Fish and Shellfish Immunology 86 (2019) 947–955

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Characterization and immunologic functions of the macrophage migration inhibitory factor from Japanese sea bass, *Lateolabrax japonicus*

Feng Xu[a], Yu-Hong Shi[a], Jiong Chen[a,b,*]

[a] Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Meishan Campus, Ningbo University, Ningbo, 315832, China
[b] Key Laboratory of Applied Marine Biotechnology of Ministry of Education, Meishan Campus, Ningbo University, Ningbo, 315832, China

ARTICLE INFO

Keywords:
Cell migration
Japanese sea bass
Macrophage migration inhibitory factor
Monocyte/macrophage functions
mRNA expression
Survival rate

ABSTRACT

Macrophage migration inhibitory factor (MIF) is a cytokine playing critical roles in inflammatory and immune responses. However, its functions have not been well studied in fish. In this study, we identified a MIF molecule from Japanese sea bass (*Lateolabrax japonicus*; LjMIF). Multiple sequence alignment showed that LjMIF has the typical structural features of MIFs. Phylogenetic tree analysis revealed that LjMIF is most closely related to the yellowfin tuna (*Thunnus albacares*), large yellow croaker (*Larimichthys crocea*), and red drum (*Sciaenops ocellatus*) homologs. Constitutive mRNA expression of LjMIF was detected in all tested tissues, with the highest level in the liver. Upon *Vibro harveyi* infection, LjMIF transcripts were altered in the tested tissues, including the liver, spleen, and head kidney. Subsequently, we prepared recombinant LjMIF (rLjMIF) and the corresponding antibody (anti-LjMIF). The *in vitro* study showed that rLjMIF inhibited the trafficking of Japanese sea bass monocytes/macrophages (MO/MΦ) and lymphocytes, but not of neutrophils, while anti-LjMIF had the opposite effect. rLjMIF also enhanced phagocytosis and intracellular killing of *V. harveyi* by MO/MΦ, while anti-LjMIF only inhibited phagocytosis by MO/MΦ. The *in vivo* study showed that rLjMIF aggravated the course of *V. harveyi* infection in Japanese sea bass, but anti-LjMIF increased the survival rate of the fish and decreased the bacterial burden. In conclusion, our observation revealed that LjMIF is closely involved in the immune responses of Japanese sea bass for combating *V. harveyi* infection.

1. Introduction

The macrophage migration inhibitory factor (MIF) was first identified as a cytokine that is released from T cells and described as an inhibitor of the random migration of macrophages from guinea pig (*Cavia porcellus*) [1,2]. MIF is an evolutionarily highly conserved molecule that contains 115 amino acids and exists as a trimer [3]. In mammals, MIF is expressed in various cell types, including T and B cells, monocytes, macrophages, and all immune-relevant tissues (*e.g.* lung and digestive epithelia and skin), and is commonly stored in cytoplasmic pools [4]. As a pleiotropic protein, MIF is involved in numerous cellular processes, such as glucose and lipid metabolism [5], neurological diseases [6], tumor angiogenesis [7–9], and autoimmune diseases [10,11]. MIF is also a key cytokine responsible for controlling the response to both parasitic and bacterial infections [12,13].

MIF has been identified in many fish species, such as green-spotted pufferfish (*Tetraodon nigroviridis*), rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), red drum (*Sciaenops ocellatus*), European sea bass (*Dicentrarchus labrax*), and large yellow croaker (*Larimichthys crocea*) [14–18]. Most fish MIFs encode a small protein of 115 amino acids. It appears that the genetic organization of fish MIFs is similar to that of mammalian MIFs, and the secondary and tertiary structures are well conserved in both mammalian and fish MIFs [19,20]. MIF is commonly found in healthy fish tissues and organs, and its tissue expression pattern differs among different fish species [14,17,20]. Lipopolysaccharide (LPS) challenge or bacterial and viral infection alter the mRNA expression of MIF [14,16–18]. Moreover, the biological functions of MIF were determined in some teleost species [14,16,20–22]. MIF inhibited the migration of fish head kidney-derived monocytes and lymphocytes [14,16], stimulated the expression of proinflammatory cytokines in head kidney cells from rock bream [20], and decreased the bacterial burden [16]. Fish MIF not only regulates immune cell trafficking and pathogen-induced immune response, but is also closely related to some physiological processes. For example, MIF has been found to influence the normal embryonic development in zebrafish [23], and the MIF

* Corresponding author. Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Meishan Campus, Ningbo University, Ningbo, 315832, China.
E-mail address: jchen1975@163.com (J. Chen).

https://doi.org/10.1016/j.fsi.2018.12.042
Received 1 November 2018; Received in revised form 16 December 2018; Accepted 21 December 2018
Available online 23 December 2018
1050-4648/

signal pathway is required for both sensory hair cell and sensory neuronal cell survival in the ear [24–26].

Japanese sea bass, *Lateolabrax japonicus*, is a commercially important fish species in China [27]. With the enlargement of marine fish farming scale, a variety of infectious diseases have become increasingly frequent, leading to a serious decline in the output and economic losses [28]. The way to improve the growth and disease resistance of fish under poor environment became a research hotspot [29,30]. Studying the immune response of Japanese sea bass will provide a better understanding of the immune response to antigenic substances and its mechanisms and may help to develop better disease management strategies in fish farming under harsh environments. Given the important roles of MIF in the inflammatory response, studying MIF and its relationship with immunity and investigating the possible role in pathological processes in fish are worthwhile. In the present study, we identified a Japanese sea bass MIF (LjMIF) and detected the relationship between LjMIF mRNA expression and *V. harveyi* infection. Moreover, we determined the effect of LjMIF on the regulation of immune cell trafficking and monocyte/macrophage (MO/MΦ) functions *in vitro*. The effect of LjMIF on the survival rate and bacterial burden of *V. harveyi*-infected Japanese sea bass were also investigated.

2. Materials and methods

2.1. Fish rearing

Japanese sea bass with a body weight of 80–100 g were obtained from a commercial farm in Xiangshan, Ningbo, China. Fish were transferred into experimental tanks at 27–28 °C, along with artificial seawater (20‰ of salinity), and acclimatized to laboratory conditions for two weeks prior to experimental use. All fish were healthy and showed no pathological signs. All experiments were conducted in accordance with the Experimental Animal Management Law of China and were approved by the Animal Ethics Committee of Ningbo University.

2.2. LjMIF sequence characterization

The cDNA sequence of LjMIF was retrieved from transcriptome data of Japanese sea bass by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and authenticated by further cloning and sequencing. The cleavage site of signal peptides was predicted using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments were performed using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/), and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 [31]. Sequences used in this study are listed in Table 1.

2.3. In vivo bacterial challenge and expression analysis of LjMIF mRNA

V. harveyi (ATCC33866) were grown in Tryptic Soy Broth (TSB) medium at 28 °C, with constant shaking at 200 rpm for 12 h, and collected at logarithmic growth phase. Japanese sea bass were infected by intraperitoneal (ip) injection of 5×10^6 colony-forming units (CFU) *V. harveyi* (in 100 μl PBS) per fish for the infected group, according to the determined 50% lethal dose (LD_{50}) in 72 h, while PBS was used for the control group. Fish were sacrificed at 0, 6, 12, 24, 36, and 48 h post infection (hpi), and the liver, spleen, and head kidney were collected for total RNA extraction, as described before [32]. The RNA of healthy fish tissues, including the muscle, skin, brain, gill, spleen, trunk kidney, head kidney, liver, and intestine, were also extracted for tissue expression pattern analysis. Real-time quantitative PCR (qPCR) was performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA), using SYBR premix Ex Taq II (TaKaRa), as follows: (1) 40 cycles of amplification at 95 °C for 30 s and 60 °C for 20 s; (2) melting curve analysis at 95 °C for 5 s, 65 °C for 15 s, and 95 °C for 15 s, and (3) cooling at 40 °C for 30 s. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with LjMIF initially normalized against Lj18S rRNA. The primers used are listed in Table 2. The experiment was repeated four times, and each qPCR was performed in triplicate.

Table 1
MIF sequences used for multiple sequence alignment and phylogenetic tree analysis.

