Fish and Shellfish Immunology 95 (2019) 277–286

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Scophthalmus maximus interleukin-1β limits *Edwardsiella piscicida* colonization *in vivo*

Shouwen Chen[a], Xin Ma[a], Di Wu[a], Dahai Yang[a,b], Yuanxing Zhang[a,b], Qin Liu[a,b,*]

[a] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China
[b] Shanghai Engineering Research Center of Marine Cultured Animal Vaccines, Shanghai, 200237, China

A R T I C L E I N F O

Keywords:
Scophthalmus maximus
Interleukin-1β
Edwardsiella piscicida
Bacterial colonization

ABSTRACT

Interleukine-1β (IL-1β) is the first identified pro-inflammatory cytokine, which is cleaved by caspase-1 following the inflammasomes activation, playing critical roles in innate immunity. However, few studies have been performed to characterize the IL-1β in lower vertebrates. Herein, we distinguished the *Scophthalmus maximus* IL-1β (*Sm*IL-1β) from three IL-1β like sequences and found that *Sm*IL-1β was cleaved by *S. maximus* caspase at a non-conserved Asp^{86}, then targeted to the plasma membrane. Moreover, during the immersion infection of *Edwardsiella piscicida*, we found that *E. piscicida* were mainly colonized in gills at early time points and invaded to systemic sites after 5 days post infection, which was consistent with the dynamic up-regulated transcription of *Sm*IL-1β. Furthermore, knockdown of *Sm*IL-1β promotes the bacterial colonization in gills at early time points and result into systemic colonization, while overexpression of *Sm*IL-1β hampers the bacterial colonization in both spleen and kidney. Taken together, these data provide new insights into the molecular mechanisms of *Sm*IL-1β and reveal its role in limiting bacterial infection *in vivo*, which will support the idea for better understanding the evolutionary of IL-1β functions in teleost.

1. Introduction

Since the cloning and expression of IL-1 DNA in 1984, interleukin 1 (IL-1) cytokines have been well studied in mammals [1]. To data, four subfamilies of IL-1 cytokines have been identified, which comprises 11 ligands including seven proinflammatory cytokines: IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ and three receptor antagonists (RA): IL-1Ra, IL-36Ra, and IL-38, and an anti-inflammatory cytokine, IL-37 [2]. As the key nodes of immune and inflammation network, these cytokines regulate a range of processes in health and disease by immune and inflammatory response [3,4]. Notably, IL-1β has an obvious effect on danger signal or pathogen infection [3]. For example, deficiency of IL-1β in mice significantly decreased the susceptibility to Lipopolysaccharide (LPS)-induced septic shock [5]. However, the reduction of IL-1β secretion results into reduced lung injury and higher survival rate during *Pseudomonas aeruginosa* infection [6]. Recent study also revealed that the inflammasome and fas-mediated IL-1β secretion could induce Th17/Th1 cells activation in mice, which directly contributes to the restriction of *Listeria monocytogenes in vivo* [7]. Collectively, IL-1β plays critical role in antimicrobial response by modulating the inflammation processes.

According to the conserved IL-1 family signature and sequence alignment analysis, Intereukin-1 genes from a myriad of fish species have been cloned [8–10]. By phylogenetic and syntenic comparisons, the fish IL-1 genes are divided into different subtypes, within a comparatively lower identity [11,12]. Thus, it is difficult to identify the homology of fish IL-1 genes by comparing to mammals. The mammalian inflammasome activation can result into maturation and secretion of IL-1β in response to various cytosolic danger signaling [13–15], and the matured IL-1β can target to the cell membrane, directly mediating its secretion [16]. In zebrafish, recent study has revealed that NLRP1-ASC-caspase A/B inflammasome is responsible for the processing of IL-1β by a two-step sequential activation mode [17]. While in seabream, stimulation or infection of macrophages cannot induce the caspase-1-dependent processing and release of IL-1β [18]. Although increasing studies have suggested that the fish IL-1β could response to a variety of stimulus [19–21], along with the lacking of a conserved caspase-1 recognition site [12,22], there are still a number of proteases in fish that can cleave IL-1β by recognizing at different sites [12]. Thus, the immune significance of IL-1β in teleost remains largely unexplored.

Edwardsiella bacteria are important zoonotic organisms that infects humans and also leading fish pathogenss causing huge losses to worldwide aquaculture industries. Previously, the genus *Edwardsiella* is classified into three species *Edwardsiella tarda*, *E. ictaluri*, and *E.*

---

[*] Corresponding author. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China.
E-mail address: qinliu@ecust.edu.cn (Q. Liu).

https://doi.org/10.1016/j.fsi.2019.10.050
Received 9 July 2019; Received in revised form 15 October 2019; Accepted 24 October 2019
Available online 31 October 2019
1050-4648/

hoshinae. However, phylogenomic analysis indicated that *E. tarda* strain display two major highly divergent genomic types (genotypes), EdwGI and EdwGII [23], and the former represents a genotype of fish-pathogenic isolates and being recently proposed as a novel species *E. piscicida* [24]. Accordingly, we named the *Edwardsiella* bacteria EIB202 used in our lab as *E. piscicida* EIB202 [25]. The infection of *E. piscicida* induced the secretion of IL-1β in murine or fish primary macrophages, dependent of its type III secretion system (T3SS) [26]. Furthermore, *E. piscicida* virulence effector trxlp was reported to promote the NLRC4 inflammasome activation and IL-1β release during infection [27]. Recently, our work has revealed that *E. piscicida* type VI secretion system (T6SS) effector, EvpP, can inhibit NLRP3 inflammasome activation by regulating the intracellular Ca^{2+} influx and Jnk phosphorylation [28]. Collectively, these results suggest that *E. piscicida* can interact with the host innate immunity through its virulence effectors to promote its pathogenesis, while the inflammasome activation-mediated IL-1β production is critical for maintaining homeostasis during infection. To better understand the function of IL-1β in *S. maximus* during *E. piscicida* infection, we distinguished *Sm*IL-1β from three IL-1β like proteins and showed that the *Sm*IL-1β could be cleaved by *Sm*Caspase at a non-conserved Asp^{86} site. Furthermore, we found that *Sm*IL-1β exerts anti-infectious roles in restricting the colonization of *E. piscicida in vivo*, which provides new insights into the evolutionary function of IL-1β in teleost, and reveal an interplay between bacterial infection and fish innate immunity.

2. Materials and methods

2.1. Strain and culture condition

E. piscicida (EIB202, CCTCC No. M208068) [29] and *V. anguillarum* MVM425 [30] were cultured as previous study. Briefly, EIB202 strain was removed from the −80 °C freezer, then streaked on the DHL selective plate (Difco, Detroit, USA) with 12.5 mg/ml Colistin (Col). The DHL plate was placed in a 28 °C incubator for 38–48 h, and the black colonies on the plate were picked and cultured in a shaker at 28 °C for 12 h with tryptic soy broth (TSB) (Difco, Detroit, USA). The secondary bacterial solution was inoculated in TSB at a ratio of 1%, and cultured at 28 °C, 200 rpm for 12–16 h for experiments. *V. anguillarum* were cultured at 28 °C in Luria broth (or on Luria agar) supplemented with 3% NaCl (LBS) likewise. Bacteria were harvested by centrifugation at 4000*g* for 15 min. Then, cells were washed twice in PBS and resuspended in seawater to indicted concentration.

2.2. Fish

Healthy turbot (*Scophthalmus maximus*) weight approx. 10 g were purchased from a commercial fish farm (Tianyuan, Shandong, China) and maintained in aerated tanks supplied with a continuous flow of sand-filtered sea water at 16 °C. Fish were fed twice daily with commercial feed and acclimated at least one week before experimental manipulation. Before experiments, fish (5% of each stock) were randomly selected for the examination of bacterial recovery from liver, kidney and spleen on thiosulfate citrate bile salts sucrose agar (DHL, Difco, USA) plates to confirm that the turbot were not infected by EIB202. All fish experiments were carried out according to the guidelines and approval of the Animal Research and Ethics Committees of East China University of Science and Technology (Protocol #2006272).

