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Short communication

Host defense peptide LEAP-2 contributes to monocyte/macrophage polarization in barbel steed (*Hemibarbus labeo*)

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ABSTRACT

The liver-expressed antimicrobial peptide 2 (LEAP-2) plays a vital role in host immunity against pathogenic organisms. In the present study, cDNA of the LEAP-2 gene was cloned and sequenced from the barbel steed (*Hemibarbus labeo*). The predicted amino acid sequence of the barbel steed LEAP-2 comprises a signal peptide and a prodomain, which is followed by the mature peptide. Sequence analysis revealed that barbel steed LEAP-2 belongs to the fish LEAP-2A cluster and that it is closely related to zebrafish LEAP-2A. We found that barbel steed LEAP-2 transcripts were expressed in a wide range of tissues, with the highest mRNA levels detected in the liver. In response to lipopolysaccharide (LPS) treatment, LEAP-2 was significantly upregulated in the liver, head kidney, spleen, gill, and mid intestine. A chemically synthesized LEAP-2 mature peptide exhibited selective antimicrobial activity against several bacteria *in vitro*. Moreover, LEAP-2, alone or in combination with LPS or phorbol 12-myristate 13-acetate, strongly induced a pro-inflammatory reaction in barbel steed monocytes/macrophages (MO/MΦ), involving the induction of iNOS activity, respiratory burst, and the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-1β. Collectively, the results of this study indicate the importance of fish LEAP-2 in the M1-type polarization of MO/MΦ.

1. Introduction

Host defence peptides (HDPs), also known as antimicrobial peptides (AMPs), are short, typically cationic, amphipathic peptides that defend the host against pathogens. Early research in the field focussed primarily on the antimicrobial functions of HDPs. In the last decade, however, HDPs have been demonstrated to possess a broad range of immunomodulatory functions [1,2]. Cathelicidins and defensins secreted at the sites of infection or injury are chemotactic for effector cells, and induce the transcription and secretion of chemokines [3,4]. HDPs also stimulate the release of specific cytokines; for example, mouse β-defensin-2-matured dendritic cells secrete the pro-inflammatory cytokines IL-12, IL-1α, IL-1β, and IL-6 [5].

In addition, several studies have shown that HDPs are involved in the polarization of macrophages [6,7]. In mammals, at least two major phenotypes of macrophages, the classical type (M1) and the alternative type (M2), are functionally polarized in response to pathogen infection and host mediators [8]. M1-type macrophages have been described as being responsive to two signal types, type 1 inflammatory cytokines and microbial products [9]. M2 macrophages include at least three subsets: M2a, induced by IL-4 or IL-13; M2b, induced by immune complexes and agonists of TLRs or IL-1 receptors; and M2c, induced by IL-10 and glucocorticoid hormones [10]. M1-and M2-type macrophages differ in terms of receptors, cytokine and chemokine expression, and effector functions [8]. The M1 type is mainly involved in pro-inflammatory responses and produces inducible nitric oxide synthase (iNOS) as well as inflammatory cytokines such as TNF-α and IL-1β. The M2 type plays essential roles in resolving inflammation by producing arginase and anti-inflammatory mediators such as IL-10 and TGF-β [8].

The human liver-expressed antimicrobial peptide 2 (LEAP-2) is predominantly expressed in the liver, and structurally comprises a cysteine-rich peptide containing four highly conserved cysteine residues that form two pairs of disulphide bonds [11–13]. Previous studies have provided evidence that human LEAP-2 disrupts the physical integrity of the bacterial membrane and binds to bacterial genomic DNA to directly kill specific bacteria [13,14]. Despite this knowledge, however, very little is currently known regarding the immunomodulatory functions of mammalian LEAP-2 relating to immune cells, or its effects on macrophage polarization.