Accession number	Species	
	Latin name	English name
MK000388	*Lateolabrax japonicus*	Japanese sea bass
FJ447490	*Thunnus albacares*	yellowfin tuna
FJ404723	*Larimichthys crocea*	large yellow croaker
FN582353	*Dicentrarchus labrax*	European sea bass
NM_001309981	*Fundulus heteroclitus*	mummichog
XM_004073247	*Oryzias latipes*	Japanese ricefish
BT075514	*Osmerus mordax*	rainbow smelt
NM_001043321	*Danio rerio*	zebrafish
BT080024	*Esox lucius*	northern pike
NM_001123609	*Salmo salar*	Atlantic salmon
NM_001124581	*Oncorhynchus mykiss*	rainbow trout
GU988719	*Epinephelus coioides*	orange spotted grouper
EU368584	*Cyprinus carpio*	common carp
KY612294	*Ctenopharyngodon idella*	grass carp
KF888661	*Ictalurus punctatus*	channel catfish
NM_001032717	*Takifugu rubripes*	tiger puffer
BT082851	*Anoplopoma fimbria*	sablefish
XM_020927268	*Boleophthalmus pectinirostris*	mudskipper
AY856134	*Tetraodon nigroviridis*	green-spotted pufferfish
JX273154	*Oplegnathus fasciatus*	rock bream
FJ447489	*Sciaenops ocellatus*	red drum
BC156031	*Xenopus tropicalis*	tropical clawed frog
BC097727	*Xenopus laevis*	African clawed frog
XM_024195529	*Terrapene mexicana triunguis*	three toed box turtle
NM_001305091	*Gallus gallus*	chicken
NM_010798	*Mus musculus*	mouse
NM_001077213	*Sus scrofa*	pig
NM_001033608	*Bos taurus*	cattle
CR541651	*Homo sapiens*	human

2.4. Isolation of peripheral blood MO/MΦ, neutrophils, and lymphocytes

Cells were isolated from the caudal vein blood of healthy Japanese sea bass according to a previously described method [33]. Briefly, heparinized blood was collected, and cells were isolated following sedimentation with 6% dextran T 500 (Sigma, St. Louis, USA). After a low-speed centrifugation at 400*g* for 25 min, the cells packed below Ficoll-Hypaque PREMIUM (*i.e.* erythrocytes and neutrophils) were subjected to hypotonic lysis with ice-cold ACK (Ammonium-Chloride-Potassium) Lysis Buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA) to eliminate the red blood cells. The resulting neutrophil suspension was washed and suspended in RPMI 1640 medium. The buffy layer above Ficoll-Hypaque PREMIUM was collected and washed, and the number of cells was determined using a hemocytometer. Cells were cultured in 35-mm dishes for 12 h, and non-adherent lymphocytes and adherent MO/MΦ were carefully collected.

2.5. Prokaryotic expression and antibody preparation

The full-length coding region of the LjMIF gene was amplified using the primer pair LjMIF-P (F) and LjMIF-P (R) (Table 2). Following digestion with *Eco*RI and *Xho*I (NEB), the amplicon was cloned into the pET-28a (+) expression vector, and the constructed plasmid was subsequently transformed into *Escherichia coli* BL21 (DE3). The expression of recombinant LjMIF protein (rLjMIF) was induced by isopropyl-β-D-thiogalactopyranoside (IPTG), and the protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (QIAGEN, Shanghai, China). Purified rLjMIF was used as an antigen to immunize mice to

Table 2
Oligonucleotide primers used in this work.

Primer	Gene	Accession number	Nucleotide sequence (5′→3′)	Amplicon size (bp)
LjMIF-P(F)	LjMIF	MK000388	GGAATTCATGCCGATGTTCGTGGTG[a]	362
LjMIF-P(R)			GCTCGAGTCAGCCAAAGGTAGTGTTGTTC[b]	
LjMIF(F)	LjMIF	MK000388	CTGCTCCCTCCACAGTATTG	158
LjMIF(R)			TGTTGTTCCAGGCCACATTG	
Lj18S (F)	Lj18S rRNA	AB089346	TCGTGCGTGACATCAAGGAG	196
Lj18S (R)			CGCACTTCATGATGCTGTTG	

[a, b] Underlined nucleotides represent the restriction site for *Eco*RI and *Xho*I, respectively.

produce antiserum. The anti-rLjMIF IgG (anti-LjMIF) and isotype IgG (IsoIgG) were purified from serum using Protein G HP SpinTrap columns (GE healthcare, New Jersey, USA), and their concentrations were measured by the Bradford method. The specificity of the antibody was tested by western blotting and visualized using an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, USA), as previously described [32].

2.6. Primary culture of Japanese sea bass MO/MΦ

Head kidney-derived MO/MΦ were isolated from healthy Japanese sea bass and cultured using a method described previously [34]. Briefly, head kidney leukocyte-enriched fractions were obtained from the Ficoll-medium interface by using a Ficoll density gradient (1.077 ± 0.001 g/ml) (Invitrogen, Shanghai, China) and incubated overnight at 24 °C. Non-adherent cells were removed by aspiration, and adherent cells were incubated in complete medium [RPMI 1640, 5% Japanese sea bass serum, 5% fetal bovine serum (FBS), 1% penicillin/streptomycin] at 24 °C with 5% CO_2.

2.7. In vitro cell chemotaxis assay

In vitro cell chemotaxis assays were performed in a 24-well transwell chamber (Corning, NY, USA). rLjMIF in complete medium was added to the lower chambers at 0, 1, and 10 μg/ml, and MO/MΦ, neutrophils, or lymphocytes were plated on the upper chambers. The chambers were incubated for 4 h at 24 °C. Cells that migrated from the upper to the lower chambers were counted using light microscopy (Nikon, Tokyo, Japan). Each migration assay was performed in quadruplicate.

2.8. Effect of rLjMIF and of anti-LjMIF on MO/MΦ phagocytosis

V. harveyi cells in the logarithmic phase of growth were collected and labeled with fluorescein isothiocyanate (FITC) (Sigma), according to the manufacturer's protocol; the cells are hereafter designated as FITC-*V. harveyi*. Japanese sea bass MO/MΦ grown on cover slips in 6-well plates (2×10^6 cells/ml) were incubated with 1.0 or 10.0 μg/ml rLjMIF for 12 h or with 1.0 or 10.0 μg/ml anti-LjMIF for 30 min. PBS was used as control. FITC-*V. harveyi* cells were added at a MOI of 10, and the cells were further incubated for 30 min before washing extensively with sterile PBS. Trypan blue (0.4%) was used to quench the fluorescence resulting from the particles, which were either outside the cells or sticking to the cell surface. Engulfed bacteria were analyzed using a Gallios flow cytometer (Beckman Coulter, Miami, USA) and the FlowJo software. The relative mean fluorescence intensity (MFI) of rLjMIF- and PBS-treated (or anti-LjMIF and IsoIgG) groups was expressed as the fold change relative to the value of the group that had not been treated with bacteria; the value of the PBS-treated (or IsoIgG) group was assigned a unit of 100.

2.9. Effect of rLjMIF and of anti-LjMIF on bacterial killing activity of MO/MΦ

After pre-incubation with rLjMIF or anti-LjMIF as previously described, Japanese sea bass MO/MΦ were infected with live *V. harveyi* at a MOI of 10. Bacterial uptake by MO/MΦ were allowed to occur for 30 min, at 28 °C in an atmosphere of 5% CO_2. Non-internalized *V. harveyi* were removed by washing with sterile PBS. One sample set (uptake group) was lysed in 1% Triton X-100 solution and plated onto thiosulfate citrate bile salts sucrose (TCBS) agar medium to assess bacterial uptake. Another sample set (kill group) was further incubated for 1.5 h to allow for bacterial killing before cell lysis. Bacterial colony numbers on TCBS plates were counted after incubation at 28 °C for 18 h. The bacterial survival rate was determined by dividing the CFU of the kill group by that of the uptake group.

