The char of migration inhibitory factor and Cathepsin B from Asian swamp eel (Monopterus albus) and their response to challenge with Aeromonas hydrophila

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Abstract

Aeromonas hydrophila causes sepsis and is responsible for significant economic losses in Asian finless eel (Monopterus albus) aquaculture. Previous studies have found that macrophage migration inhibitory factor (MIF) and Cathepsin B (CTSB) may be expressed in many vertebrate tissues and organs. However, there have been no reports discussing the importance of MIF and CTSB in Asian swamp eel. The complete ORF sequences of MAMIF and MACTSB were found to be 348 and 993 bp, respectively. The adaptive analysis showed that the sequences of MAMIF and MACTSB were highly conserved, and only two positive selection sites were identified in MAMIF. Quantitative real time polymerase chain reaction (qRT-PCR) demonstrated that MAMIF and MACTSB genes were expressed in all tissues evaluated with the highest expression occurring in the head kidney, spleen and liver. Challenge with Aeromonas hydrophila resulted in a significant increase in the expression of MAMIF and MACTSB in skin, liver, head kidney and spleen that peaked at 12-h post-infection. The results show that MAMIF and...
Immunity is one of the most significant physiological responses of animals. MIF is an important mediator of innate immunity and macrophage reaction and plays an important role in amplifying LPS-driven cytokine responses and controlling infection (Das et al., 2013; Kudrin & Ray, 2008). MIF can cause the aggregation, proliferation, and activation of inflammatory cells such as T cells and macrophages (Schwartz et al., 2009). Many downstream processes support the immune functions of MIF, including regulation of glucocorticoid-counter regulating activity, inhibition of EGF (Epidermal growth factor) binding to its receptor, and inhibition of p53-dependent apoptosis (Mitchell et al., 2002; Zheng et al., 2015). MIF has also been found to upregulate macrophage TLR4 (Toll-like receptor 4) expression, which can initiate excessive inflammatory reactions and cause septicemia (Calandra & Roger, 2003). MIF production by dendritic cells is also differentially regulated by TLR and TNF-α (Tumor necrosis factor-α) and is increased during inflammation (Popa et al., 2006). In obesity and wound repair, MIF contributes to adipose tissue inflammation by modulating the functions of adipose tissue macrophages (Kim, Pallua, Bernhagen, & Bucala, 2015). The release of MIF has been demonstrated to increase due to stimulation of macrophages and lymphocytes with either cytokines, microbial endotoxins, or exotoxins (Xie et al., 2016).

During an immune reaction, endo-lysosomal proteases are important (Conus & Simon, 2010). Cathepsin B (CTSB) is considered an intracellular lysosomal protease, which is responsible for intracellular and extracellular proteolysis, and associated with inflammatory response, antigen processing (Brix, 2005), protein activation, and degradation (Mort & Buttle, 1997). CTSB is required in the TLR9 responses, production of TNF-α, and activation of autophagy initiation kinase (ULK1) for clearance of bacteria (Qi et al., 2016). CTSB is inhibited by Spi2A (Serine protease inhibitor 2A) and controls the persistence of memory CD8+ T lymphocytes (Byrne et al., 2012). Earlier studies have shown that CTSB is associated with various diseases, such as malignant tumors, cancer, and acute pancreatitis (Halangk et al., 2000). CTSB induces proteolytic cleavage, which is both a prerequisite for TLR signaling and TNF-α production (Conus & Simon, 2010). Hence, the functional studies of MIF and CTSB in inflammatory responses may further reveal the immune function in aquatic animals.

Asian swamp eel (Monopterus albus) was tentatively identified as belonging to the synbranchid genus Monopterus and was regarded as the unique representative of Synbranchidae (Collins, Trexler, Nico, & Rawlings, 2002). It is one of the most economically important freshwater fishes in East Asia because of its medicinal and food value (Zhou et al., 2002). M. albus is affected by the bacterial pathogen Aeromonas hydrophila (A. hydrophila), causing disease that could be responsible for major economic losses in the aquaculture industry (Chen, Lai, Wang, Wei, & Zhong, 2018). A. hydrophila infection can be treated with antibiotics, but this has raised concerns regarding antibiotic resistance and public health security due to antibiotic residue in fish and the environment. Improving the function of the immune system using immune stimulants has become an important tool for preventing bacterial disease in fish (Veenstra et al., 2018). Many studies have reported the genes involved in immune response, such as MHC I and MHC II (major histocompatibility complex I and II), chitinase 1, complement component, β-galactoside-binding lectin, and C-type lysozyme (Li, Sun, Tang, Li, & Liu, 2011). However, there is little known about the function of MIF and CTSB in fish immune responses, which could contribute to a broader understanding of the specific infection mechanism of M. albus.

