERK to inhibit its phosphorylation by MEK, and subsequently inhibits Serine$^{276}$ phosphorylation of Dorsal. Finally, the activation of the Toll pathway is limited to prevent an inappropriate inflammatory response.
Table 1. Sequences of the primers used in this study

| Primer       | Sequence (5’-3’)                          |
|--------------|------------------------------------------|
| **Tissue distribution and expression pattern analysis** |                                |
| Mj-βarr1-RT-F | TTTCACGCTGACGCCACT                      |
| Mj-βarr1-RT-R | AGCAACCAGATCACCACACTAG                   |
| Mj-βarr2-RT-F | TGGCTCTATTCTCTCTGCG                      |
| Mj-βarr2-RT-R | TGGCTCTATTCTCTCTGCG                      |
| Mj-ERK-RT-F   | GTGCTGAACCTCTAAGGGCTAC                   |
| Mj-ERK-RT-R   | GCTGGGTCATAATACGTGCT                    |
| Mj-Cactus-RT-F | CCGCTGACCTAACAACCATATGA                |
| Mj-Cactus-RT-R | TGGCTCACCACCATCTTT                    |
| Mj-Dorsal-RT-F | GCAATGCTGGTGACAACCTGCTA                |
| Mj-Dorsal-RT-R | CATATGAGATGGTCACCAGTTTA                |
| Mj-MEK-RT-F   | CCGAGCCATCTTATAGG                      |
| Mj-MEK-RT-R   | GAGGGAGCGAGGGCAGTGATT                   |
| β-actin F     | CAGCCTTCCTTCCTGGGTATAGG                 |
| β-actin R     | GAGGGAGCGAGGGCAGTGATT                   |
| Mj-ALF-C2-RT-F | TCTGGTGAGAGGCTGCT                      |
| Mj-ALF-C2-RT-R | TGCGGTCTCGGCTGCTCCT                    |
| Mj-ALF-D1-RT-F | GTCTTTATTTTGGGGGTACGTG                  |
| Mj-ALF-D1-RT-R | CTTTGGCGTGAACAGTGAGG                   |
| Mj-CruI-1-RT-F | TGTCGAGAATCCCTCACC                      |
| Mj-CruI-1-RT-R | TGAAATCAGCCATCGTCT                     |
| Mj-TNFSF-RT-F | GGAATCGAGGGACTTGTGAGGT                  |
| Mj-TNFSF-RT-R | CTTCTCTCCCTCTCTGCG                      |
| **Recombinant expression** |                                      |
| Mj-βarr1-ExF  | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-βarr1-ExR  | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-βarr1-C-ExF | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-βarr1-C-ExR | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-βarr1-N-ExF | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-βarr1-N-ExR | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-ERK-ExF    | TACTCAGGATCCATAATGGAGGACAACAG          |
| Mj-ERK-ExR    | TACTCAGGATCCATAATGGAGGACAACAG          |
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| Mj-Cactus-ExF     | TACTCAGGATCCATGTGGGCACATTGGCAGTGCC |
|-------------------|-----------------------------------|
| Mj-Cactus-ExR     | TACTCAGTGCACCTTAGAAGTAGCGATCTGC  |
| Mj-Cactus-N-ANK-ExF | TACTCAGTGCACGGGCGTCCCCAGGAGGC |
| Mj-Cactus-N-ExR   | TACTCAGTGCACCTGGAAAGATCTCCTGGAG |
| Mj-Cactus-ANK-ExF | TACTCAGGATCCCTCCAGGAGATCTTTCAG |
| Mj-Cactus-ANK-ExR | TACTCAGTGCACGGGCTCCCCAGGAGGC |
| Mj-Cactus-PEST-ExF| TACTCAGGATCCGCTCTGGGGAGCC |
| Mj-Dorsal-ExF     | TACTCAGGATCCGACCCTGATCTGGAGAGT |
| Mj-Dorsal-ExR     | TACTCAGGATCCGACCTGGGATCTGAGTC |
| Mj-Dorsal-IPT-ExR | TACTCAGGATCCGACATACAGATCTGA   |
| Mj-Dorsal-RHD-ExF | TACTCAGGATCCGATCCCATATTGACAAG |

**RNA interference**

| Mj-βarr1-Fi       | GCGTAATACGACTCACTATAGGCTGATTTCAAGTCAAAA |
|-------------------|-----------------------------------------|
| Mj-βarr1-Ri       | GCGTAATACGACTCACTATAGGGAATCTTCTCTTCTGT |
| Mj-βarr2-Fi       | GCGTAATACGACTCACTATAGGCTCTGCTGGAGACAT |
| Mj-βarr2-Ri       | GCGTAATACGACTCACTATAGGCTCTTATCTCTCT |
| Mj-ERK-Fi         | GCGTAATACGACTCACTATAGGCCATGCAAGAAGAC |
| Mj-ERK-Ri         | GCGTAATACGACTCACTATAGGACATACACTTTATTTTC |
| Mj-Cactus-Fi      | GCGTAATACGACTCACTATAGGCCAGGGCTGCTGCA |
| Mj-Cactus-Ri      | GCGTAATACGACTCACTATAGGGAAGTACGGAGATCTGCA |
| Mj-Dorsal-Fi      | GCGTAATACGACTCACTATAGGCCATAGAGCTAGATA |
| Mj-Dorsal-Ri      | GCGTAATACGACTCACTATAGGCTAGTACCCAAAGGTG |
| Mj-MEK-Fi         | GCGTAATACGACTCACTATAGGATCCAGGAAGAACAC |
| Mj-MEK-Ri         | GCGTAATACGACTCACTATAGGAGTGTCTGTCTATTTG |
| GFP-Fi            | GCGTAATACGACTCACTATAGGTTAGGTGCTCCTG |
| GFP-Ri            | GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTG |

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Figure 1

A. S. aureus - + + + Mj-Dorsal
β-actin 0 1 3 6 h
Hemocytes

B. S. aureus - + + + Mj-Dorsal
β-actin 0 1 3 6 h
Intestine

C. S. aureus - + + + Mj-Dorsal
β-actin 0 1 3 6 h
Gill

D. DAPI Fluorescence Merge
PBS
S. aureus

E. S. aureus - + + + Mj-Dorsal
Loading control
Cytoplasm Nucleus
Intestine

F. S. aureus - + + + Mj-Dorsal
Loading control
Cytoplasm Nucleus
Gill

G. Hemocytes
Relative expression level
Normal dsGFP Mj-Dorsal dsMj-Dorsal TNF-s

H. Hemocytes
β-actin
Relative expression level
Normal dsGFP Mj-Dorsal dsMj-Dorsal

I. Hemocytes
Relative expression level
Normal dsGFP + S. aureus dsMj-Dorsal + S. aureus

J. Intestine
Relative expression level
Normal dsGFP Mj-Dorsal dsMj-Dorsal TNF-s

K. Intestine
β-actin
Relative expression level
dsGFP Mj-Dorsal

L. Intestine
Relative expression level
Normal dsGFP + S. aureus dsMj-Dorsal + S. aureus

M. Gill
Relative expression level
Normal dsGFP Mj-Dorsal dsMj-Dorsal TNF-s

N. Gill
β-actin
Relative expression level
dsGFP Mj-Dorsal

O. Gill
Relative expression level
Normal dsGFP + S. aureus dsMj-Dorsal + S. aureus

P. The clearance of S. aureus
The number of bacteria
dsGFP + S. aureus dsMj-Dorsal + S. aureus

Q. Survival rate
Survival rate
1 2 3 4 5 d
Time post challenge

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Figure 2

A1 and B1 show the relative expression levels of Mj-βarr1 and Mj-βarr2 in the intestine of shrimp infected with S. aureus compared to dsGFP controls. The graphs indicate a negative regulation by Mj-βarrs on the expression of Mj-Dorsal in response to S. aureus infection.

A2 and B2 display the relative expression levels in the cytoplasm of Mj-βarrs in control and S. aureus-infected conditions. The graphs support the negative regulation observed in the intestine.

A3 and B3 exhibit the relative expression levels in hemocytes under similar conditions. The graphs again confirm the negative regulation by Mj-βarrs.

A4 and B4 illustrate the fluorescent images of hemocytes stained with DAPI, showing the distribution of Mj-βarrs and their impact on S. aureus infection.

A5 and A6 depict the clearance of S. aureus by different treatments over time, highlighting the role of Mj-βarrs in the immune response.

B5 and B6 present the survival rates and bacterial load over time, further emphasizing the protective role of Mj-βarrs against S. aureus infection.
Figure 3

Beta-Arrestins negatively regulate the Toll pathway in shrimp
Beta-Arrestins negatively regulate the Toll pathway in shrimp

Figure 4
Figure 5
Beta-Arrestins negatively regulate the Toll pathway in shrimp

Figure 6

A

| Input | Pull down by GST-Mj-ERK |
|-------|------------------------|
| GST-Mj-ERK | GST-Mj-ERK |
| Mj-βarr1 | Mj-βarr2 |
| Mj-βarr1 | Mj-βarr2 |

B

| Input | IP |
|-------|----|
| Mj-ERK | IB |
| Mj-βarr1 | Mj-βarr2 |
| Mj-ERK-P | S. aureus |

C

| Input | IP |
|-------|----|
| Mj-ERK | IB |
| Mj-βarr1 | Mj-βarr2 |
| Mj-ERK-P | S. aureus |

D

| dsGFP | dsMj-βarr1 |
|-------|------------|
| dsGFP | dsMj-βarr2 |

E

| dsGFP | dsMj-βarr1 |
|-------|------------|
| dsGFP | dsMj-βarr2 |

F

| Protein relative expression level |
|----------------------------------|
| Cytoplasm                         |
| Normal                           |
| dsGFP                            |
| dsMj-βarr1                       |
| dsMj-βarr2                       |
| Nucleus                           |
| Normal                           |
| dsGFP                            |
| dsMj-βarr1                       |
| dsMj-βarr2                       |

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Figure 7

Beta-Arrestins negatively regulate the Toll pathway in shrimp
Beta-Arrestins negatively regulate the Toll pathway in shrimp

Figure 8
Figure 9

Beta-Arrestins negatively regulate the Toll pathway in shrimp
Beta-Arrestins negatively regulate the Toll pathway in shrimp by preventing Dorsal translocation and inhibiting Dorsal transcriptional activity

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