2.10. Fish survival and bacterial burden assays

Japanese sea bass were divided into two groups for survival assays. Fish were ip-injected with 1×10^4 CFU/fish live *V. harveyi* and injected with rLjMIF (10 mg/kg) 30 min after bacterial infection or with anti-LjMIF or IsoIgG (10 mg/kg) 1 h before bacterial infection. Morbidity was monitored for 9 d after challenge, and the mortality was recorded every 24 h. The Kaplan-Meier method was used to assess the survival rate. The liver, spleen, head kidney, and blood were aseptically collected at 24 hpi for bacterial burden assay. Tissue homogenates (0.1 g fresh tissue weight) and blood (0.1 ml) samples were serially diluted in sterile PBS, plated onto TCBS agar plates, and incubated for 24 h at 28 °C. CFUs were calculated by multiplying the number of colonies on the plate by the dilution factor.

2.11. Statistical analysis

Data are reported as mean ± SEM. Statistical analysis of the results was performed using One-Way Analysis of Variance (ANOVA) with the SPSS version 13.0 (SPSS Inc., Chicago, USA). *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Sequence analysis of LjMIF

The nucleotide sequence of LjMIF has been deposited in the GenBank Data Library under the accession number MK000388. The isolated sequence, 964 nucleotides (nts) in length, possessed an open reading frame (ORF) of 348 nts, which encodes a polypeptide precursor of 115 amino acids (aa) with a molecular mass of 12.4 kDa. Sequence analysis revealed that LjMIF lacks a signal peptide. Multiple alignment with other known MIF aa sequences showed that LjMIF has conserved domains similar to its mammalian counterparts. Additionally, alignment analysis showed that LjMIF is highly similar to its fish and mouse counterparts and that it has the characteristic features of MIF proteins (Fig. 1). LjMIF contains three conserved cysteine residues (C^{57}, C^{60}, and C^{81}), and the first two form a typical $C^{57}XXC^{60}$ motif. Furthermore, there are two conserved catalytic residues, P^2 and K^{33}, similarly to known MIFs (Fig. 1).

Sequence comparison showed that LjMIF shared the highest amino acid identity (97%) with the yellowfin tuna homolog. Phylogenetic tree

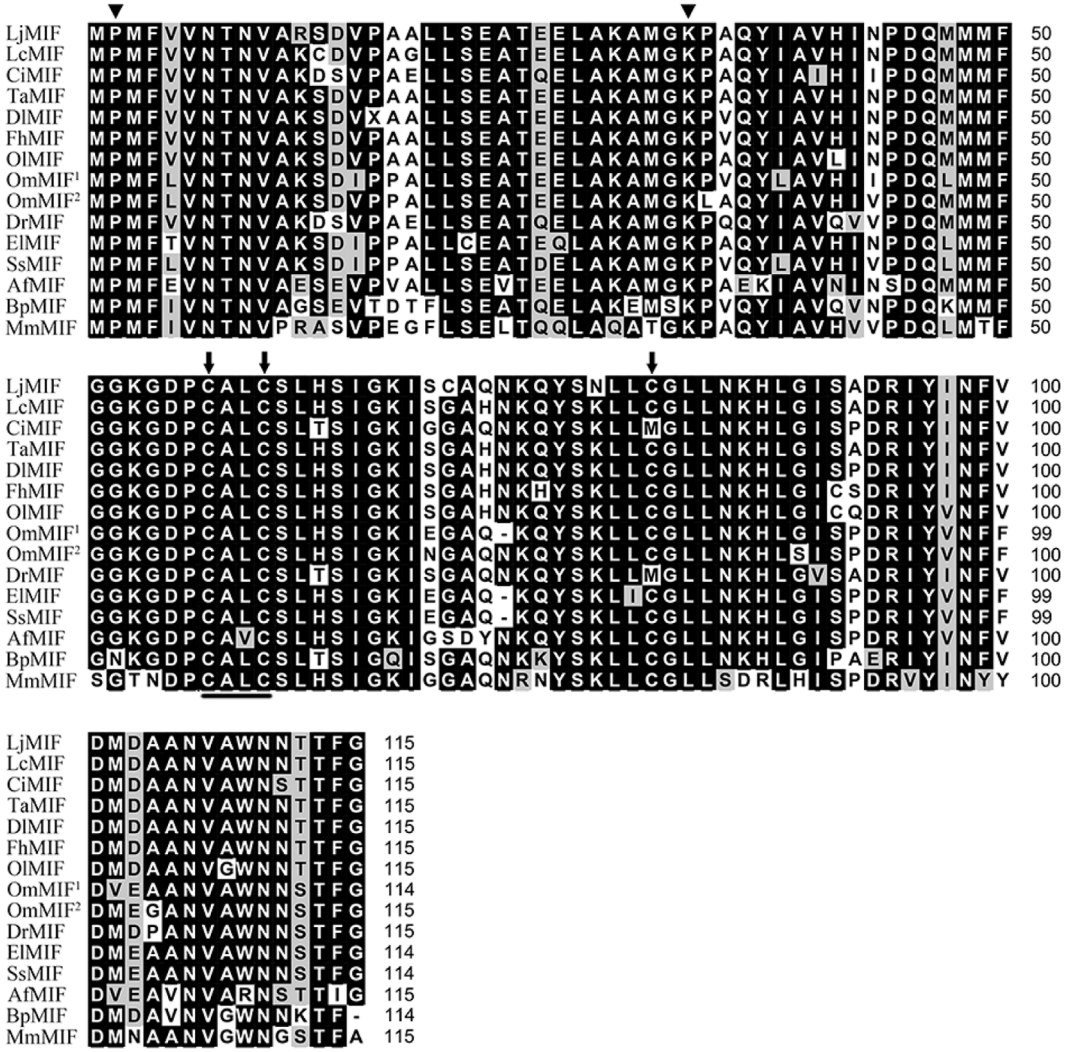

Fig. 1. Multiple alignment of the amino acid sequences of Japanese sea bass MIF with other related MIF homologs. The threshold for shading was 60%. Identical residues are highlighted in black, similar residues are highlighted in gray, and alignment gaps are marked as "-". LjMIF: Japanese sea bass MIF; LcMIF: large yellow croaker MIF; CiMIF: grass carp MIF; TaMIF: yellowfin tuna MIF; DlMIF: European sea bass MIF; FhMIF: mummichog MIF; OlMIF: Japanese ricefish MIF; OmMIF^{1}: rainbow trout MIF; OmMIF^{2}: rainbow smelt MIF; DrMIF: zebrafish MIF; ElMIF: northern pike MIF; SsMIF: Atlantic salmon MIF; AfMIF: sablefish MIF; BpMIF: mudskipper MIF; MmMIF: mouse MIF. The inverted triangles (▼) indicate the conserved residues essential for catalytic activity (P^{2}) and isomerase activity (K^{33}). The downward arrows (↓) indicate the three conserved cysteine residues involved in the oxidoreductase activity (C^{57}, C^{60}, and C^{81}). The "CXXC" motif is underlined. Species names are abbreviated on the left, and GenBank accession numbers of amino acid sequences are listed in Table 1.

analysis revealed that fish MIFs grouped together to form a distinct cluster related to the cluster of higher vertebrates (Fig. 2). LjMIF is most closely related to yellowfin tuna, large yellow croaker, and red drum homologs (Fig. 2).

3.2. Tissue LjMIF transcripts in response to V. harveyi infection

qPCR was performed to analyze the mRNA expression levels of LjMIF in Japanese sea bass tissues. The amplification efficiency of 18SrRNA and LjMIF were 0.99 and 1.01, respectively (Fig. S1). In healthy fish, LjMIF transcripts were detected in all tested tissues, including spleen, intestine, head kidney, trunk kidney, liver, and gill, with the highest expression in the liver (Fig. 3A). Upon *V. harveyi* infection, LjMIF mRNA expression was shown to be altered in the tested immune tissues, including liver, spleen, and head kidney (Fig. 3B–D). In the liver, LjMIF mRNA expression was significantly upregulated at all tested time points and peaked at 12 hpi (2.62 fold) (Fig. 3B). In the spleen, a remarkable increase in LjMIF transcript was observed at 12, 24, and 36 hpi and peaked at 24 hpi (2.27-fold) (Fig. 3C). In the head kidney, LjMIF mRNA expression was upregulated at 6 and 12 hpi and peaked at 12 hpi (4.53-fold) but returned to the control level at 24 hpi (Fig. 3D).