In the present study, we first cloned the full-length cDNA of MAMIF and MACTSB from M. albus and determined their structures. Moreover, to understand the fish immune system and a possible role in immune responses, we examined the expression levels of these genes after injection with A. hydrophila. Our results are the first to report in...
detail the expression characteristics of MAMIF and MACTSB from M. albus exposed to A. hydrophila. It therefore provides a theoretical basis for further exploration of the mechanism of action of these two genes in hemorrhagic septicemia and provides a guide for M. albus molecular breeding and development of immune vaccines.

2 | MATERIALS AND METHODS

2.1 | Fish and challenge experiments

Healthy M. albus (100.00 ± 10 g mean weight) was obtained from the department of Zoology, Sichuan Agricultural University (Chengdu, China). All fish were cultivated in eight tanks (80 × 80 × 50 cm³) with 200 L of domestic water with aeration (17–20 °C) for a week. Fish were fed twice a day, at 8:00 a.m. and 6:00 p.m., with commercial floating pellets (Tongwei, China). Three healthy, disease-free, robust eels were randomly selected for the gene cloning and tissue expression analysis of MAMIF and MACTSB genes. Tissues, including heart, head kidney, spleen, skin, muscle, liver, and intestine were sampled and frozen immediately in liquid nitrogen and stored at −80 °C.

A. hydrophila (ATCC 7966, Microbial Culture Collection Center, Beijing, China) was cultured in LB broth at 28 °C for 18–24 h with constant shaking. The cells were harvested by centrifugation at 10,000g for 10 min at 4 °C followed by two washes in phosphate buffered saline (PBS, pH 7.2). Viable cell count was determined by counting colony forming units (CFU) from 10-fold serial dilutions plated on nutrient agar.

A total of 108 M. albus were randomly and equally divided into two groups, with three tanks (80 cm × 80 cm × 50 cm) per group and 18 fish per tank. The M. albus in one sample group was intramuscularly injected with 0.2 mL of A. hydrophila (1.5 × 10⁶ CFU/mL). The second group was injected with an equal volume of normal saline to serve as a control. At 0, 4, 8, 12, 24, and 48 hr post-injection (hpi), head kidney, spleen, liver, and skin were sampled (three biological replicates at each time point in each group, three fish per replicate). During the bacterial challenge, the fish were cultured in a fishbowl at 17–20 °C. All samples from each group were frozen in liquid nitrogen and stored below −80 °C prior to being used for RNA isolation.

All animals used during the study were treated in compliance with the requirements of the Animal Ethics Committee of Sichuan Agricultural University, China.

2.2 | RNA isolation and first cDNA synthesis

Total RNA was prepared from 50 to 100 mg frozen tissue samples by grinding them to a powder with liquid nitrogen. Total RNA was extracted using the RNeasy Plus kit as per the manufacturer’s recommendations. The quality and quantity of RNA of each sample was analyzed using a Nano Vue™ Plus (GE, England). The A260/280 ratio of each of the isolated samples was between 1.8 and 2.1. cDNA was synthesized using a PrimeScript™ RT Reverse Transcription kit with gDNA Eraser as per the supplied protocol.

2.3 | The complete open reading frame (ORF) sequence clone

The complete ORF sequences of MAMIF and MACTSB were obtained directly by homology-based cloning. Premier 5.0 software was used to design the primers for MAMIF based on MIF sequences from Oplegnathus fasciatus (JX273154.1), Epinephelus coioides (GU988719.1), Larimichthys crocea (FJ404723.1), and Sciaenops ocellatus (FJ447488.1) (Table 1). The primer design for MACTSB was based on the 906 bp EST sequence (GW584813) obtained from NCBI, which is homologous to CTSB sequences from Oplegnathus fasciatus (HM060314), Scophthalmus maximus (KM261797), and Larimichthys crocea (KF753237) (Table 1). Each 25 μL reaction was composed of 0.5 μL Ex Taq® (Takara, Dalian, China), 1 μL each forward and reverse primers (10 mM), 1.5 μL cDNA, 2.5 μL of 10 × PCR buffer (Mg²⁺ plus), 0.7 μL dNTPs, and 17.8 μL
ultrapure water. The reaction conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s and extension at 72°C for 30 s; the last cycle was followed by 10 min incubation at 72°C for final extension. The PCR products were subsequently sequenced (Invitrogen, Shanghai, China).