2.3. Bacterial infection

For immersion infection, EIB202 was cultured in TSB medium as described above and resuspended in seawater to indicated bacterial concentration. Healthy turbot ($n = 20$ per group) were immersed in bacterial bath divided for 0.5, 0.75, 1 or 2 h as indicated, while control groups were immersed in seawater containing PBS. After the immersion treatment, the fish were moved to new tanks containing fresh seawater to remove unattached EIB202 and then reared under normal conditions as described above. Survival rate was recorded for lasting 21 days. For intraperitoneal infection, the fish ($n = 3$ per group) were injected with 100 μL PBS containing 1 mg LPS (Sigma), 2×10^3 CFU EIB202 or 1×10^7 CFU *V. anguillarum*, after one day infection, the spleen was harvested for RNA isolation. All the experiments were conducted for three biological repeats.

2.4. Bacterial enumeration

Fish ($n = 3$ per group) were euthanized with MS-222 (tricaine methanesulfonate with 100 ng/ml; Sigma, St Louis, MO, USA) for tissue collection at indicated time points (0, 0.5, 1, 2, 5, 7, 9 and 11 dpi). Subsequently, the tissues were sufficiently grinded to homogenate, properly diluted and dropped on the TSA plate. Then the TSA plate was placed in a 28 °C incubator for 38–48 h, and the black colonies on the plate were counted. All the experiments were conducted for two biological repeats.

2.5. RNA isolation and q-PCR

At indicated time points, tissues were pooled in Rnase-free centrifuge tube, added 200 μl RNAstore Reagent (Tiangen, Beijing, China) and stored at −80 °C. mRNA from indicated samples were isolated by Trizol (Invitrogen, USA) and chloroform. Purified total RNA (2 μg) was used for cDNA synthesis by utilizing FastQuant RT Kit (Tiangen, Beijing, China), then analyzed with real-time quantitative PCR (Bio-rad, USA) by using SuperReal PreMix Plus (SYBR Green) (Tiangen, Beijing, China) according to procedures. The results were analyzed by $2^{-\Delta\Delta Ct}$ method. Primers (*Smil-1β, Smil-1Fm2, Smnil*-1Fm,*β-actin*) used in this study were listed in Table S1.

2.6. Western blot analysis

HEK293T cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl 5 mM EDTA, 10% glycerol, and 0.1% Triton X-100; PH = 7.4) containing protease inhibitor cocktail and phosphatase inhibitor (Roche Applied Science, Penzberg, Germany), then mixed with cell supernatants. Protein lysates were obtained by organic solvent precipitation method. Subsequently, protein precipitate was mixed with protein loading buffer, boiled for 10 min and centrifuged (12,000 rpm, 5 min). Protein lysates were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore Sigma, Burlington, MA). The membranes were blocked in 5% w/v non-fat dry milk in TBST. Signals were detected with mouse anti-Actin antibody (Ab) (1:5000, Sigma-Aldrich), rabbit anti-GFP antibody (1:3,000, HUABIO, Hangzhou, China) overnight at 4 °C, followed by incubation with the appropriate secondary HRP-conjugated-anti-rabbit Abs (1:2,000, Beyotime Biotechnology, Shanghai, China) and detection with ECL (Cell Signaling Technology). The signal intensities were quantitatively analyzed using NIH ImageJ.

2.7. Cell culture and transfection

The HEK293T cells were seeded and grown to a density of $\sim 1 \times 10^5$ cells per well in 24-well plates with DMEM, which has been fortified with 10% FBS (Fetal Bovine Serum, Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin, with 5% CO_2 at 37 °C overnight. Replacing the culture medium with Opti-MEM 1 h before the transfection, the HEK293T cells were transfected with 1 μg GFP-tagged *Sm*IL-1β, *Sm*IL-$1\beta^{D86A}$ (D86A mutant of *Sm*IL-1β), *Sm*IL-$1\beta^{D100A}$ (D100A mutant of *Sm*IL-1β) and vector plasmid with 1 μg FLAG-tagged *Sm*Caspase or vector plasmid by using Lipofectamine 2000™ (Invitrogen). Cell supernatants and pellets were harvested after 48 h post-transfection for western blot analysis. All the experiments were conducted for three biological repeats.

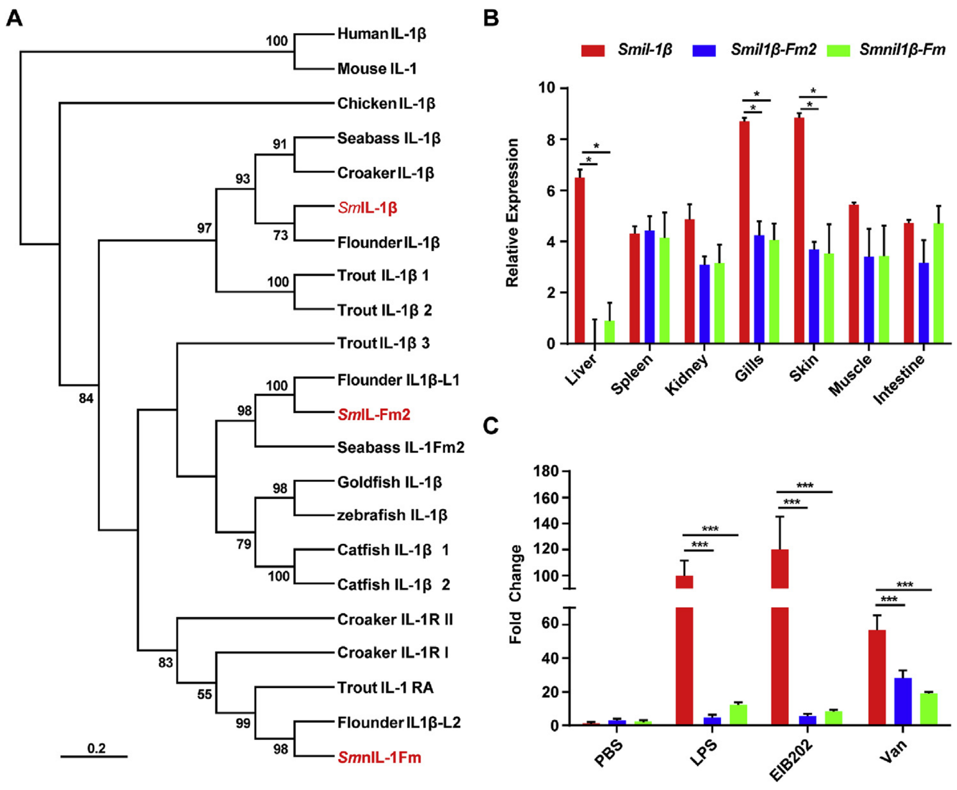

Fig. 1. Identification of *S. maximus* IL-1β. (A) Phylogenetic tree showing relationship between *S. maximus* IL-1 family members and other known IL-1 family members. The tree was constructed by neighbor-joining method using the CLUSTALW and was bootstrapped 1000 times. All the sequences were gained from the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and UniProt (https://www.uniprot.org). The sequence accession numbers are listed in Table S3 **(B)** Transcripts of *Smil-1β*, *Smil-1Fm2* and *Smnil*-1Fm compared to liver *Smil-1Fm2* in normal tissues as indicated. **(C)** The expression levels of *Smil-1β*, *Smil-1Fm2, Smnil*-1Fm in spleen from *S. maximus* were injected with LPS, *E. piscicida* EIB202 or *V. anguillarum* (Van) for 12 h. Data are presented as mean ± SEM of three independent experiments (n = 3 per group at each time).

2.8. Microscopy

The coding sequences of *S. maximus* IL-1β were cloned into the pCDH mammalian expression vector. The following IL-1β coding sequences were used: pro-*Sm*IL-1β (matching the full coding sequence), mature *Sm*IL-1β (residues 87–246, mimicking the caspase cleavage product, m*Sm*IL-1β), pre-*Sm*IL-1β (residues 1–86). All constructs encoded a N-terminal GFP tag. The HEK293T cells were seeded and grown to a density of ~1 × 10^5 cells per well in 24-well plates as mentioned above, and then transfected with indicated plasmids. After 20 h transfection, the cells were washed twice with sterile PBS and fixed with 4% paraformaldehyde (PFA) at room temperature for 2 h, then washed in PBS and permeabilized with Triton X-100 (0.1% in PBS, 10 min at room temperature). Actin was counterstained with TRITC phalloidin (Yeasen Biotech, Shanghai, China) and nuclei with DAPI (Beyotime). Fixed samples were observed under a confocal microscope (Nikon, Tokyo, Japan; A1R).