3.3. Preparation of recombinant LjMIF (rLjMIF) and corresponding antibody

The complete ORF sequence of LjMIF was amplified, digested, and directionally cloned into the pET28a (+) vector to construct the pET-28a-LjMIF plasmid. The plasmid was, then, transformed into *E. coli* BL21 pLysS, and protein expression was induced with IPTG. The expressed rLjMIF, of approximately 14.6 kDa (12.4 kDa LjMIF plus 2.2 kDa His-tag), was purified using a Ni^{2+}-NTA Sefinose column (Fig. 4A). Antiserum was generated by immunizing mice with purified rLjMIF. By using this antiserum, a band with the molecular mass of 12.4 kDa was recognized by western blotting in a liver sample of Japanese sea bass; this band has a molecular weight similar to that predicted from the sequence (Fig. 4B).

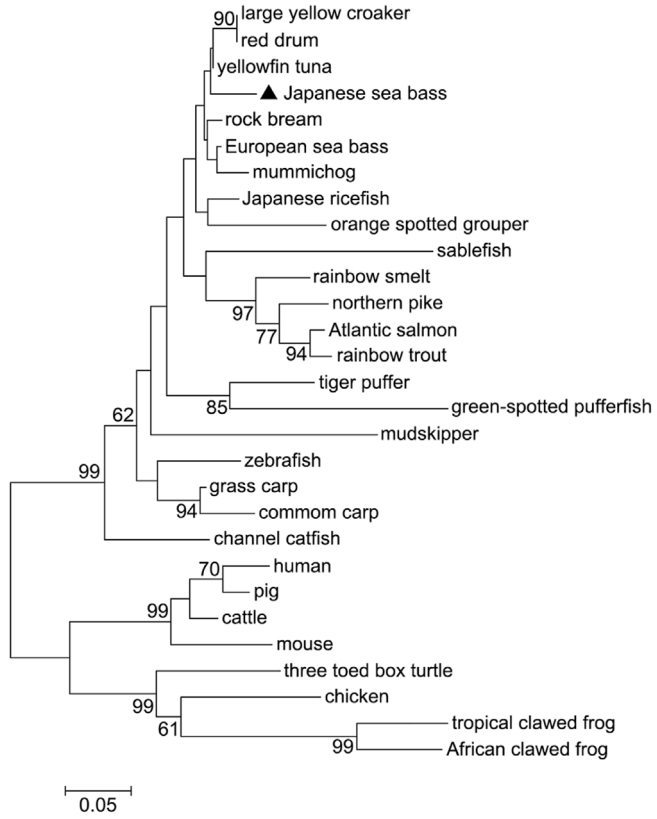

Fig. 2. Phylogenetic (neighbor-joining) analysis of the complete amino acid sequence of LjMIF with other related sequences, using the MEGA 7 program. The values at forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; shown only when > 60%). The scale bar represents the number of substitutions per base position. The site of Japanese sea bass MIF is marked with "▲". The GenBank accession numbers of the sequences used are listed in Table 1.

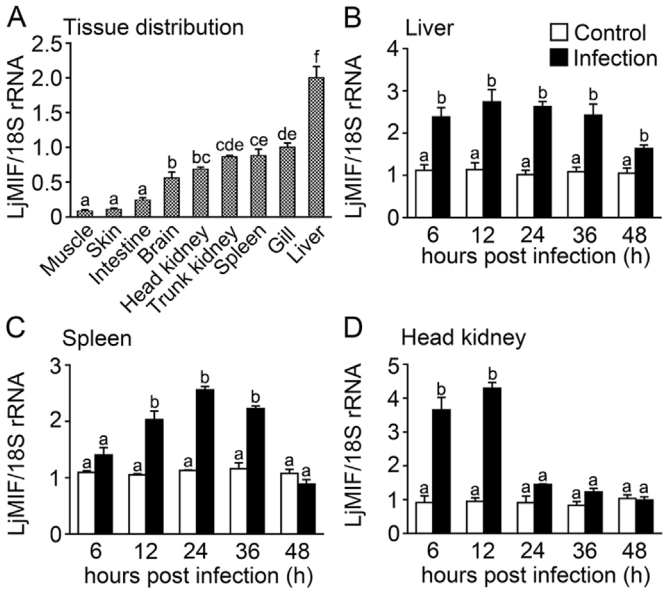

Fig. 3. qPCR analysis of LjMIF mRNA expression in different tissues. (A) LjMIF mRNA expression levels in healthy Japanese sea bass tissues. (B-D) LjMIF mRNA expression at different time points following *V. harveyi* infection (5×10^6 CFU/fish). Tissues were collected at 6, 12, 24, 36, and 48 hpi. LjMIF mRNA expression was normalized to that of Lj18S rRNA. Date are expressed as mean ± SEM. n = 4; Values denoted by different letters are significantly different when compared by ANOVA ($P < 0.05$).

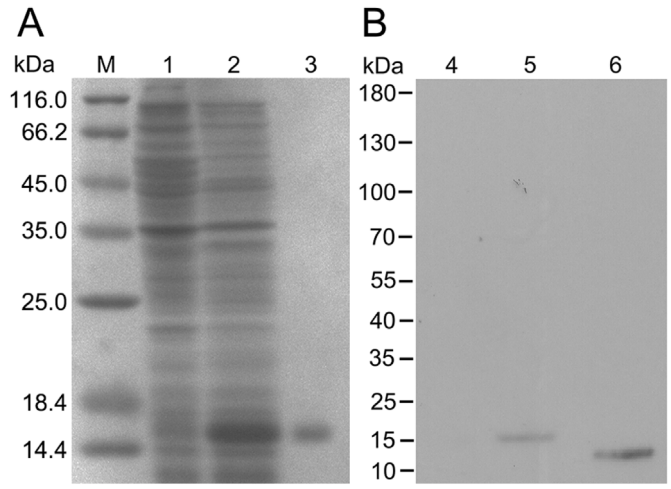

Fig. 4. Prokaryotic expression and western blot analysis of LjMIF. (A) SDS-PAGE analysis of rLjMIF. Line M: protein marker; 1 and 2: protein from *E. coli* BL21 (DE3) transformed with the pET-28a-LjMIF plasmid before and after IPTG induction, respectively; 3: purified rLjMIF. (B) Western blot analysis of rLjMIF and native LjMIF. 4: negative control; 5: purified rLjMIF; 6: Japanese sea bass total liver protein.

3.4. Effect of rLjMIF and of anti-LjMIF on cell migration

Since MIFs have been reported to inhibit the random movement of immune cells in various species [35], an *in vitro* transwell cell migration assay was conducted to test the effect of rLjMIF and of anti-LjMIF on Japanese sea bass MO/MΦ, lymphocytes, and neutrophils trafficking. The results showed that rLjMIF treatment had an inhibitory effect on the migration of MO/MΦ and lymphocytes compared with the control, while anti-LjMIF treatment increased the migration of MO/MΦ and lymphocytes (Fig. 5A–B). The higher concentrations of rLjMIF and of anti-LjMIF had a stronger effect than the lower ones. However, neither rLjMIF nor anti-rLjMIF treatment had a significant effect on the migration of neutrophils (Fig. 5C).

3.5. Effect of rLjMIF and of anti-LjMIF on the phagocytic and bacterial killing activity of head kidney-derived MO/MΦ

Since phagocytosis and intracellular killing of pathogens are the most important functions of MO/MΦ in fish innate immune response, the effects of rLjMIF and of anti-LjMIF on Japanese sea bass MO/MΦ were further investigated. Phagocytosis of FITC-*V. harveyi* by 10 μg/ml rLjMIF-pretreated MO/MΦ was significantly higher than that of the PBS group (increased by 1.42-fold) and of the 1 μg/ml rLjMIF-treated group (increased by 1.27-fold), while there was no significant difference between the latter two (Fig. 6A). Phagocytosis of FITC-*V. harveyi* by 10 μg/ml anti-LjMIF-pretreated MO/MΦ was significantly lower than that of the IsoIgG- (decreased by 0.46-fold) or 1 μg/ml anti-LjMIF-treated group (decreased by 0.43-fold); no significant difference was observed between the latter two (Fig. 6B). In addition, direct measurement of intracellular *V. harveyi* CFUs in Japanese sea bass MO/MΦ showed that the bacterial survival rate of the 10.0 μg/ml rLjMIF-treated group (20.52 ± 1.91%) was significantly lower than that of the control group (84.65 ± 2.33%) (Fig. 7A). However, there was no significant difference between the bacterial survival rate of the 10.0 μg/ml anti-LjMIF-treated group (80.61 ± 2.53%) and the control group (83.81 ± 2.57%) (Fig. 7B).