2.4 | Bioinformation analysis of MAMIF and MACTSB

ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to analyze the obtained sequences. The deduced amino acid sequences from the ORFs were aligned by BLASTP against the NCBI protein sequence data bank with no repetitions (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The molecular mass (MM) and theoretical isoelectric point (pl) were calculated using ProtParam (http://www.expasy.ch/tools/protparam.html). The signal peptide was predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). The deduced amino acid sequences were checked for conserved domains using the SMART algorithm (http://smart.embl-heidelberg.de/). Multiple protein sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Identity and similarity matrices were calculated using the EMBOSS Needle Series in Pair Array instrument (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Phylogenetic and molecular evolutionary analyses were performed by the adjacent method in the Molecular Evolutionary Genetics Analysis (MEGA7) software with 1,000 bootstraps. Adaptive evolution analysis was performed using the PAML software package analysis, specifically the CODEML program (which is widely used in estimating the rate of synonymous and non-synonymous substitutions of protein coding sequences and detecting whether a sequence has undergone positive selection). This study used the CODEML program to perform site model analysis to detect changes in the selection pressure that genes have undergone during evolution.

2.5 | Expression analysis of MAMIF and MACTSB by real-time PCR

Primer 5 software was used to design gene specific primers based on MAMIF and MACTSB sequences (Table 1). GAPDH (FJ873738.1) and β-actin (AY345056) of M. albus were used as reference genes for standardization.
FIGURE 1  Legend on next page.
RT-qPCRs were performed on a CFX96™ Real-time PCR detection system with SYBR® Premix Ex Taq™ II (Takara, Dalian, China) as per the manufacturer's instructions. The reaction conditions were: 95°C for 30 s, followed by 35 cycles of 95°C for 5 s and 61°C for 30 s. A melting point curve was generated and analyzed, and the specificity of the PCR was verified by the presence of a single peak. Serial dilutions of cDNA were used to plot a standard curve and to measure the amplification efficiency of the system (Bustin et al., 2009). The expression levels of MAMIF and MACTSB were analyzed by an optimized comparative Ct (2−ΔΔCt) value method and analyzed using ANOVA in the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard error.

3 | RESULTS

3.1 | Characterization of MAMIF and MACTSB

The ORF of MAMIF (KY318075) was 348 bp long and encoded a 115 amino acid, with an estimated molecular weight (MW) of 12.49 kDa, and theoretical isoelectric point of 6.89. No signal peptide or conserved domain could be predicted. The ORF of MACTSB (KY318074) was 993 bp long and encoded a peptide of 330 amino acids, with an estimated MW of 36.28 kDa, and theoretical isoelectric point of 5.54. The deduced amino acid sequence contained a signal peptide from residues 1 to 18 and a C1-peptidase from amino acids 79 to 328.

3.2 | Amino acid sequence alignment

The predicted amino acid sequence of MAMIF showed highest identity with Oplegnathus fasciatus (90.5%), followed by Oreochromis niloticus (81.7%), Danio rerio (81.0%), and Homo sapiens (69.8%). Multiple sequence alignment analysis revealed that conserved proline (P) and lysine (K) residues were present in each of the analyzed MIFs (Figure 1(a)). MACTSB shared approximately 70–90% identity and general characteristics with Cathepsin B of several vertebrates, such as Epinephelus coioides (84.8%), Larimichthys crocea (87%), and D. rerio (87%). Furthermore, multiple sequence alignment showed that MACTSB had the active site residues (C107, H277, and N297) required for protease activity (Figure 1(b)).