2.9. SmIL-1β knockdown and overexpression in vivo

To select small interfering RNA (siRNA) for *Sm*IL-1β knockdown, the fragments were inserted into pRNAT-CMV3.1 (GenScript, Piscataway, USA) as described previously [31]. Briefly, psiIL-1β-1, psiIL-1β-2, psiIL-1β-3 were constructed for knockdown, corresponding to non-specific siRNAs, psiC1, psiC2, psiC3 as controls. For overexpression, pIL-1β was constructed with vector plasmid pCN3 as the control [32]. Endotoxin-free plasmids were prepared using Endo-Free plasmid Kit (Tiangen, Beijing, China). To examine the knockdown efficiency, fish were retroorbitally injected with three psiIL-1β mixture or psiC plasmids (10 μg/fish) or with PBS. To examine the overexpression efficiency, fish were retroorbitally injected with pIL-1β or pCN3 (10 μg/fish). At 3- or 7-day post-plasmid administration, liver, spleen and kidney, gills were sampled under aseptic conditions and the transcription of *Sm*IL-1β by qRT-PCR were analyzed as described above. To examine the effects of *Sm*IL-1β knockdown or overexpressed during infection, fish were administered with psiIL-1β, psiC, pIL-1β, pCN3 or PBS (control) as above, and at 3 days post-plasmid administration, the fish were immersion-infected with EIB202 with 2 × 10^8 CFU/mL. Liver, kidney, spleen and gills were sampled under aseptic conditions and examined for bacterial numbers by plate count. All the primers associated are listed in Table S2.

2.10. ELISA

The tail venous blood (n = 3 per group) was collected from fish at indicated time pionts post the immersion infection and then clotted at room temperature for 1–2 h. Serum was isolated after centrifugation at 1000*g* for 10 min, and divided into several aliquots to store at −80 °C. Briefly, each well of a microtiter plate was coated with 100 μl of tenfold diluted serum in a coating buffer (50 mM carbonate buffer, pH 9.6) overnight at 4. Then, the wells were washed with PBS containing 0.05% Tween-20 (PBSTB) once and blocked with PBST containing 1% BSA (PBST) at room temperature for 2 h. After three times washing, the wells were added diluted *Sm*IL-1β polyclonal antibody (1:100; Genscript Biotech, Piscataway, NJ, USA) in PBSTB and incubated at 37 °C for 2 h. Subsequently, the microplate was washed three times with PBST, and 100 μL diluted secondary anti-HRP-conjugated-rabbit antibody (1:200; Sigma) was added to each well. After rewashing four times with PBST, the plate was developed with TMB. Then, the reaction was stopped with 2 M H_2SO_4, and absorbance was read at 450 nm wavelength. All the experiments were conducted for two biological repeats.

2.11. Statistical analysis

Statistical analysis was performed by Prism 7.0 (Graphpad, USA). Each experiment was performed at least two times. The statistical significance was determined by one-way ANOVA or two-tailed *t*-test. The results were presented as mean ± SEM, $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

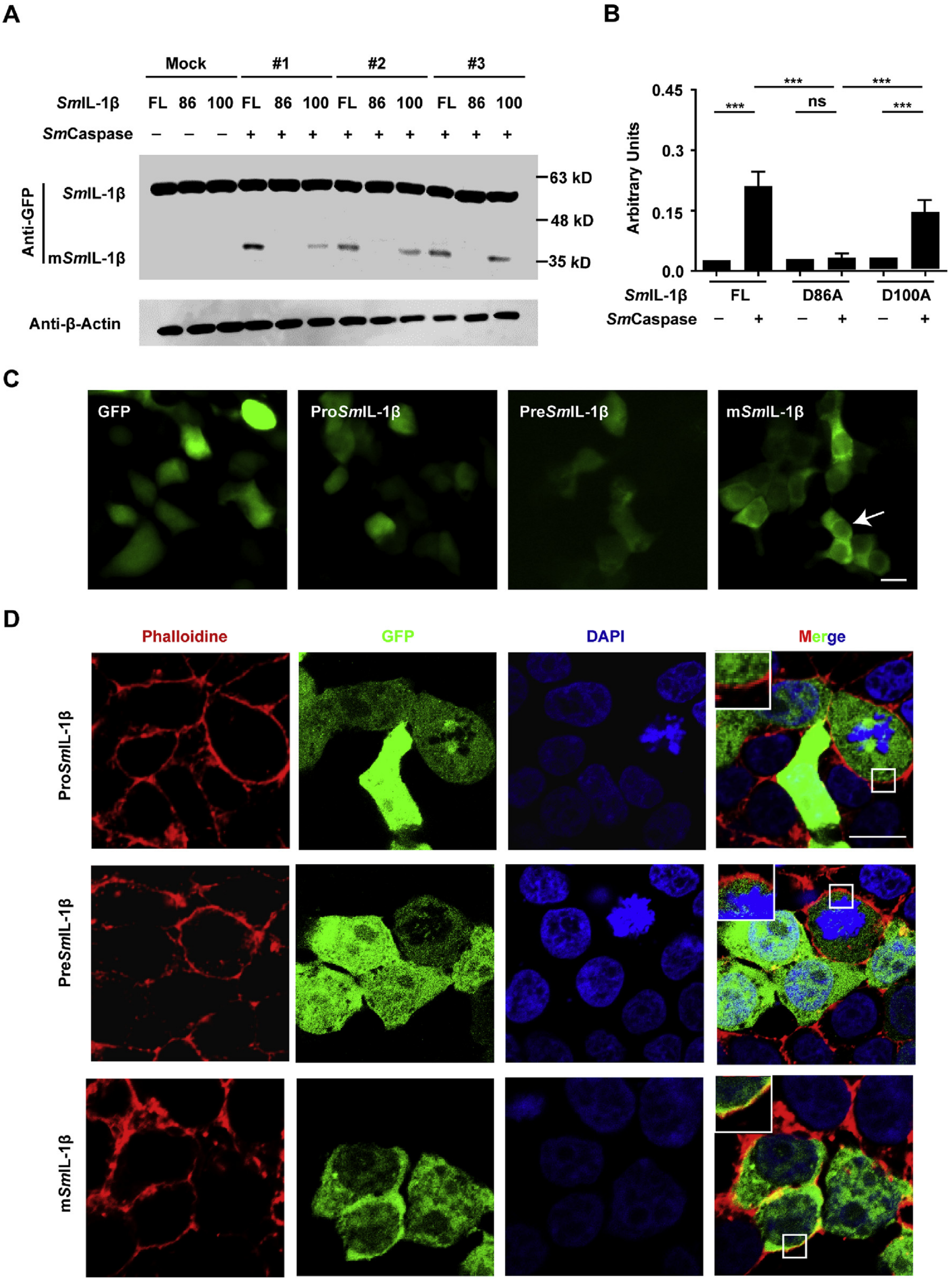

Fig. 2. *Sm*IL-1β processing enables its relocation to plasma membrane. (A) N-terminal GFP-tagged *Sm*IL-1β (FL), *Sm*IL-1β^{D86A} (86, D86A) or *Sm*IL-1β^{D100A} (100, D100A) with FLAG-tagged *Sm*Caspase or vector plasmid (Mock) were co-transfected in HEK293T cells by Lipofectamine 2000™, respectively. After 48 h, mixtures of cell lysates and supernatants were subjected to immunoblotting. #1, #2, #3 represent three independent experiments. The abbreviation mSmIL-1β represents the caspase cleavage product, **(B)** Grayscale analysis of western blot strips in (A), then calculated grayscale value ratio of m*Sm*IL-1β/*Sm*IL-1β. **(C)** HEK293T cells were transfected with plasmid pCDH-*gfp*, pCDH-*Smil-1β-gfp* (pro*Sm*IL-1β), pCDH-*Smil-1β (1–86)-gfp* (pre*Sm*IL-1β) and pCDH-*Smil-1β (87–246)-gfp* (m*Sm*IL-1β). After 20 h, cells were observed under a confocal microscope. The white arrow indicates cells with significant green fluorescence intensity difference between the nuclear and plasmic area. **(D)** Cells were transfected with plasmid as in (C), fixed with 4% paraformaldehyde (PFA) for immunofluorescence. The white box indicates the co-localized signal between IL-1β and cytomembrane. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; NS, not significant (two-tailed *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