In fish, LEAP-2 was initially identified in rainbow trout (*Oncorhynchus mykiss*) [15], and was subsequently isolated and characterized from channel catfish (*Ictalurus punctatus*) [16], grass carp

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(*Ctenopharyngodon idella*) [17], blunt snout bream (*Megalobrama amblycephala*) [18], large yellow croaker (*Larimichthys crocea*) [19], miiuy croaker (*Miichthys miiuy*) [20], yellow catfish (*Pelteobagrus fulvidraco*) [21], common carp (*Cyprinus carpio* L.) [22], and mudskipper (*Boleophthalmus pectinirostris*) [23]. In contrast to mammals that contain only a single homologue of LEAP-2, some fish species, like rainbow trout, large yellow croaker, and common carp have multiple homologues, which are generally classified into three groups, namely LEAP-2A, LEAP-2B, and LEAP-2C [15,19,22]. The tissue expression pattern of LEAP-2 can vary in different fish species. For example, grass carp LEAP-2A gene is expressed in a wide range of tissues, with the highest level of transcripts found in liver [17], whereas the levels of LEAP-2A and LEAP-2C transcripts in large yellow croaker are considerably lower in the liver than in the intestine [19]. LEAP-2 expression is closely related to the immune response of fish against pathogenic organisms [19,21]. For example, in mudskippers, the expression level of LEAP-2 is instantaneously upregulated in the liver, spleen, kidney, and gill in response to infection with *Edwardsiella tarda* [23].

Barbel steed (*Hemibarbus labeo*) is a freshwater bottom dwellers cyprinid living in streams and feeding on aquatic insects. *H. labeo* are distributed in major drainage of east mainland China, Japan, and Korea. The development of barbel steed aquaculture has been hampered, to a certain extent, by bacterial fish diseases adversely affecting the production, although to date there has been no research on the innate immunity of this fish species. In the present study, we cloned a LEAP-2 gene from the barbel steed and examined the expression patterns of this gene in various tissues under both physiological conditions and after treatment with lipopolysaccharide (LPS). The antibacterial activity of barbel steed LEAP-2 was determined using a two-fold microdilution method. Additionally, for the first time, we report the immunomodulatory effect of a fish LEAP-2 on the polarization of monocytes/macrophages (MO/MΦ).

2. Materials and methods

2.1. Experimental fish

Barbel steed (70–80 g) were obtained from a commercial farm in Lishui City, China, and maintained at 23–25 °C in a recirculation system in 100-L tanks. Dissolved oxygen was maintained at > 5 mg/mL. The fish were fed twice daily with a commercial diet and acclimatized to laboratory conditions for 2 weeks before experiments. Before the experiments, fish were randomly sampled for examination of bacterial recovery from blood, liver, kidney, and spleen. No bacteria were detected from the sampled fish. In addition, only fish with no visible pathological signs were used for experiments. All the experiments were performed in accordance with the Experimental Animal Management Law of China and were duly approved by the Animal Ethics Committee of Lishui University.

2.2. Molecular characterization of barbel steed LEAP-2 cDNA

The cDNA sequence of barbel steed LEAP-2 was obtained from the transcriptome data of barbel steed intestine. To date, only a single type of LEAP-2 cDNA had been identified in barbel steed by high-throughput transcriptome analysis. Specific primers were designed based on sequences in the 5′- and 3′-untranslated regions (UTR) and used for PCR amplification of the complete coding region using a mixed tissue cDNA sample, followed by cloning and sequencing. The similarity of the sequence obtained with other known sequences was assessed via BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cleavage sites for the signal peptides were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). The sequence of the mature peptide of barbel steed LEAP-2 was deduced using the ProtParam tool (http://us.expasy.org/tools/protparam.html). Multiple alignments were analysed using ClustalW (http://clustalw.ddbj.nig.ac.jp/), and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.

2.3. Barbel steed LEAP-2 expression profiles in response to LPS

Thirty-two fish were evenly divided into two groups and cultured under the same conditions. An LPS stimulation experiment was performed as previously described [24]. The barbel steed were intraperitoneally injected with LPS, derived from *Escherichia coli* 0127:B8 (Sigma, St. Louis, MO, USA) and dissolved in 200 μL sterile phosphate-buffered saline (PBS), at a dose of 0.5 μg/g body weight. Saline was used as the control. At 4, 8, 12, and 24 h post treatment (hpt), the liver, spleen, head kidney, mid intestine, and gill were collected and stored at −80 °C until use. Healthy fish tissues, including the liver, head kidney, trunk kidney, and mid intestine were also collected for analysis of the expression patterns in different tissues. Four samples from each tissue were collected, frozen in liquid nitrogen, and stored at −80 °C until use.