3.6. Effect of rLjMIF and of anti-LjMIF on the survival of V. harveyi-infected Japanese sea bass

We investigated the effect of rLjMIF and of anti-LjMIF on the 9-day

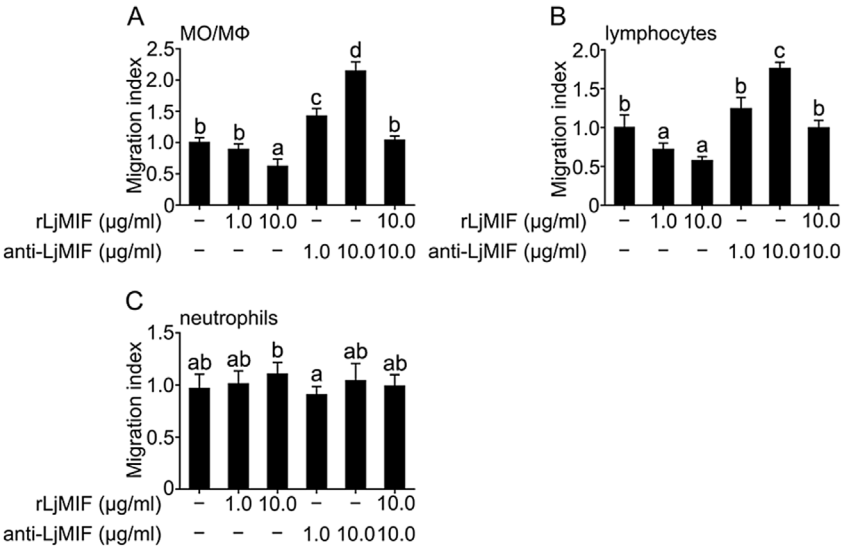

Fig. 5. Effect of rLjMIF and of anti-LjMIF on the migration of MO/MΦ (A), lymphocytes (B), and neutrophils (C) from Japanese sea bass. Cells were examined in a transwell chamber in the presence or absence of 1.0 or 10.0 μg/ml rLjMIF or anti-LjMIF serum. The migration index of the control was defined as 1.0. Each bar represents the mean ± SEM. n = 4; Values denoted by different letters are significantly different when compared by ANOVA ($P < 0.05$).

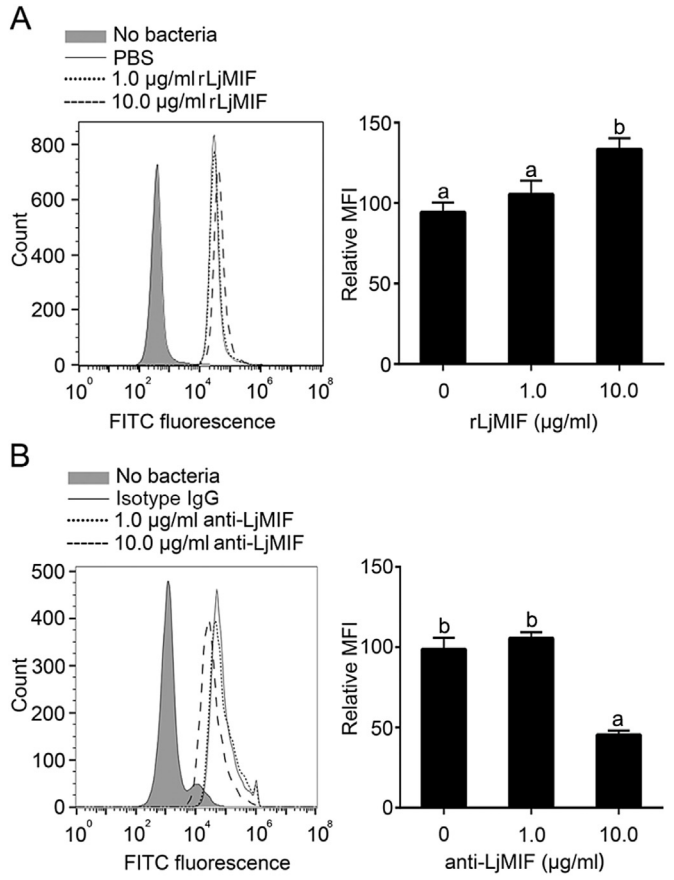

Fig. 6. Effect of rLjMIF (A) and of anti-LjMIF (B) on the phagocytic activity of Japanese sea bass MO/MΦ. MO/MΦ were pretreated with 1.0 or 10.0 μg/ml rLjMIF for 12 h or with 1.0 or 10.0 μg/ml anti-LjMIF for 30 min. After 30 min of incubation with FITC-*V. harveyi* at a MOI of 10, the effect of rLjMIF on bacteria uptake by MO/MΦ was determined using a Gallios Flow Cytometer. MFI is presented as fold-change relative to the PBS- or IsoIgG-treated group, to which a value of 100 was assigned. Data are expressed as mean ± SEM. n = 4; Values denoted by different letters are significantly different when compared by ANOVA ($P < 0.05$).

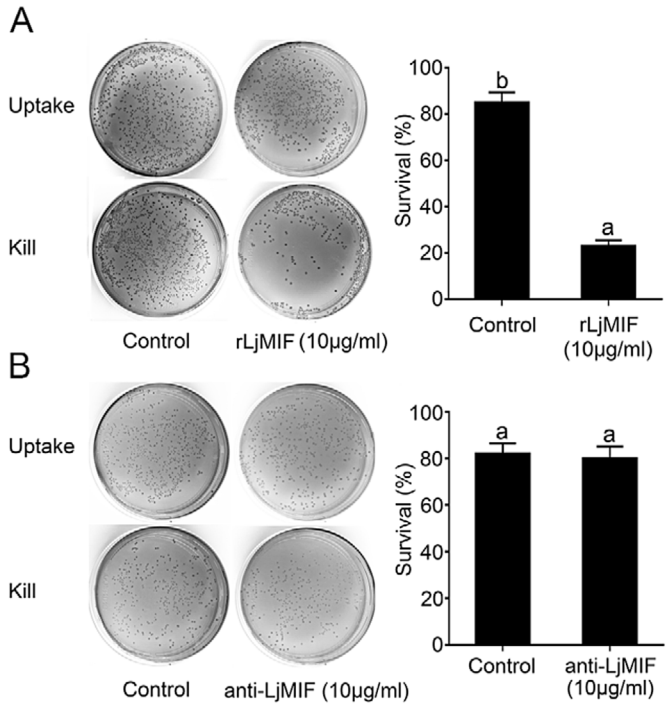

Fig. 7. Effect of rLjMIF (A) and of anti-LjMIF (B) on the bactericidal activity of Japanese sea bass MO/MΦ. MO/MΦ were pretreated with 10.0 μg/ml rLjMIF for 12 h or with 10.0 μg/ml anti-LjMIF for 30 min before live *V. harveyi* were added at a MOI of 10. Bacterial phagocytosis was allowed to proceed for 30 min, and the samples of the kill group were further incubated for 1.5 h to allow for the killing of bacteria. Histograms represent the effect of rLjMIF and of anti-LjMIF on the killing of bacteria by MO/MΦ. Data are expressed as mean ± SEM. n = 4; Values denoted by different letters are significantly different when compared by ANOVA ($P < 0.05$).

survival rate of *V. harveyi*-infected Japanese sea bass. Compared with the IsoIgG-treated group, treatment with anti-LjMIF improved fish survival upon *V. harveyi* infection, but treatment with rLjMIF accelerated the death of bacteria-infected fish (Fig. 8). Fish in the anti-LjMIF-treated group achieved a survival rate of approximately 53.3% by day 9 post infection (pi), while fish in IsoIgG-treated group were all dead by day 8 pi (Fig. 8).