3.3 | Phylogenetic analysis of the MAMIF and MACTSB

Phylogenetic trees were constructed using MAMIF and MACTSB sequences from selected vertebrates. The proteins were classified into two primary groups, one contained fish's proteins, while the other comprised mammalian, amphibian, reptilian, and avian proteins. The distribution of the phylogenetic trees confirmed the developmental relationship among different groups (Figure 2).

FIGURE 1 Multiple sequence alignment of MAMIF and MACTSB. (a) Multiple sequence alignment of MAMIF with different species with the following accession numbers: Oreochromis niloticus (XP_003444421), Danio rerio (NP_001036786), Gallus gallus (NP_001292020), and Homo sapiens (CAG30406). C27ALC60 for oxidoreductase activity is marked with a box. The sites of catalytic activity (P) and isomerase activity (K) are marked with “▲.” (b) Multiple sequence alignment of MACTSB with other species with the following accession numbers: O. niloticus (XP_003454569), D. rerio (NP_998501), G. gallus (NP_990702), and H. sapiens (AAH10240). The predicted signal peptide is marked with a box and the occluding loop is underlined. The active site residues (C107, H277, N297) required for protease activity are marked with “▲.” Sequence identities are shown in black (100%), dark gray (≥75%), light gray (≥50%), and white (≥33%), as determined by ClustalW and DNAMAN.
Phylogenetic trees of MAMIF and MACTSB were constructed by MEGA7 software, and PAML software was used to analyze the selection pressures that MAMIF and MACTSB experienced during evolution (Table 2). The M0 and M3 likelihood ratio tests of the first pair of models show that $2\Delta\ln L = 69.39$, $df = 4$, and $p < .01$. Therefore, model M3 is significantly better than model M0, indicating that the selection pressure experienced between sites is heterogeneous. Model M7 and M8 comparison results show that model M8 is significantly better than M7 ($2\Delta\ln L = 9.14$, $df = 2$, $p = .014$), so the alternative hypothesis model M8 was established, and its $\omega = 1.48681 > 1$, indicated that the MAMIF gene is under selection pressure during evolution, and that there are positive selection sites (28C, 92T). However, no positive selection sites were found in the MACTSB analysis.

**FIGURE 2** The neighborhood-joining phylogenetic tree of MIF (a) and Cathepsin B (b) based on their amino acid sequences

### 3.4 Positive selected sites analysis of MAMIF and MACTSB
## TABLE 2  MAMIF and MACTSB protein adaptive evolution analysis and positive selected sites

| Gene | Models | np | lnL  | Estimates of parameters | Model compared | df | 2ΔlnL | p-value | Positive sites |
|------|--------|----|------|--------------------------|----------------|----|-------|---------|----------------|
| MIF  | M0     | 32 | -2,823.20 | $\omega_0 = 0.78630$ | M0/M3 | 4  | 69.39 | < .01  | Not allowed    |
|      | M3     | 36 | -2,788.51 | $p_0 = 0.49196, p_1 = 0.49102, p_2 = 0.01703$  
$\omega_0 = 0.34645, \omega_1 = 1.38897, \omega_2 = 2.41614$ | | | | | |
|      | M1a    | 33 | -2,792.38 | $p_0 = 0.40439, p_1 = 0.59561$  
$\omega_0 = 0.25497, \omega_1 = 1.00000$ | M1a/M2a | 2  | 7.70  | .021  | Not allowed    |
|      | M2a    | 35 | -2,788.53 | $p_0 = 0.48208, p_1 = 0.08145, p_2 = 0.43646$  
$\omega_0 = 0.34258, \omega_1 = 1.00000, \omega_2 = 1.48240$ | | | | | |
|      | M7     | 33 | -2,792.85 | $p = 0.11281, q = 0.02641$ | M7/M8 | 2  | 9.14  | .0104  | 28C 0.983$^a$  
92T 0.989$^a$ |
|      | M8     | 35 | -2,788.28 | $p_0 = 0.54088, p = 3.21566, q = 4.88740 (p_1 = 0.45912), \omega = 1.48681$ | | | | | |
| CTSB | M0     | 26 | -2,315.74 | $\omega_0 = 0.09753$ | M0/M3 | 4  | 150.07 | < .01  | Not allowed    |
|      | M3     | 30 | -2,315.70 | $p_0 = 0.76886, p_1 = 0.23114, p_2 = 0.00000$  
$\omega_0 = 0.02111, \omega_1 = 0.44977, \omega_2 = 1.17823$ | | | | | |
|      | M1a    | 27 | -2,256.28 | $p_0 = 0.79339, p_1 = 0.20661$  
$\omega_0 = 0.03420, \omega_1 = 1.00000$ | M1a/M2a | 2  | 0  | 1.0  | Not allowed    |
|      | M2a    | 29 | -2,256.28 | $p_0 = 0.79339, p_1 = 0.10951, p_2 = 0.09710$  
$\omega_0 = 0.03420, \omega_1 = 1.00000, \omega_2 = 1.00000$ | | | | | |
|      | M7     | 33 | -2,258.76 | $p = 0.41410, q = 1.95981$ | M7/M8 | 2  | 0  | .9997  | Not allowed    |
|      | M8     | 35 | -2,258.76 | $p_0 = 0.99999, p = 0.41411, q = 1.95988 (p_1 = 0.00000), \omega = 1.00000$ | | | | | |