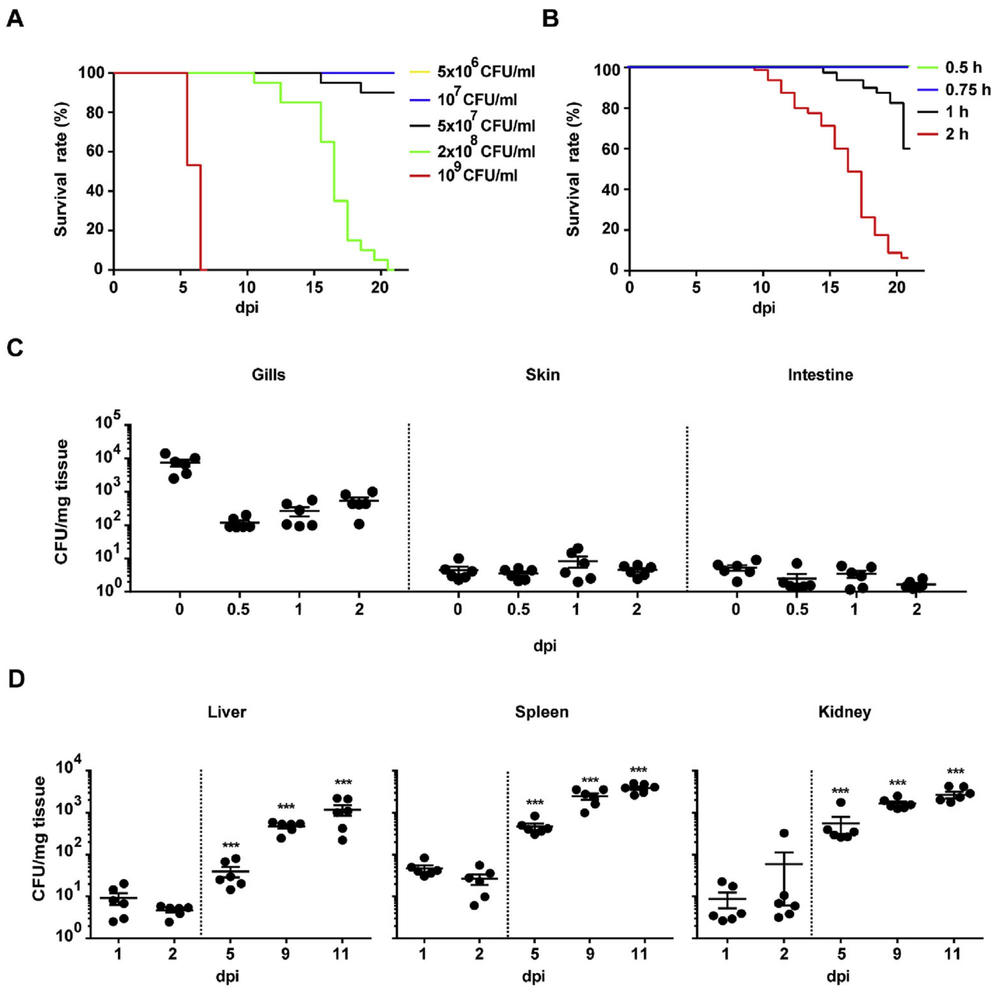

Fig. 3. Immersion infection of *E. piscicida* in turbot. (A–B) Survival rate of *S. maximus* (n = 20 per group) immersed with indicated doses (A) for 2 h or with 2×10^8 CFU/mL (repeated for three times) for indicated hours (B)·**(C–D)** Bacterial burdens of *S. maximus* immersed with 2×10^8 CFU/mL for 1 h, in mucosal sites at early time points (C) or in systemic sites at indicated time points (D). The dotted line is used to distinguish the stages of infection, of which the left and right side represent mucosal infection and systemic infection, respectively (n = 3 per group, repeated for two times). Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed *t*-test).

3. Results

3.1. Identification of *S. maximus* IL-1β

As an important component of inflammatory response in vertebrates during bacterial infection, IL-1 family proteins have been identified in many fish species [12]. Herein, three genes from *S. maximus* genome [33] were annotated and named as *Sm*IL-1β, *Sm*IL-1Fm2 and *Sm*nIL-1Fm. Alignment with other identified IL-1 family genes, we constructed the phylogenic tree (Fig. 1A), and found that *S. maximus* IL-1 genes are relatively closer to those from Japanese flounder. Unlike in catfish and rainbow trout, *Sm*IL-1Fm2 and *Sm*nIL-Fm were distinct from *Sm*IL-1β (Fig. 1A). Notably, *Sm*IL-1β was clustered with mammalian IL-1β in contrast to zebrafish IL-1β. Further, *Sm*IL-1Fm2 is clustered with seabass IL-1Fm2, suggesting that *Sm*IL-1Fm2 may be the second IL-1β in *S. maximus*. Meanwhile, the *Sm*nIL-Fm is clustered with miiuy croaker RAs, also suggesting that *Sm*nIL-Fm may be the receptor antagonist of *S. maximus*.

To better characterization the *S. maximus* IL-1 genes, we utilized the real-time PCR analysis to detect their expression patterns in seven different tissues. The results showed that *Sm*IL-1β, *Sm*IL-1Fm2 and *Sm*nIL-1Fm exhibits different expression levels in different tissues (Fig. 1B). Notably, *Sm*IL-1β mRNA levels were significantly higher than other two genes in liver, gills and skin (Fig. 1B). To further evaluate the regulatory effects of these genes during infection, we examined the transcriptional changes in spleen from *S. maximus* which injected with LPS, *E. piscicida* EIB202, *V. anguillarum* (Van) for 12 h, respectively (Fig. 1C), and found that *Sm*IL-1β could significantly response to infection compared with *Sm*IL-1Fm2 and *Sm*nIL-1Fm, which suggest that *Sm*IL-1β is essential for antibacterial responses.

3.2. *Sm*IL-1β processing enables its relocation to plasma membrane

The maturation and release of inflammatory cytokines is a critical event downstream of inflammsome activation [34]. In mammals, the IL-1β precursor can be cleaved by caspase-1 and result into the active form released to the extracellular space [13]. In our study, we expect that the *Sm*IL-1β might not be cleaved at a unique site in *S. maximus*, because when we aligned the *Sm*IL-1β with human, mouse and zebrafish IL-1β, which have confirmed caspase-1 recognition sites, *Sm*IL-1β showed a comparatively lower sequence identity (Fig. S1A). However, through homology remodeling, *Sm*IL-1β was predicted to possess a similar 3D structure with human IL-1β (Fig. S1B). To analyze the precise cleavage site of caspase-1 in *Sm*IL-1β, we speculate that Asp^{100} and Asp^{86} might be the potential cleavage sites. To test this, GFP-tagged *Sm*IL-1β was co-transfected with FLAG-tagged caspase in HEK293T cells to detect the processing, we found that mutation of Asp^{86} abolished the generation of mature fragment, whereas mutating Asp^{100} did not affect *Sm*IL-1β processing (Fig. 2A and B). Thus, the results suggest that cleavage of *Sm*IL-1β by caspase occurs at the Asp^{86}, which is not conserved within mammalian IL-1β and reported cleavage site of caspase-1 in fish IL-1β.

In contrast to the IL-1β precursor, previous study has revealed that the mature IL-1β enables its relocation from the cytosol to the plasma membrane in macrophages [16]. Thus, we stepped forward to analyze whether the *S. maximus* mature IL-1β can target to the plasma membrane or not, through overexpression of GFP-tagged pro*Sm*IL-1β and mature *Sm*IL-1β in HEK293T cells, we found that the mature *Sm*IL-1β extensively located in cytoplasm, whereas the pro*Sm*IL-1β was