3.7. Effect of rLjMIF and of anti-LjMIF on the bacterial load of *V. harveyi*-infected Japanese sea bass

To determine the effect of rLjMIF and of anti-LjMIF on the *in vivo*

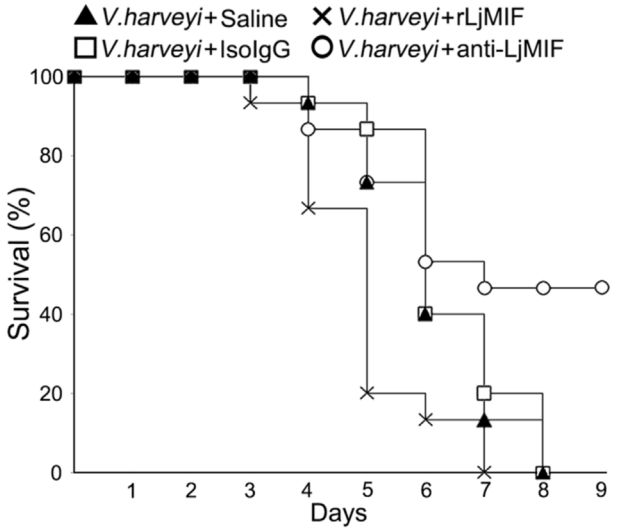

Fig. 8. Effect of rLjMIF and of anti-LjMIF on the survival rate of *V. harveyi*-infected Japanese sea bass. Fish were ip-injected with *V. harveyi* (1×10^4 CFU/fish). Ten mg/kg of rLjMIF was ip-injected 30 min after bacterial infection. Ten mg/kg of anti-LjMIF or of IsoIgG were ip-injected 1 h before bacterial infection. Fish were monitored for signs of sickness and mortality every 24 h for 9 days. n = 15.

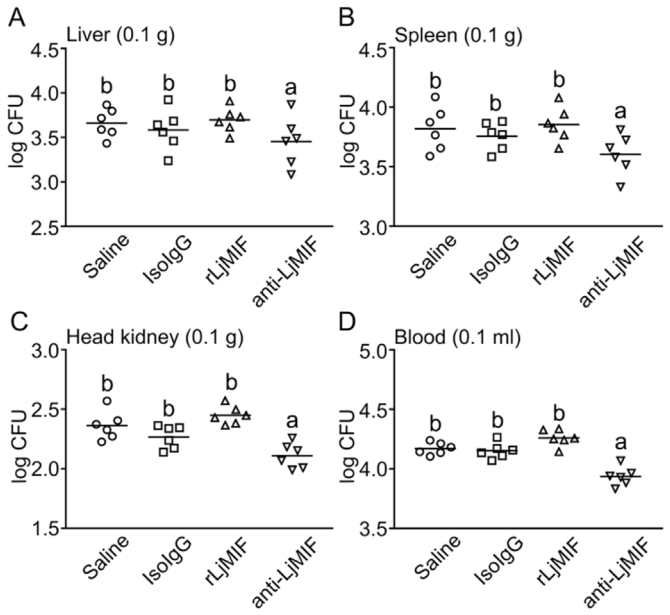

Fig. 9. Effect of rLjMIF and of anti-LjMIF on the bacterial burden in Japanese sea bass tissues. Fish were ip-injected with live *V. harveyi* and received the same volume of 10 mg/kg rLjMIF 30 min after injection or 10 mg/kg anti-LjMIF 1 h before injection. The control group received an equal volume of sterile saline or 10 mg/kg IsoIgG. Fish were euthanized 24 h later, and the liver (A), spleen (B), head kidney (C), and blood (D) were collected. Homogenates were cultured on TCBS plates. CFUs were normalized to 0.1 g of tissue weight or 0.1 ml of blood. Data represent the bacterial load in the liver, spleen, head kidney, and blood. n = 6; Values denoted by different letters are significantly different when compared by ANOVA ($P < 0.05$).

bacterial proliferation and dissemination, bacterial loads were quantitated in the tissues and blood following ip injection with rLjMIF or anti-LjMIF in *V. harveyi*-infected fish. Fish in the anti-LjMIF-treated group showed a significant reduction in the bacterial load in the liver, spleen, head kidney, and blood 24 h after *V. harveyi* infection in comparison with the IsoIgG-treated control group, while fish in the rLjMIF-treated group showed no significant changes in comparison with the saline-treated control group (Fig. 9A–D).

4. Discussion

MIF, an anciently discovered cytokine widely expressed in both immune and non-immune cells, plays an essential role in the pathophysiology of host immune and inflammatory responses [36,37]. Most mammalian MIFs are 115 aa in length and exhibit a highly conservative structure among species. Recent studies show that the enzyme and immunomodulatory active sites identified in mammalian MIFs are also conserved in fish MIFs [17]. However, there are relatively less studies on the biologic functions and mechanisms of fish MIFs. In this study, we identified a MIF homologue from Japanese sea bass. LjMIF also lacks an N-terminal guidance sequence or a distinct internal signal peptide sequence related to secretion, and it may be released from cells via a non-classical protein secretion pathway [38]. MIFs are evolutionary well conserved, and LjMIF also has the typical structural features identified in other known MIFs. The phylogenetic tree analysis revealed that LjMIF is most closely related to the yellowfin tuna, large yellow croaker, and red drum homologs. Considering the conservation of MIF sequence and structure among vertebrates [39], LjMIF might have a function similar to that reported for MIFs of mammalian species.

The expression pattern of a gene commonly reflects its biological function. In mammals, MIF is constitutively expressed in both immune and non-immune tissues and cells, and its tissue distribution is almost ubiquitous [40]. Furthermore, pathogen infection upregulated the mRNA expression of mammalian MIF [41]. To date, some reports have described the gene expression pattern of MIF in fish under normal or pathological conditions [14,16–18,20]. In green-spotted pufferfish, the MIF transcript was shown to be expressed at a higher level in the gill and gonads than in other selected tissues from healthy fish, and the expression of MIF increased sharply after fish had been stimulated with LPS [14]. In large yellow croaker, the MIF transcript was detected in all the tested tissues, with the highest level being observed in the brain, and the mRNA expression of MIF was significantly upregulated in the liver upon *V. harveyi* infection [17]. In European sea bass, the highest mRNA level was observed in the thymus [18], and, in rock bream, the highest expression of MIF was observed in the liver [20]. In red drum, the highest MIF mRNA expression was observed in the gill, and *E. tarda* or megalocytivirus infection significantly increased MIF mRNA expression in the head kidney and spleen [16]. Those studies suggested that MIF is constitutively expressed and that the tissue expression pattern of MIF differs among different fish species. In this study, the LjMIF transcript was detected in all tested tissues, with the highest expression level being observed in the liver. Upon *V. harveyi* infection, the LjMIF mRNA expression was dramatically upregulated in the immune tissues of Japanese sea bass, which is in agreement with what was previously reported in other animals [16,17,41]. This result suggests that LjMIF is closely involved in the process of *V. harveyi* infection in Japanese sea bass.

MIF is well known to have a chemokine-like function to regulate the trafficking and recruitment of macrophages and lymphocytes to the inflammatory site [42–44]. For example, mouse MIF promoted neutrophil trafficking in inflammatory arthritis via facilitation of chemokine-induced migratory responses and MAP kinase activation [45]. In green-spotted pufferfish, MIF inhibited the migration of macrophages [14]. In red drum, MIF exhibited an inhibitory effect on the migration of both head kidney-derived monocytes and lymphocytes in a dose-dependent manner [16]. In this study, we found that rLjMIF exerted an inhibitory effect on MO/MΦ and lymphocyte trafficking, which is consistent with the above-mentioned reports on other fish MIFs [14,16]. However, rLjMIF had no effect on neutrophil migration, which is not consistent with that reported in mice [45]. Anti-LjMIF had an effect opposite to rLjMIF. This result confirmed that LjMIF has a chemokine-like function by regulating the trafficking of MO/MΦ and lymphocytes.

Phagocytosis with subsequent bacterial digestion is the primary defense program orchestrated by macrophages [46,47]. As a cytokine, MIF was shown to influence immuno-regulatory processes. Deficiency of MIF did not affect bacterial phagocytosis but strongly impaired the killing of gram-negative bacteria by mouse macrophages [48]. In this study, we found that *in vitro* rLjMIF treatment enhanced phagocytosis and intracellular killing of *V. harveyi* by Japanese sea bass MO/MΦ. In addition, *in vitro* anti-LjMIF treatment decreased phagocytosis by MO/MΦ but had no effect on bacterial killing. Therefore, LjMIF is a pro-inflammatory cytokine, but the enhanced bacterial killing of MO/MΦ is likely not the primary target of the rLjMIF-mediated effect.