Abbreviations: 2ΔlnL, twice the log likelihood difference of the models compared; np, number of free parameters.  
$^a$95% levels inferred positive selection sites.
MAMIF was observed in all the tissues examined, with higher expression levels in liver and head kidney, moderate levels in heart, spleen, intestine, and muscle, and the lowest levels in the skin (Figure 3(a)). The MACTSB was highly expressed in liver, spleen, and head kidney, but with lower expression levels in intestine, muscle, heart, and skin (Figure 3(b)).
3.6 Temporal expression analysis of MAMIF and MACTSB after A. hydrophila challenge

The expression of MAMIF in skin was upregulated at 8 hpi ($p < .05$) and peaked at 12 hpi reaching a 7.9-fold increase ($p < .01$). There after levels decreased until there was no significant difference at 24 hpi when compared with the control group (Figure 4(a)). The expression of MAMIF in liver and skin was similar (Figure 4(b)). The expression of MAMIF in head kidney was upregulated at 4 hpi ($p < .05$), and peaked at 12 hpi reaching 11.3-fold ($p < .01$). Although levels then decreased, the difference was still extremely significant at 24 hpi ($p < .01$) and 48 hpi ($p < .01$) when compared with the control group (Figure 4(c)). The expression of MAMIF in spleen was increased significantly at 8 hpi ($p < .01$) and peaked at 15.13-fold at 12 hpi ($p < .01$), subsequently, the expression level decreased at 24 hpi and still significantly higher than the control group, but at 48 hpi, there was no significant difference from the control group ($p < .01$) (Figure 4(d)).

The expression of MACTSB in skin was upregulated at 4 hpi ($p < .05$), and peaked at 8.0-fold at 12 hpi ($p < .01$), and then decreased although the difference was still significant at 24 hpi ($p < .01$) and 48 hpi ($p < .05$) when compared with the control group (Figure 5(a)). The expression of MACTSB in liver was upregulated at 4 hpi ($p < .05$) and peaked at 10-fold at 12 hpi ($p < .01$), and then decreased slightly but the difference was still extremely significant at 24 hpi ($p < .01$) and 48 hpi ($p < .01$) when compared with the control group (Figure 5(b)). The expression of MACTSB in head kidney had a similar trend to that seen in the liver (Figure 5(c)). The expression of MACTSB in spleen was increased significantly at 8 hpi ($p < .01$) and peaked at 15.13-fold at 12 hpi ($p < .01$), subsequently, the expression level decreased at 24 hpi and still significantly higher than the control group, but at 48 hpi, there was no significant difference from the control group ($p < .01$) (Figure 5(d)).
**DISCUSSION**

*MIF* has previously been shown to be conserved among vertebrates, which may result from its functional significance. *MIF* was considered to be a unique cytokine produced by T cells and involved in the immune response to inflammation and promoted the secretion or expression of many other pro-inflammatory markers, such as IL-6, IL-1β, and TNF-α (Calandra & Roger, 2003; Jankauskas, Wong, Bucala, Djudjaj, & Boor, 2019). In this study, the identified *MAMIF* gene encodes a protein having structural features that are similar to the vertebrate *MIF* family due to the presence of sites for catalytic (P2) and isomerase activity (K35), and the CXXC motif C57-ALC60, thus conferring certain immunologic properties to *MIF* (Kleemann et al., 1998) (Figure 1(a)). In addition, *MAMIF* does not have a signal peptide cleavage site for secretion or an internal secretory sequence, which indicates that it is secreted by a non-conventional leaderless pathway (Lue, Kleemann, Calandra, Roger, & Bernhagen, 2002). The presence of *MAMIF* in this study further demonstrated that *MIF* is evolutionarily conserved.