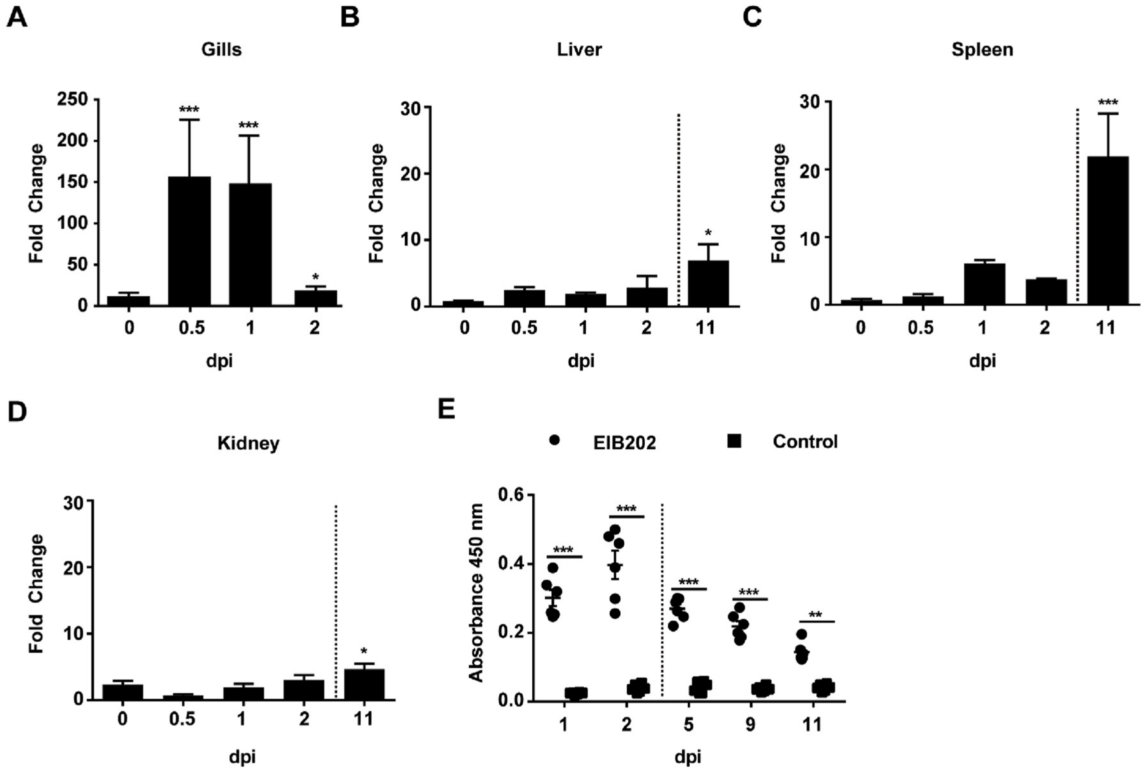

Fig. 4. Expression of *Sm*IL-1β during *E. piscicida* infection. (A–D) The upregulated expression levels of *Smil-1β* in gills at 0, 0.5, 1 and 2 dpi (A) or liver (B), spleen (C), kidney (D) at indicated time points after immersion infection of *E. piscicida* compared with uninfected controls (n = 3 per group, repeated for three times). **(E)** Sem-quantitative analysis of secreted *Sm*IL-1β in serum of fish infected with EIB202 or not at indicated days post the infection by ELISA (n = 3 per group, repeated for two times). Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed *t*-test).

distributed throughout the intracellular space including the nuclear region (Fig. 2C). Moreover, we labeled the plasma membrane with rhodamine-phalloidin, and found an apparent colocalization between mature *Sm*IL-1β and cytomembrane, but not in pro*Sm*IL-1β (Fig. 2D). These data suggest that the cleavage of *Sm*IL-1β could be mediated by caspase and enable its relocation to the plasma membrane.

3.3. Immersion infection of S. maximus by E. piscicida

To further evaluate the role of *Sm*IL-1β in response to infection, we established an immersion infection model by optimizing the duration of infection by indicated doses of *E. piscicida* (Fig. 3A and B). After 2 h immersion challenge with 2×10^8 CFU/mL *E. piscicida,* the infection caused a high accumulated mortality rate of *S. maximus*, compared with other groups (Fig. 3B), suggesting that a systemic infection could be induced. Furthermore, we detected the colonization of *E. piscicida* in mucosal sites at 0, 0.5, 1 and 2 days post the challenge. The results showed that the bacterial loads in the gills have a higher order of magnitude, compared with in skin and intestine (Fig. 3C); however, the bacterial loads in systemic sites at early time points (1 and 2 dpi) were comparatively lower (Fig. 3D). After 5-day infection, the bacterial loads were observed in systemic sites were significantly increased (Fig. 3D), but not in muscle (Fig. S2). Taken together, these results suggest that the *E. piscicida* were mainly colonized in mucosal organs at the early time points and metastasized to systemic sites later in turbot.

3.4. SmIL-1β is induced during E. piscicida infection

To better characterize the role of *Sm*IL-1β in response to *E. piscicida* infection, we take the advantage of the immersion infection above, and found that at 0.5, 1 dpi, the expression levels of *Sm*IL-1β was significantly induced in gills compared to control groups (Fig. 4A), which was consistent with higher bacterial burdens (Fig. 3C). After 11-day post immersion infection, the *Sm*IL-1β showed a significantly higher transcriptional upregulation in liver (Fig. 4B), spleen (Fig. 4C) and kidney (Fig. 4D), which was consistent with the bacterial burdens happened from gills to systemic sites (Fig. 3D). Moreover, by detecting the *Sm*IL-1β production in serum (Fig. 4E) during infection, we found that *E. piscicida* remarkably induced the secretion of *Sm*IL-1β (Fig. 4E), and interestingly, the *Sm*IL-1β production were higher at the early time points than that at the late phase (Fig. 4E), which suggest that the *Sm*IL-1β can be induced during *E. piscicida* infection.

3.5. SmIL-1β restricts the E. piscicida colonization in vivo

Recently, increasing data indicate that the maturation and secretion of mammalian IL-1β were important for pathogens clearance [35], however, whether *Sm*IL-1β plays a role in antibacterial response has not yet been elucidated. To test this, we knockdowned and overexpressed the *Sm*IL-1β respectively *in vivo* to examine its potential effects during *E. piscicida* infection. The knockdown and overexpression efficiency of IL-1β were analyzed in liver, spleen, kidney and gills at 3- and 7-day post-plasmid injection. The results showed that the expression levels of IL-1β was significantly reduced in tissues from the fish administered with pSiIL-1β compared to that control groups (Fig. 5A). Moreover, fish administered with pIL-1β had an upregulated expression of IL-1β was in indicated tissues compared with in PBS or pCN3 vector treated groups (Fig. 5B).

To further investigate the effects of *Sm*IL-1β in response to *E. piscicida* infection, we designed an infection model as shown in Fig. 6A, in which fish were immersion challenged with 2×10^8 CFU/mL *E. piscicida* for 2 h at 3-day post-plasmid administration, and found that the *Sm*IL-1β production in sera were significantly lower in pSi*Sm*IL-1β administrated fish, while in *Sm*IL-1β overexpression fish, the *Sm*IL-1β production were comparatively higher in response to *E. piscicida* infection (Fig. 6B). Consequently, the bacterial loads in gills from fish