The survival rate is one of the most important measurements in infection research, and bacterial clearance in organs is beneficial for animal survival [49,50]. In mammals, some reports showed that MIF is closely associated with the innate immune response to a wide range of infectious and inflammatory diseases [13], and it can aggravate disease pathology [51]. LPS toxicity was exacerbated by co-injection of recombinant MIF and LPS, whereas neutralization of MIF or MIF knockout could save mice from lethal endotoxic shock [48,52–55]. On the other hand, MIF deficiency strongly impaired the host defenses against gram-negative bacteria infection, which was shown by an increased mortality in a *Klebsiella pneumonia* model [48]. Some of those results are conflicting. However, not many studies exist on fish MIFs. A study showed that bacterial recoveries from tissues of recombinant MIF-administered *E. tarda*-infected red drum were significantly lower than those from the control fish (*E. tarda*-infected) [16]. In this study, we investigated the effect of rLjMIF and of anti-LjMIF on the survival rate and bacterial burden of *V. harveyi*-infected Japanese sea bass. We found that anti-LjMIF increased the survival rate and reduced the bacterial burden, which coincides well with what was previously reported in mammals [51,55]. In contrast, rLjMIF decreased the survival rate and had no significant effect on the bacterial burden, which is not the same as previously reported in red drum [16]. This result revealed that LjMIF could aggravate the course of the infectious disease in Japanese sea bass.

In summary, we identified a MIF gene in Japanese sea bass. The transcripts of the LjMIF gene were dramatically upregulated in immune tissues upon *V. harveyi* infection. LjMIF has a chemokine-like function by regulating the trafficking of MO/MΦ and lymphocytes, but not of neutrophils. LjMIF regulates phagocytosis by MO/MΦ, but the enhanced bacterial killing of MO/MΦ is likely not the primary target of the rLjMIF-mediated effects. LjMIF could aggravate the course of the infectious disease in Japanese sea bass. Our finding supports that LjMIF is required for Japanese sea bass to fight serious infections; however, high-level production of LjMIF may be harmful during acute infections.

Acknowledgements

This project was supported by the Program for the Natural Science Foundation of China (31772876; 31372555), the Natural Science Foundation of Zhejiang Province (LZ18C190001), the Scientific Innovation Team Project of Ningbo (2015C110018), and the KC Wong Magna Fund in Ningbo University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.042.

References

[1] J.R. David, Delayed hypersensitivity *in vitro*: its mediation by cell-free substances formed by lymphoid cell-antigen interaction, Proc. Natl. Acad. Sci. U.S.A. 56 (1966) 72–77.
[2] B.R. Bloom, B. Bennett, Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity, Science 153 (1966) 80–82.
[3] H.W. Sun, J. Bernhagen, R. Bucala, E. Lolis, Crystal structure at 2.6-Å resolution of human macrophage migration inhibitory factor, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 5191–5196.
[4] T. Calandra, T. Roger, Macrophage migration inhibitory factor: a regulator of innate immunity, Nat. Rev. Immunol. 3 (2003) 791–800.
[5] M.C. Morrison, R. Kleemann, Role of macrophage migration inhibitory factor in obesity, insulin resistance, type 2 diabetes, and associated hepatic co-morbidities: a comprehensive review of human and rodent studies, Front. Immunol. 6 (2015) 308.
[6] M.F. Leyton-Jaimes, J. Kahn, A. Israelson, Macrophage migration inhibitory factor: a multifaceted cytokine implicated in multiple neurological diseases, Exp. Neurol. 301 (2018) 83–91.
[7] J.A. Chesney, R.A. Mitchell, 25 years on: a retrospective on migration inhibitory factor in tumor angiogenesis, Mol. Med. 21 (2015) S19–S24.
[8] C.C. Nobre, J.M. de Araújo, T.A. Fernandes, R.N. Cobucci, D.C. Lanza, V.S. Andrade, et al., Macrophage migration inhibitory factor (MIF): biological activities and relation with cancer, Pathol. Oncol. Res. 23 (2017) 235–244.
[9] B.A. Castro, P. Flanigan, A. Jahangiri, D. Hoffman, W. Chen, R. Kuang, et al., Macrophage migration inhibitory factor downregulation: a novel mechanism of resistance to anti-angiogenic therapy, Oncogene 36 (2017) 3749–3759.
[10] T. Lang, A. Foote, J.P. Lee, E.F. Morand, J. Harris, MIF: implications in the pathoetiology of systemic lupus erythematosus, Front. Immunol. 6 (2015) 577.
[11] K.W. Kim, H.R. Kim, Macrophage migration inhibitory factor: a potential therapeutic target for rheumatoid arthritis, Korean J. Intern. Med. 31 (2016) 634–642.
[12] JdeD. Rosado, M. Rodriguez-Sosa, Macrophage migration inhibitory factor (MIF): a key player in protozoan infections, Int. J. Biosci. 7 (2011) 1239–1256.
[13] J. Hertelendy, G. Reumuth, D. Simons, C. Stoppe, B.S. Kim, J.P. Stromps, et al., Macrophage migration inhibitory factor - a favorable marker in inflammatory diseases? Curr. Med. Chem. 25 (2018) 601–605.
[14] H.J. Jin, L.X. Xiang, J.Z. Shao, Molecular cloning and identification of macrophage migration inhibitory factor (MIF) in teleost fish, Dev. Comp. Immunol. 31 (2007) 1131–1144.
[15] J.S. Leong, S.G. Jantzen, K.R. von Schalburg, G.A. Cooper, A.M. Messmer, N.Y. Liao, et al., *Salmo salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome, BMC Genomics 11 (2010) 279.
[16] R. Qiu, J. Li, Z.Z. Xiao, L. Sun, Macrophage migration inhibitory factor of *Sciaenops ocellatus* regulates immune cell trafficking and is involved in pathogen-induced immune response, Dev. Comp. Immunol. 40 (2013) 232–239.
[17] Y. Mao, B. Xu, Y.Q. Su, Z.W. Zhang, S.X. Ding, D. Wang, et al., Cloning and mRNA expression of macrophage migration inhibitory factor (MIF) gene of large yellow croaker (*Pseudosciaena crocea*), Acta Oceanol. Sin. 29 (2010) 63–73.
[18] F. Buonocore, E. Randelli, A.M. Facchiano, A. Pallavicini, M. Modonut, G. Scapigliati, Molecular and structural characterisation of a macrophage migration inhibitory factor from sea bass (*Dicentrarchus labrax* L.), Vet. Immunol. Immunopathol. 136 (2010) 297–304.
[19] A. Sato, T.S. Uinuk-ool, N. Kuroda, W.E. Mayer, N. Takezaki, R. Dongak, et al., Macrophage migration inhibitory factor (MIF) of jawed and jawless fishes: implications for its evolutionary origin, Dev. Comp. Immunol. 27 (2003) 401–412.
[20] M. Oh, S.R. Kasthuri, Q. Wan, S.D. Bathige, I. Whang, B.S. Lim, et al., Characterization of MIF family proteins: MIF and DDT from rock bream, *Oplegnathus fasciatus*, Fish Shellfish Immunol. 35 (2013) 458–468.
[21] L. Passantino, M. Altamura, A. Cianciotta, F. Jirillo, M.R. Ribaud, E. Jirillo, et al., Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with *Candida albicans* and release of cytokine-like factors active upon autologous macrophages, Immunopharmacol. Immunotoxicol. 26 (2004) 573–585.
[22] F. Jirillo, G. Passantino, M.A. Massaro, A. Cianciotta, A. Crasto, A. Perillo, et al., *In vitro* elicitation of intestinal immune responses in teleost fish: evidence for a type iv hypersensitivity reaction in rainbow trout, Immunopharmacol, Immunotoxicol 29 (2007) 69–80.
[23] K. Ito, Y. Yoshiura, M. Ototake, T. Nakanishi, Macrophage migration inhibitory factor (MIF) is essential for development of zebrafish, *Danio rerio*, Dev. Comp. Immunol. 32 (2008) 664–672.
[24] Y.C. Shen, D.L. Thompson, M.K. Kuah, K.L. Wong, K.L. Wu, S.A. Linn, et al., The cytokine macrophage migration inhibitory factor (MIF) acts as a neurotrophin in the developing inner ear of the zebrafish, *Danio rerio*, Dev. Biol. 363 (2012) 84–94.
[25] K.F. Barald, Y.C. Shen, L.M. Bianchi, Chemokines and cytokines on the neuroimmunoaxis: inner ear neurotrophic cytokines in development and disease prospects for repair? Exp. Neurol. 301 (2018) 92–99.
[26] L.J. Weber, H.K. Marcy, Y.C. Shen, S.E. Tomkovich, K.M. Brooks, K.E. Hilk, et al., The role of jab1, a putative downstream effector of the neurotrophic cytokine macrophage migration inhibitory factor (MIF) in zebrafish inner ear hair cell development, Exp. Neurol. 301 (2018) 100–109.
[27] Y. Li, Q.H. Ai, K.S. Mai, W. Xu, Z.Y. Cheng, Effects of the partial substitution of dietary fish meal by two types of soybean meals on the growth performance of juvenile Japanese seabass, *Lateolabrax japonicus* (Cuvier 1828), Aquacult. Res. 43 (2012) 458–466.
[28] Z.Y. Xie, C.Q. Hu, L.P. Zhang, C. Chen, C.H. Ren, Q. Shen, Identification and pathogenicity of *Vibrio ponticus* affecting cultured Japanese sea bass, *Lateolabrax japonicus* (Cuvier in Cuvier and Valenciennes), Lett. Appl. Microbiol. 45 (2007) 62–67.
[29] J.B. Xiong, L. Nie, J. Chen, Current understanding on the roles of gut microbiota in fish disease and immunity, Zool. Res. (2018), https://doi.org/10.24272/j.issn.2095-8137.2018.069.
[30] C. Zhao, M.J. Fu, C.Y. Wang, Z.Y. Jiao, L.H. Qiu, RNA-Seq analysis of immune-relevant genes in *Lateolabrax japonicus* during *Vibrio anguillarum* infection, Fish Shellfish Immunol. 52 (2016) 57–64.