*CTSB* is a lysosomal cysteine protease with the form of zymogen, which can be involved in protein hydrolysis and other physiological processes especially in the body’s immune response (Qiu, Liu, Hu, & Sun, 2013). In all identified vertebrates, including *M. albus*, residues P179 to G199 in *CTSB* contain a closed occluding loop with 21 amino acid residues. This includes a histidine residue that creates a positive charge at the C-terminus, restricting *CTSB* active nick sites after tertiary structure formation, thus conferring peptidase activity on *MACTSB* (Zhang et al., 2008). In addition, multiple sequence alignment indicated that *MACTSB* has conserved active site residues (C107, H277, and N297) (Figure 1(b)). Previous studies have shown that these active sites play crucial roles in the formation and stabilization of the catalytic site of the activated enzyme (Lecaille, Kaleta, & Brömme, 2002). Moreover, bioinformatics analysis indicated that *MACTSB* is a secreted protein and has a signal peptide of 18 amino acid residues. The signal peptide is thought to transport the ribosomal expressed proenzyme to the endoplasmic reticulum (Turk, Turk, & Turk, 2000).

The phylogenetic trees showed that *MAMIF* and *MACTSB* were both first clustered with Perciformes, and then clustered with Cyprinodontidae and Cypriniformes (Figure 2). The sequence analysis confirms the structural conservation between *MAMIT* and *MACTSB* and other vertebrates. These results were consistent with other reported genes of *M. albus* (Li, Sun, et al., 2011; Li, Sun, Meng, & Hong, 2014) and traditional taxonomy of fish (http://fishdb.sinica.edu.tw/AjaxTree/tree.php). Conservation of amino acid sequence and genomic structure of MIFs and CTSBs between *M. albus* and teleost fish suggests that they might have a similar immunological function. This study used site model to detect selection pressure. The results showed that only two positive selection sites were detected in MIF (Table 2). It can be seen that the protein encoded by the MIF gene is mainly affected by neutral drift and purification selection at the overall level. No positive selection site was identified in CTSB. It is speculated that the functions encoded by the MIF and CTSB genes are important, and the amino acid sequence is relatively conserved.

*MAMIF* and *MACTSB* were both ubiquitously expressed in various tissues (Figure 3), especially *MAMIF* shows constitutive expression and is highly expressed in immune-related organs, such as head kidney and spleen. These organs are considered to be the most important immune organs of fish, suggesting that these genes may be important in immunological supervision (Huang et al., 2016; Ito, Yoshiura, Ototake, & Nakanishi, 2008; Zhan, Jakovlic, & Wang, 2019). Immune supervision is one of the most basic functions of the immune system, responsible for identifying, resisting, and killing pathogens. These results were consistent with most previous studies in various fish (Huang et al., 2016; Jin, Xiang, & Shao, 2007; Wang et al., 2013). Antigen-presenting cells largely reside in the kidney and spleen, which are major sites of innate and adaptive immune responses in fish (Luo et al., 2016). In addition, high level expression of *MAMIF* and *MACTSB* was detected in the liver in the present study. Liver is not a typical immune organ in vertebrates, although it contains a large amount of innate lymphocytes, including both natural killer T cells and T cells (Wang et al., 2013; Whang et al., 2011). Moreover, a moderate level expression of *MAMIF* and *MACTSB* was detected in muscle. This result was different from other reported immune-related genes in *M. albus*, such as MHC Ia, MHC IIb, and hepcidin, which are only minimally or negligibly expressed in the muscle (Li et al., 2011; Li et al., 2013; Li et al., 2015; Mao et al., 2010). This was also different from *MIF* and *CTSB* expression in other fish.
species, such as sea cucumber *Apostichopus japonicus* and striped murrel *Channa striatus* (Arockiaraj et al., 2014; Chen et al., 2017; Parisi et al., 2012). The reason may be involved in the special evolution pattern of the immune system. It is possible that a small number of phagocytes are dotted sporadically throughout these tissues, but this requires future demonstration by histological studies. This study verified the role of the MIF and CTSB genes in sepsis, and further studies will provide strategies for the treatment of sepsis.