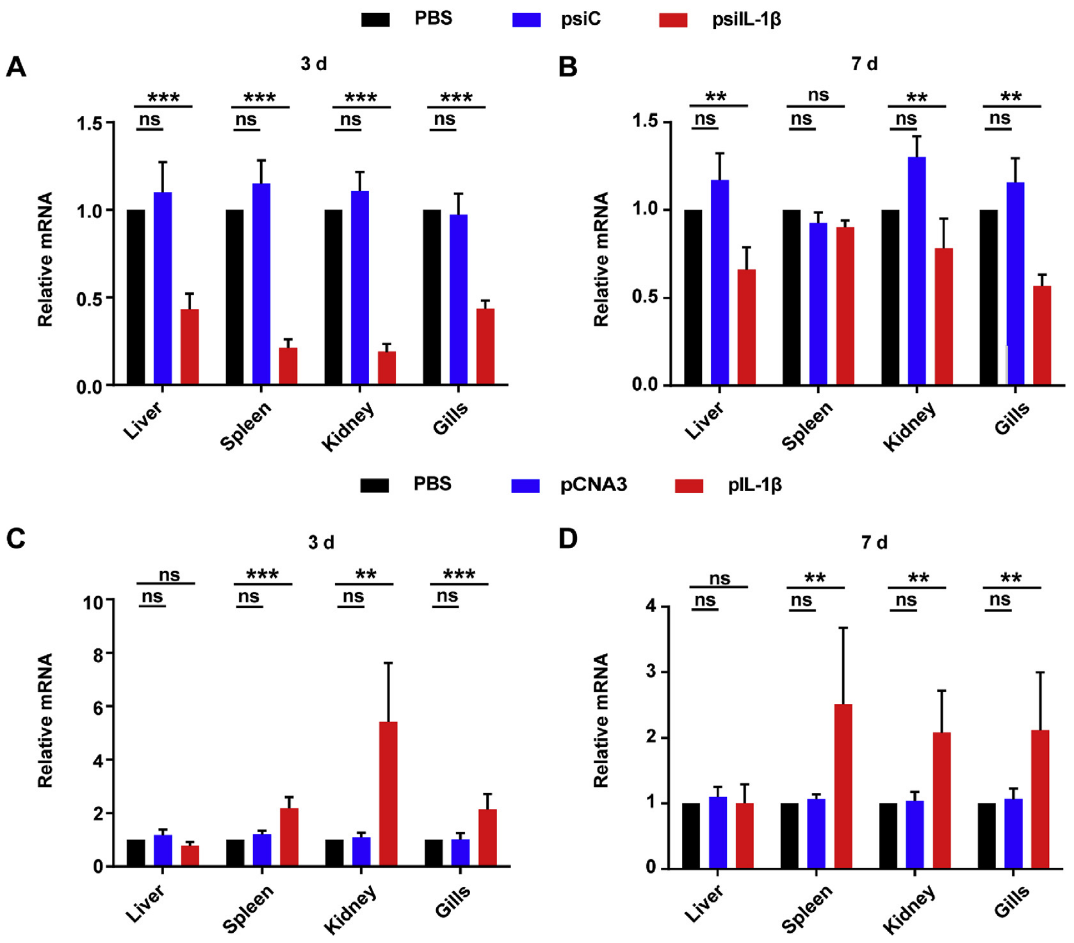

Fig. 5. Efficiency of *Smil-1β* knockdown and overexpression *in vivo*. (A and B) *S. maximus* were randomly selected and injected with PBS, pSi*Sm*IL-1β or pSiC (n = 3 per group and repeated for three times) for knockdown. At 3 (A) or 7 (B) days post-plasmid administration, liver, spleen and kidney, gills were sampled under aseptic conditions and examined for the transcription of *Smil-1β* by qRT-PCR. **(C and D)** *S. maximus* were randomly selected and injected with PBS, pCN3-*Sm*IL-1β or pCN3 for overexpression. At 3 (C) or 7 (D) days post-plasmid administration, liver, spleen and kidney, gills were sampled under aseptic conditions and examined for the transcription of *Smil-1β* by qRT-PCR. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed *t*-test).

administered with pSi*Sm*IL-1β were significantly higher compared to those in PBS-treated fish at 0, 12 hpi, whereas administration with psiC did not affect the colonization of *E. piscicida* (Fig. 6C). Conversely, bacterial loads in *Sm*IL-1β overexpression fish gills were comparable to those in PBS or pCN3 treated fish (Fig. 6C). Furthermore, bacterial loads in spleen, and kidney from *Sm*IL-1β knockdown or overexpression fish are significantly increased or reduced correspondingly, compared to those in PBS, psiC, or pCN3 treated groups, but the bacterial loads in liver were not changed (Fig. 6D). Thus, these results indicate that the *Sm*IL-1β plays critical role in restricting *E. piscicida* colonization *in vivo.*

4. Discussion

In mammals, IL-1β is released as mature form following its conversion from pro-IL-1β by proteolytic cleavage [36]. The well-known IL-1β converting enzyme is caspase-1, which cleaves the bioactive IL-1β domain at Asp^{116} [37]. The caspase-1 recognition site is present in almost all mammalian IL-1β sequences; however, this caspase-1 recognition site seems not that conserved in fish [10]. In zebrafish, IL-1β was sequentially cleaved by caspase orthologues, caspase-A and caspase-B at Asp^{104} and Asp^{112} correspondingly [17]. While in seabass, the IL-1β can be cleaved into a 18 kDa isoform by *in vitro* incubation with caspase-1, and the cleavage site is homologous to zebrafish caspase-B recognition site [22], which is presented in all vertebrate IL-1β sequences [10]. In addition to caspase-1, there are many other protease proteins that can recognize and cleave pro-IL-1β. Recent studies have demonstrated that murine caspase-11, homologous to the human caspase-4/5 and zebrafish caspase-B, also could cleave pro-IL-1β [38]. Moreover, pro-IL-1β is also reported to be processed by extracellular proteases at different sites, such as collagenase, cathepsin G or elastase [39], chymotrypsin [40], and granzyme A [41]. In this study, we revealed that the cleavage site in *Sm*IL-1β by *S. maximus* caspase is not conserved comparing with previously identified fish caspase recognition site. This results indicate that the cleavage of IL-1β is functionally conserved, but the cleavage site might not that conserved in different species, which will provide us a better understanding of innate immunity in teleost.

Mucosal immunity constitutes an important component of innate immune system and plays important role in response to pathogens infection [36]. Since fish are continuously exposed to a microbial-rich environment and reaches every epithelial barrier of their body, facing a greater challenge coping with high microbial loads compared to terrestrial animals. Thus, it is reasonable to explore the function of mucosal immunity in response to infection [36]. Here, by using an immersion challenge model, we revealed that the *E. piscicida* were mainly colonized in mucosal sites, especially in gills at early time points. Moreover, as one of the central components of inflammation response, IL-1β regulates both innate and adaptive immune mediated anti-bacterial defense in mammals [34,41], and in this study, we provided the evidence that *Sm*IL-1β expression in gills might play critical role in mucosal immunity in teleost.