2.4. Real-time quantitative PCR (RT-qPCR)

Total RNAs were extracted from tissues using RNAiso reagent (TaKaRa, Dalian, China), and subsequently digested with deoxyribonuclease I (TaKaRa). The first-strand cDNA was then synthesized using AMV reverse transcriptase (TaKaRa) primed by Oligo(dT) and a random primer (TaKaRa) [25]. Gene-specific primers were designed based on the sequences of LEAP-2 and 18S rRNA, respectively (Table 1). Primer efficiency was determined from a standard curve of cDNA samples according to the MIQE guidelines for RT-qPCR. Primer specificity was determined by both gel electrophoresis and melting curve analysis. For gene expression analysis, 1 μL of 1:10-diluted cDNA was added to 10 μL 2 × SYBR premix Ex Taq (Perfect Real Time; TaKaRa), and the final concentration of each primer was 400 nM in a 20-μL total volume. The amplification was performed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA), using the following programme: 94 °C for 5 min, followed by (1) 40 cycles of 94 °C for 30 s and 60 °C for 20 s; (2) melting curve analysis performed at 94 °C for 5 s,

Table 1
The sequences of oligonucleotide primers used in this study.

Gene	Accession number	Primers	Sequence (5′–3′)	Amplicon size (bp)
LEAP-2	MH843149	LEAP-2-t(+)	TGCAGGATCACAGTCACAGA	243
		LEAP-2-t(−)	GGCCTCTCCTGCATATTCCT	
IFN-γ	MH843152	IFN-γ-t(+)	AAACTACTTCCCGGGCAAGA	169
		IFN-γ-t(−)	TCGTCTCCTGCGGTCTTTAT	
IL-1β	MH843151	IL-1β-t(+)	GCCGAGTCTGATGAGATGGA	162
		IL-1β-t(−)	CTTGGGTTTGACGTGCTTCA	
TNF-α	MH843150	TNF-α-t(+)	CGAGATTCACATTCCTGCCG	197
		TNF-α-t(−)	TGTGAGATTGGCAGACGGAT	
18S rRNA	MH843153	18S rRNA-t(+)	AGAAACGGCTACCACATCCA	247
		18S rRNA-t(−)	CCGAGATCCAACTACGAGCT	

65 °C for 15 s, and 95 °C for 15 s; and (3) cooling at 40 °C for 30 s. For determination of gene expression, a threshold cycle (Ct) was obtained from each amplification curve. Calculation of the relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method as previously described [26], using barbel steed 18S rRNA for normalization. For each group, tissue samples were taken from four fish. All samples were measured in duplicate.

2.5. Antibacterial assay

A mature LEAP-2 peptide containing two disulphide bonds (between Cys67 and Cys78, and between Cys73 and Cys83) was chemically synthesized with over 95% purity (SynPeptide, Shanghai, China). The antibacterial activity of the peptide was assayed against a panel of bacteria, including *Vibrio vulnificus, Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio anguillarum*, *Aeromonas hydrophila, Staphylococcus aureus*, and *Streptococcus iniae*. A modified two-fold microdilution method was used to determine the minimal inhibitory concentration (MIC) of various agents as previously described [27]. Two-fold serial dilutions of the peptide were made to give final concentrations ranging from 100 to 1.563 μg/mL in a 96-well plate. Chloramphenicol (Sigma) was used as a positive control and was diluted using the same method as described for the peptide. For each series of experiments, PBS was employed as a negative control. An 80-μL aliquot of each peptide solution was added to the corresponding well of the 96-well plate. The bacteria were cultured to mid-logarithmic phase, and were subsequently diluted to a final concentration of 1×10^5 colony-forming units (CFU)/mL in suitable media (Table 2). Aliquots (20 μL) of these bacterial suspensions were added to each well of the 96-well plate and incubated at an appropriate temperature (Table 2) for 24 h. Minimal inhibitory concentrations (MICs) were determined by measuring the absorbance of bacterial sedimentation at 600 nm using a microplate reader (Varioskan Flash Multimode Reader; Thermo Fisher Scientific, USA).