In the process of antigen presentation, macrophages recognize antigens through non-specific immune responses, and endocytose antigens to form phagosomes, after entering the lysosome, phagosomes fuse with lysosomes to form phagolysosomes; the antigen can be degraded in phagolysosomes to form immunogenic peptides (Lah et al., 2000). When an organism is stimulated by antigens such as gram-negative bacteria, lipopolysaccharide, or immune, factors such as TNF-α, IFN-γ, and macrophages release MIF, a multifunctional pro-inflammatory factor, which can regulate innate immunity and specific immunity. MIF can promote the activation of macrophages, and activated macrophages may secrete more MIF, in a positive feedback loop. However, the overexpression of MIF can aggravate fish sepsis to a certain extent (Noels, Bernhagen, & Weber, 2009; Schwartz et al., 2009). CTSB participates in the antigen presentation process through the degradation of endocytic antigen protein processing and li chain processing (Villadangos et al., 1999).

In the present study, challenge with *A. hydrophila* resulted in a significant increase in the expression of MIF and CTSB in the liver, spleen, head kidney, and skin within 12 hpi, followed by a decrease from 12 to 48 hpi (Figure 4 and Figure 5). The expression levels of MAMIF and MACTSB in the liver, spleen, and head kidney tissues increased first and then decreased after *A. hydrophila* challenge. MIF is constitutively expressed when stimulated by an antigen from *A. hydrophila*. Such stimuli cause macrophages in tissues such as the head kidney, spleen, and liver to release MIF with a bell-shaped structure. The initial dose is relatively small, and the release afterwards shows a trend of rising first and then falling (Buonocore et al., 2010; Oh et al., 2013). These results were similar to other studies on fish species such as *O. fasciatus* (Whang et al., 2011), orange-spotted grouper, *Epinephelus coioides* (S. Wei et al., 2014), Chinese giant salamanders *Andrias davidianus* (Wang et al., 2013), and blunt snout bream (Luo et al., 2014). Collectively, the wide distribution and upregulation of MAMIF and MACTSB suggested not only a role involved in the acute inflammatory responses but also key responses in the pathogen triggered immune response after pathogen entry and recognition (Chen et al., 2017). In the present study, significant increases of expression in liver, spleen, and head kidney imply that these organs are important in defending against pathogenic antigens. These results indicated that MAMIF and MACTSB were respond to *A. hydrophila* infection in all tested organs, but the temporal expression and duration of MAMIF and MACTSB for immune response triggered by the pathogen are different in various organs.

*Monopterus albus* is a fish species without scales, and the naked skin has numerous glands that can secrete mucus. In this work, it was also found that the expression of MACTSB in the skin was significantly increased from 4 to 48 hpi, while MAMIF increased significantly from 8 to 12 hpi, indicating that MACTSB plays a more important role in skin immunity than MAMIF. Interestingly, CTSB extracted from skin mucus has been shown to be a potential bacteriolysin, which is involved in nonspecific immunity of fish (Aranishi, 1999). Generally, the intestinal tract, skin, and gills have all been proposed as natural routes of entry for bacterial infections (Chen, Yan, Wang, Zhuang, & Wang, 2008; Ringø et al., 2007). In this study, the application of intramuscular injection was equivalent to simulating the skin route.

In summary, we successfully cloned the full-length cDNA of MIF and CTSB genes from *M. albus* and investigated the expression of MIF and CTSB in various tissues in response to infection with the pathogenic bacteria *A. hydrophila*. Collectively, MAMIF and MACTSB were not only involved in the acute inflammatory responses but also played key roles in the pathogen triggered immune response after *A. hydrophila* infection. Further study concerning the mechanism of action of MAMIF and MACTSB within the host immune system may elucidate their exact immunological roles and provide a vehicle for the prevention of viral and bacterial infections among aquaculture stocks, and provide technical support for future investigation of molecular vaccines for disease resistance, or screening of *M. albus* molecular breeding.

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