In mice, the interleukin-1 receptor deficient exhibits significant

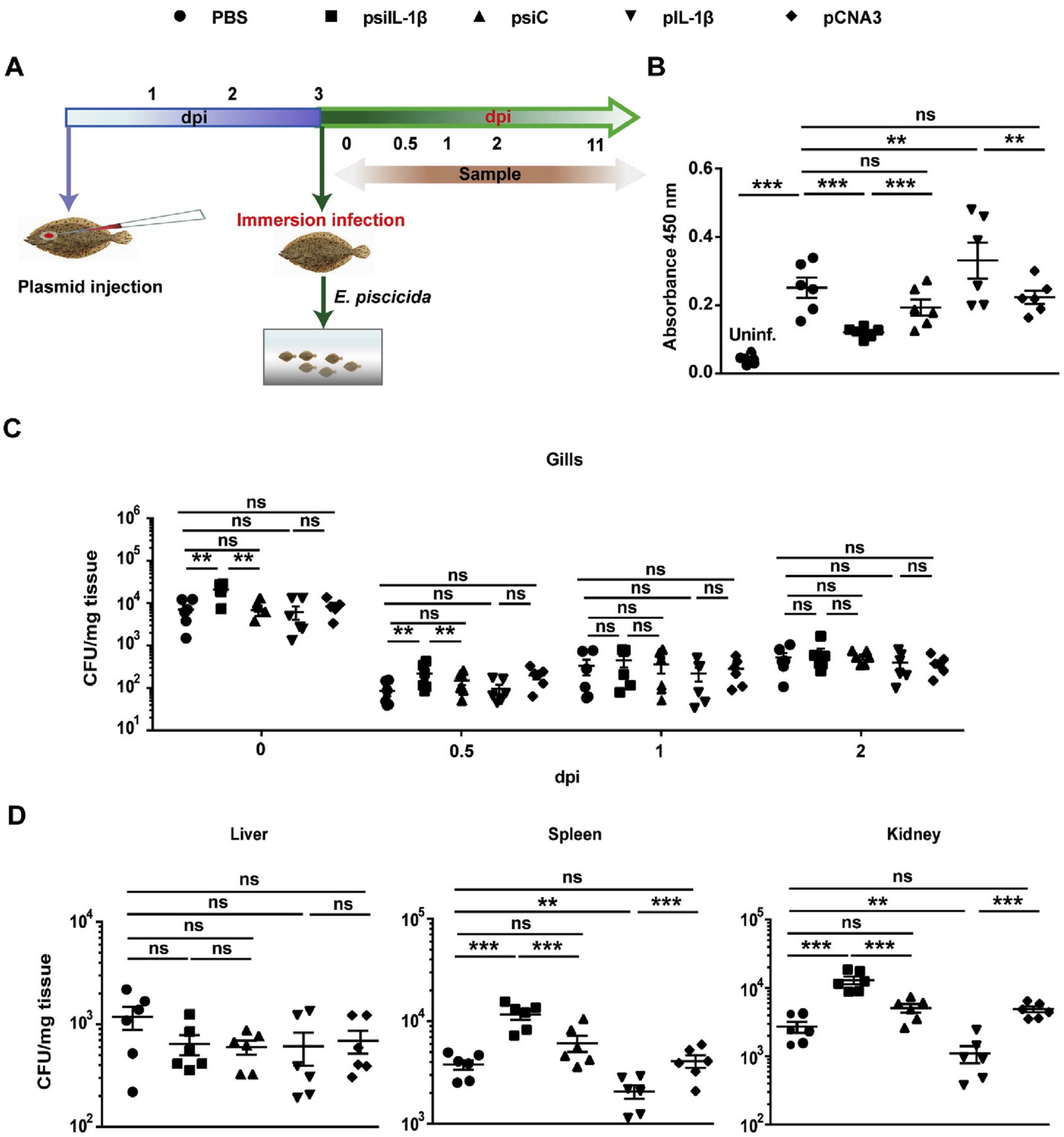

Fig. 6. *Sm*IL-1β restricts the *E. piscicida* colonization *in vivo*. (A) Diagram of *S. maximus* immersion infection model. Briefly, healthy fish were administered with psiIL-1β, psiC, pIL-1β, pCN3 or PBS (control), and at 3 days post-plasmid administration, the fish were immersion-infected with EIB202 with 2 × 10^8 CFU/mL, then liver, kidney, spleen and gills were sampled under aseptic conditions at indicated time points. **(B)** Sem-quantitative analysis of secreted serum *Sm*IL-1β in (A) at 11-day post the infection by ELISA, the uninfected group (Uninf.) as the mock. **(C)** Bacterial loads in liver, spleen, kidney at 11-day post infection. **(D)** Bacterial loads in gills at 0, 0.5, 1 and 2-day post-infection. Data are presented as mean ± SEM (n = 3 for per group and repeated for two times). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, not significant (one-way ANOVA).

defects in generating vaccine-induced protective immunity, whereas IL-18 deficient were protected compared with wild-type mice, suggest that IL-1β production governs adaptive immunity in response to *Helicobacter pylori* [42]. Moreover, *P. aeruginosa*-infected IL-1 receptor type 1 (IL-1R$^{-/-}$) deficient mice displayed a significantly decreased IL-1β production and contained comparable lower bacterial loads in lung, comparing with *P. aeruginosa*-infected wild-type mice [43]. Furthermore, intraperitoneal injection of IL-1β antibodies into wild-type mice prior to infection with *P. aeruginosa,* the bacterial loads in lungs were significantly reduced [44]. Besides, IL-1R$^{-/-}$ mice also have reduced numbers of neutrophils during the early phase of infection, and reduced macrophages during the late phase in carriage, which result into prolonged bacterial colonizations [45]. Taken together, these studies suggest that mammalian IL-1β might play an important role in anti-microbial responses through restricting the bacterial colonizations *in vivo*. In teleost, accumulating data has revealed that IL-1β can be induced by different stimuli, such as LPS, poly I:C [46–48]. There also has been abundant evidence that fish immunocytes secrete IL-1β during immune responses [49–51]. Interestingly, it was reported that *Sm*IL-1β gene showed a significant increase in expression in the kidney of the nucleotide-supplemented group compared to control [52]. Further, infection of cultivated turbot with *V. anguillarum* induced rapid synthesis and secretion of *Sm*IL-1β, which initiates the innate immune response. Movever, the vesicle-associated membrane protein-2 (VAMP-2) was found involved in regulating the exocytosis of cytokines in turbot immunocytes [53]. Herein, we uncovered the ‘mask’ of *Sm*IL-1β in restricting the colonization of *E. piscicida* in turbot gills at early time points of infection, resulting into a reduced bacterial load in systemic immune organs, including spleen and kidney. Taken together, these results will provide us a new insight into the immune mechanism of fish IL-1β in anti-bacterial infection *in vivo*, and help us better understand the evolutionary function of IL-1β in teleost.

Authors contribution

Q. L. and S.C. conceived the study; S.C. conducted the majority of experiments with help from X.M. and D.W.; Y.Z., Q.L. and D.Y. provided expert advice and critical review of the manuscript. S.C. and D.Y. wrote the manuscript; all authors discussed the results and commented on the manuscript.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31622059 to Q.L.) and the Young Elite Scientists Sponsorship Program by CAST (2016QNRC001 to D.Y.), the Fundamental Research Funds for the Central Universities, and Shanghai Engineering Research Center (19DZ2284400 to Q.L.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.050.

References

[1] C.J. March, B. Mosley, A. Larsen, D.P. Cerretti, G. Braedt, V. Price, et al., Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs, Nature 315 (1985) 641–647.

[2] C. Garlanda, C.A. Dinarello, A. Mantovani, The interleukin-1 family: back to the future, Immunity 39 (2013) 1003–1018.

[3] N. Munoz-Wolf, E.C. Lavelle, A Guide to IL-1 family cytokines in adjuvanticity, FEBS J. 285 (2018) 2377–2401.

[4] C.A. Dinarello, Immunological and inflammatory functions of the interleukin-1 family, Annu. Rev. Immunol. 27 (2009) 519–550.

[5] B.T. Vanden, D. Demon, P. Bogaert, B. Vandendriessche, A. Goethals, B. Depuydt, et al., Simultaneous targeting of IL-1 and IL-18 is required for protection against inflammatory and septic shock, Am. J. Respir. Crit. Care Med. 189 (2014) 282–291.

[6] B. Wonnenberg, M. Bischoff, C. Beisswenger, T. Dinh, R. Bals, B. Singh, et al., The role of IL-1beta in Pseudomonas aeruginosa in lung infection, Cell Tissue Res. 364 (2016) 25–29.

[7] R. Uchiyama, S. Yonehara, S. Taniguchi, S. Ishido, K.J. Ishii, H. Tsutsui, Inflammasome and fas-mediated IL-1beta contributes to Th17/Th1 cell induction in pathogenic bacterial infection in vivo, J. Immunol. 199 (2017) 1122–1130.

[8] A. Taechavasonyoo, H. Kondo, R. Nozaki, Y. Suzuki, I. Hirono, Identification of novel interleukin 1 beta family genes in Japanese flounder Paralichthys olivaceus, Fish Shellfish Immunol. 34 (2013) 393–396.

[9] Q. Yang, Q. Chu, X. Zhao, T. Xu, Characterization of IL-1beta and two types of IL-1 receptors in miiuy croaker and evolution analysis of IL-1 family, Fish Shellfish Immunol. 63 (2017) 165–172.

[10] S. Bird, J. Zou, T. Wang, B. Munday, C. Cunningham, C.J. Secombes, Evolution of interleukin-1beta, Cytokine Growth Factor Rev. 13 (2002) 483–502.

[11] D. Angosto, J. Montero, A. Lopez-Munoz, F. Alcaraz-Perez, S. Bird, E. Sarropoulou, et al., Identification and functional characterization of a new IL-1 family member, IL-1Fm2, in most evolutionarily advanced fish, Innate Immun. 20 (2014) 487–500.

[12] N.V. Ogryzko, S.A. Renshaw, H.L. Wilson, The IL-1 family in fish: swimming through the muddy waters of inflammasome evolution, Dev. Comp. Immunol. 46 (2014) 53–62.

[13] J.M. Platnich, D.A. Muruve, NOD-like receptors and inflammasomes: a review of their canonical and non-canonical signaling pathways, Arch. Biochem. Biophys. 670 (2019) 4–14.

[14] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Mol. Cell 10 (2012) 417–426.

[15] S. Kesavardhana, T.D. Kanneganti, Mechanisms governing inflammasome activation, assembly and pyroptosis induction, Int. Immunol. 29 (2017) 201–210.

[16] M. Monteleone, A.C. Stanley, K.W. Chen, D.L. Brown, J.S. Bezbradica, J.B. von Pein, et al., Interleukin-1beta maturation triggers its relocation to the plasma membrane for gasdermin-D-dependent and -independent secretion, Cell Rep. 24 (2018) 1425–1433.

[17] J.Y. Li, K. Gao, T. Shao, D.D. Fan, C.B. Hu, C.C. Sun, et al., Characterization of an NLRP1 inflammasome from zebrafish reveals a unique sequential activation mechanism underlying inflammatory caspases in ancient vertebrates, J. Immunol. 201 (2018) 1946–1966.

[18] D. Angosto, G. Lopez-Castejon, A. Lopez-Munoz, M.P. Sepulcre, M. Arizcun, J. Meseguer, et al., Evolution of inflammasome functions in vertebrates: inflammasome and caspase-1 trigger fish macrophage cell death but are dispensable for the processing of IL-1beta, Innate Immun. 18 (2012) 815–824.

[19] Z.L. Mo, J. Li, G.Y. Li, P. Xiao, Phenotypic characterization, virulence, and immunogenicity of *Edwardsiella tarda* LSE40 aroA mutant, Appl. Microbiol. Biotechnol. 97 (2013) 6325–6335.