[31] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[32] Y. Ren, S.F. Liu, L. Nie, S.Y. Cai, J. Chen, Involvement of ayu NOD2 in NF-κB and MAPK signaling pathways: insights into functional conservation of NOD2 in antibacterial innate immunity, Zool. Res. (2018), https://doi.org/10.24272/j.issn.2095-8137. 2018.066.
[33] H. Liu, X.J. Lu, J. Chen, Full-length and a smaller globular fragment of adiponectin have opposite roles in regulating monocyte/macrophage functions in ayu, *Plecoglossus altivelis*, Fish Shellfish Immunol. 82 (2018) 319–329.
[34] Q. Chen, X.J. Lu, M.Y. Li, J. Chen, Molecular cloning, pathologically-correlated expression and functional characterization of the colony-stimulating factor 1 receptor (CSF-1R) gene from a teleost, *Plecoglossus altivelis*, Zool. Res. 37 (2016) 96–102.
[35] C.J. DiCosmo-Ponticello, D. Hoover, F.D. Coffman, S. Cohen, M.C. Cohen, MIF inhibits monocytic movement through a non-canonical receptor and disruption of temporal Rho GTPase activities in U-937 cells, Cytokine 69 (2014) 47–55.
[36] J. Bloom, S. Sun, Y. Al-Abed, MIF, a controversial cytokine: a review of structural features, challenges, and opportunities for drug development, Expert Opin. Ther. Targets 20 (2016) 1463–1475.
[37] R. Bucala, J. Bernhagen, MIF Family Cytokines in Innate Immunity and Homeostasis, Springer International Publishing, Cham, 2017.
[38] M. Merk, J. Baugh, S. Zierow, L. Leng, U. Pal, S.J. Lee, et al., The golgi-associated protein p115 mediates the secretion of macrophage migration inhibitory factor, J. Immunol. 182 (2009) 6896–6906.
[39] A. Sparkes, P. de Baetselier, K. Roelants, C. de Trez, S. Magez, J.A. van Ginderachter, et al., Reprint of: the non-mammalian MIF superfamily, Immunobiology 222 (2017) 858–867.
[40] H.Q. Lue, R. Kleemann, T. Calandra, T. Roger, J. Bernhagen, Macrophage migration inhibitory factor (MIF): mechanisms of action and role in disease, Microb. Infect. 4 (2002) 449–460.
[41] M. Bacher, A. Meinhardt, H.Y. Lan, W. Mu, C.N. Metz, J.A. Chesney, et al., MIF expression in experimentally-induced endotoxemia, Am. J. Pathol. 150 (1997) 235–246.
[42] R. Abe, T. Peng, J. Sailors, R. Bucala, C.N. Metz, Regulation of the CTL response by macrophage migration inhibitory factor, J. Immunol. 166 (2001) 747–753.
[43] J. Bernhagen, R. Krohn, H. Lue, J.L. Gregory, A. Zernecke, R.R. Koenen, et al., MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment, Nat. Med. 13 (2007) 587–596.
[44] A. Schober, J. Bernhagen, C. Weber, Chemokine-like functions of MIF in atherosclerosis, J. Mol. Med. 86 (2008) 761–770.
[45] L.L. Santos, H. Fan, P. Hall, D. Ngo, C.R. Mackay, G. Fingerle-Rowson, et al., Macrophage migration inhibitory factor regulates neutrophil chemotactic responses in inflammatory arthritis in mice, Arthritis Rheum. 63 (2011) 960–970.
[46] S. Salazar, C. Oliver, A.J. Yáñez, R. Avendaño-Herrera, Comparative analysis of innate immune responses to *Streptococcus phocae* strains in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 51 (2016) 97–103.
[47] P.S. Pires, R.L. Santos, T.A. da Paixão, L.C. de O. Bernardes, A.A. de Macêdo, L.A. Gonçalves, et al., Intracellular survival of *clostridium chauvoei* in bovine macrophages, Vet. Microbol. 199 (2017) 1–7.
[48] T. Roger, J. Delaloye, A.L. Chanson, M. Giddey, D.L. Roy, T. Calandra, Macrophage migration inhibitory factor deficiency is associated with impaired killing of gram-negative bacteria by macrophages and increased susceptibility to *Klebsiella pneumoniae* sepsis, J. Infect. Dis. 207 (2013) 331–339.
[49] R.N. Gomes, M.G.A. Teixeira-Cunha, R.T. Figueiredo, P.E. Almeida, S.C. Alves, P.T. Bozza, et al., Bacterial clearance in septic mice is modulated by MCP-1/CCL2 and nitric oxide, Shock 39 (2013) 63–69.
[50] J. Chen, Q. Chen, X.J. Lu, C.H. Li, LECT2 improves the outcomes in ayu with *Vibrio anguillarum* infection via monocytes/macrophages, Fish Shellfish Immunol. 41 (2014) 586–592.
[51] S.U. Noh, W.Y. Lee, W.S. Kim, Y.T. Lee, K.J. Yoon, Expression of macrophage migration inhibitory factor in footpad skin lesions with diabetic neuropathy, Mol. Pain 14 (2018) 1–15.
[52] M. Bozza, A.R. Satoskar, G. Lin, B. Lu, A.A. Humbles, C. Gerard, et al., Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis, J. Exp. Med. 189 (1999) 341–346.
[53] T. Calandra, B. Echtenacher, D.L. Roy, J. Pugin, C.N. Metz, L. Hültner, et al., Protection from septic shock by neutralization of macrophage migration inhibitory factor, Nat. Med. 6 (2000) 164–170.
[54] T. Roger, M.P. Glauser, T. Calandra, Macrophage migration inhibitory factor (MIF) modulates innate immune responses induced by endotoxin and gram-negative bacteria, J. Endotoxin Res. 7 (2001) 456–460.
[55] S. Jose, A. Mukherjee, M.M. Abhyankar, L. Leng, R. Bucala, D. Sharma, et al., Neutralization of macrophage migration inhibitory factor improves host survival after *Clostridium difficile* infection, Anaerobe 53 (2018) 56–63.