[20] M. Varela, A. Romero, S. Dios, M. van der Vaart, A. Figueras, A.H. Meijer, et al., Cellular visualization of macrophage pyroptosis and interleukin-1beta release in a viral hemorrhagic infection in zebrafish larvae, J. Virol. 88 (2014) 12026–12040.

[21] L.N. Vojtech, N. Scharping, J.C. Woodson, J.D. Hansen, Roles of inflammatory caspases during processing of zebrafish interleukin-1beta in *Francisella noatunensis* infection, Infect. Immun. 80 (2012) 2878–2885.

[22] M.I. Reis, V.A. Do, P.J. Pereira, J.E. Azevedo, S.N. Dos, Caspase-1 and IL-1beta processing in a teleost fish, PLoS One 7 (2012) e50450.

[23] Q. Yang, Q. Chu, X. Zhao, T. Xu, Characterization of IL-1beta and two types of IL-1 receptors in miiuy croaker and evolution analysis of IL-1 family, Fish Shellfish Immunol. 63 (2017) 165–172.

[24] T. Abayneh, D.J. Colquhoun, H. Sørum, *Edwardsiella piscicida* sp. nov., a novel species pathogenic to fish, J. Appl. Microbiol. 114 (2013) 644–654.

[25] S. Shao, Q. Lai, Q. Liu, H. Wu, J. Xiao, Z. Shao, Q. Wang, Y. Zhang, Phylogenomics characterization of a highly virulent *Edwardsiella* strain ET080813(T) encoding two distinct T3SS and three T6SS gene clusters: propose a novel species as *Edwardsiella anguillarum* sp. nov, Syst. Appl. Microbiol. 38 (2015) 36–47.

[26] H.X. Xie, J.F. Lu, N. Rolhion, D.W. Holden, P. Nie, Y. Zhou, et al., *Edwardsiella tarda*-Induced cytotoxicity depends on its type III secretion system and flagellin, Infect. Immun. 82 (2014) 3436–3445.

[27] W. Xu, Z. Gu, L. Zhang, Y. Zhang, Q. Liu, D. Yang, *Edwardsiella piscicida* virulence effector trxlp promotes the NLRC4 inflammasome activation during infection, Microb. Pathog. 123 (2018) 496–504.

[28] H. Chen, D. Yang, F. Han, J. Tan, L. Zhang, J. Xiao, et al., The bacterial T6SS effector EvpP prevents NLRP3 inflammasome activation by inhibiting the Ca(2+)-dependent MAPK-Jnk pathway, Cell Host Microbe 21 (2017) 47–58.

[29] Q. Wang, M. Yang, J. Xiao, H. Wu, X. Wang, Y. Lv, et al., Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches, PLoS One 4 (2009) e7646.

[30] P. Bao, X. Sun, Q. Liu, Y. Zhang, X. Liu, Synergistic effect of a combined live *Vibrio anguillarum* and *Edwardsiella piscicida* vaccine in turbot, Fish Shellfish Immunol. 88 (2019) 84–90.

[31] J.J. Wang, L. Sun, *Edwardsiella tarda*-regulated proteins in Japanese flounder (*Paralichthys olivaceus*): identification and evaluation of antibacterial potentials, J. Proteomics. 124 (2015) 1–10.

[32] M. Guo, X. Tang, X. Sheng, J. Xing, W. Zhan, The effects of IL-1beta, IL-8, G-CSF and TNF-alpha as molecular adjuvant on the immune response to an *E. tarda* subunit vaccine in flounder (Paralichthys olivaceus), Fish Shellfish Immunol. 77 (2018) 374–384.

[33] A. Figueras, D. Robledo, A. Corvelo, M. Hermida, P. Pereiro, J.A. Rubiolo, et al., Whole genome sequencing of turbot (*Scophthalmus maximus*; Pleuronectiformes): a fish adapted to demersal life, DNA Res. 23 (2016) 181–192.

[34] C. Garlanda, C.A. Dinarello, A. Mantovani, The interleukin-1 family: back to the future, Immunity 39 (2013) 1003–1018.

[35] I. Jorgensen, M. Rayamajhi, E.A. Miao, Programmed cell death as a defence against infection, Nat. Rev. Immunol. 17 (2017) 151–164.

[36] S. Koshio, Immunotherapies targeting fish mucosal immunity - current knowledge and future perspectives, Front. Immunol. 6 (2015) 643.

[37] D.K. Miller, J.M. Ayala, L.A. Egger, S.M. Raju, T.T. Yamin, G.J. Ding, et al., Purification and characterization of active human interleukin-1 beta-converting enzyme from THP.1 monocytic cells, J. Biol. Chem. 268 (1993) 18062–18069.

[38] N. Kayagaki, M.T. Wong, I.B. Stowe, S.R. Ramani, L.C. Gonzalez, S. Akashi-Takamura, et al., Noncanonical inflammasome activation by intracellular LPS independent of TLR4, Science 341 (2013) 1246–1249.

[39] D.J. Hazuda, J. Strickler, F. Kueppers, P.L. Simon, P.R. Young, Processing of precursor interleukin 1 beta and inflammatory disease, J. Biol. Chem. 265 (1990) 6318–6322.

[40] R.A. Black, S.R. Kronheim, M. Cantrell, M.C. Deeley, C.J. March, K.S. Prickett, et al., Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor, J. Biol. Chem. 263 (1998) 9437–9442.

[41] N. Munoz-Wolf, E.C. Lavelle, A Guide to IL-1 family cytokines in adjuvanticity, FEBS J. 285 (2018) 2377–2401.

[42] I. Hitzler, A. Sayi, E. Kohler, D.B. Engler, K.N. Koch, W.D. Hardt, et al., Caspase-1 has both proinflammatory and regulatory properties in *Helicobacter* infections, which are differentially mediated by its substrates IL-1beta and IL-18, J. Immunol. 188 (2012) 3594–3602.

[43] M.J. Schultz, A.W. Rijneveld, S. Florquin, C.K. Edwards, C.A. Dinarello, T. van der Poll, Role of interleukin-1 in the pulmonary immune response during *Pseudomonas aeruginosa* pneumonia, Am. J. Physiol. Lung Cell Mol. Physiol. 282 (2002) 285–290.

[44] J. Palomo, T. Marchiol, J. Piotet, L. Fauconnier, M. Robinet, F. Reverchon, et al., Role of IL-1beta in experimental cystic fibrosis upon *P. aeruginosa* infection, PLoS One 9 (2014) e114884.

[45] J.K. Lemon, M.R. Miller, J.N. Weiser, Sensing of interleukin-1 cytokines during *Streptococcus pneumoniae* colonization contributes to macrophage recruitment and bacterial clearance, Infect. Immun. 83 (2015) 3204–3212.

[46] M. Seppola, A.N. Larsen, K. Steiro, B. Robertsen, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.), Mol. Immunol. 45 (2008) 887–889.

[47] J. Zou, P.S. Grabowski, C. Cunningham, C.J. Secombes, Molecular cloning of interleukin 1beta from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ice cut site, Cytokine 11 (1999) 552–560.

[48] G. Scapigliati, F. Buonocore, S. Bird, J. Zou, P. Pelegrin, C. Falasca, et al., Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1beta, Fish Shellfish Immunol. 11 (2001) 711–726.

[49] C.J. Secombes, S. Bird, C. Cunningham, J. Zou, Interleukin-1 in fish, Fish Shellfish

Immunol. 9 (1999) 335–343.

[50] P. Pelegrin, E. Chaves-Pozo, V. Mulero, J. Meseguer, Production and mechanism of secretion interleukin-1b from the marine fish gilthead seabream, Dev. Comp. Immunol. 28 (2004) 229–237.

[51] E. Chaves-Pozo, P. Pelegrin, J. Garcia-Castillo, A. Garcia-Ayala, V. Mulero, J. Meseguer, Acidophilic granulocytes of the marine fish gilthead seabream (*Sparus aurata* L.) produce interleukin-1b following infection with *Vibrio anguillarum*, Cell Tissue Res. 316 (2004) 189–195.

[52] Y. Chai, X. Huang, B. Cong, S. Liu, K. Chen, G. Li, et al., Involvement of VAMP-2 in exocytosis of IL-1 beta in turbot (*Scophthalmus maximus*) leukocytes after *Vibrio anguillarum* infection, Biochem. Biophys. Res. Commun. 342 (2006) 509–513.

[53] C. Low, S. Wadsworth, C. Burrells, C.J. Secombes, Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet, Aquaculture 221 (2003) 23–40.