2.6. Primary culture of barbel steed head kidney-derived MO/MΦ

Barbel steed head kidney-derived MO/MΦ were isolated and cultured as previously described [28]. In brief, the head kidney was isolated and washed in RPMI 1640 medium (Invitrogen, Shanghai, China), supplemented with 2% foetal bovine serum (FBS) (Invitrogen), penicillin (100 U/mL), streptomycin (100 μg/mL), and heparin (20 U/mL). The cells were separated using Ficoll-Hypaque PREMIUM (1.077 g/mL; GE Healthcare) after centrifugation, according to the manufacturer's instructions. The cells were then seeded in 35-mm dishes at a density of 2×10^7/mL. The non-adherent cells were washed off, and the attached cells were incubated in complete medium (RPMI 1640, 5% barbel steed serum, 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) at 24 °C in a 5% CO_2 atmosphere.

2.7. Inducible nitric oxide synthase (iNOS) assay

Barbel steed MO/MΦ were treated with LEAP-2 (0, 1, or 10 μg/mL), LPS (10 μg/mL), or a combination of these for 12 h, with PBS being used in the control treatment. Cellular iNOS activity was measured based on the conversion of L-arginine to NO using a nitric-oxide synthase assay kit (Beyotime Biotech, Beijing, China).

2.8. Respiratory burst assay

The respiratory burst for barbel steed MO/MΦ was determined by measuring the concentration of intracellular O^{2-} using the nitroblue tetrazolium (NBT) reduction test, as described previously [23]. In brief, MO/MΦ were pre-treated with or without LEAP-2 (0, 1, or 10 μg/mL) for 12 h. The cells were then washed with sterile PBS and treated with 0.1 μg/mL phorbol 12-myristate 13-acetate (PMA). NBT was added to each plate at a final concentration of 1 mg/mL and the cells were incubated for 1 h at 24 °C. The reaction was stopped by the addition of 400 μL of 70% methanol, and the cells were washed and air-dried. Formazan was dissolved in 120 μL of 2 M KOH and 140 μL of dimethyl sulphoxide (DMSO). The optical density at 620 nm (OD620) was measured using an Ultrospec 1100 Pro UV/visible spectrophotometer (Amersham Biosciences, Piscataway, USA).

2.9. Analysis of cytokine expression in MO/MΦ

Barbel steed MO/MΦ were treated with LEAP-2 (0, 1, or 10 μg/mL), LPS (10 μg/mL), or a combination of these for 12 h, with PBS being used in the control treatment. RT-qPCR analyses were performed as described in section 2.4 using the primers listed in Table 1. The expression of IFN-γ, TNF-α, and IL-1β genes was normalized to that of 18S rRNA.

2.10. Statistical analysis

All the data are reported as means ± SEM. Statistical analyses of the results were conducted using SPSS version 13.0 (SPSS Inc., Chicago, USA). For RT-qPCR measurements, the arbitrary units for each sample were obtained after normalization to the lowest expression level in a data set that was defined as 1, and were log 2 transformed to improve the normality of data distribution. One-way ANOVA and the least significant difference (LSD) post hoc test were then used to analyse the expression data, with *P* values less than 0.05 considered to be statistically significant.

3. Results

3.1. Molecular characterization of barbel steed LEAP-2

The cDNA sequence of LEAP-2 has been submitted to the GenBank database under the accession number MH843149. The open reading frame (ORF) of LEAP-2, which was 276 nt in length, is predicted to encode a polypeptide of 91 amino acids. The protein has a calculated

Table 2
Minimum inhibitory concentration (MIC) values of LEAP-2 against bacteria.

Bacteria	Strains	Culture medium	Culture Temperature (°C)	LEAP-2 MIC (μg/mL)	Chloramphenicol MIC (μg/mL)
Vibrio vulnificus	ATCC279562	TSB	28	50	1.563
Vibrio alginolyticus	ATCC17749	TSB	28	100	3.125
Vibrio parahaemolyticus	ATCC33847	TSB	28	–	1.563
Vibrio anguillarum	ATCC19264	TSB	28	12.5	6.25
Staphylococcus aureus	ATCC6538	LB	37	100	3.125
Aeromonas hydrophila	ATCC7966	LB	37	12.5	1.563
Streptococcus iniae	ATCC29178	BHI	37	–	1.563

- no inhibition detected at 100 μg/mL.

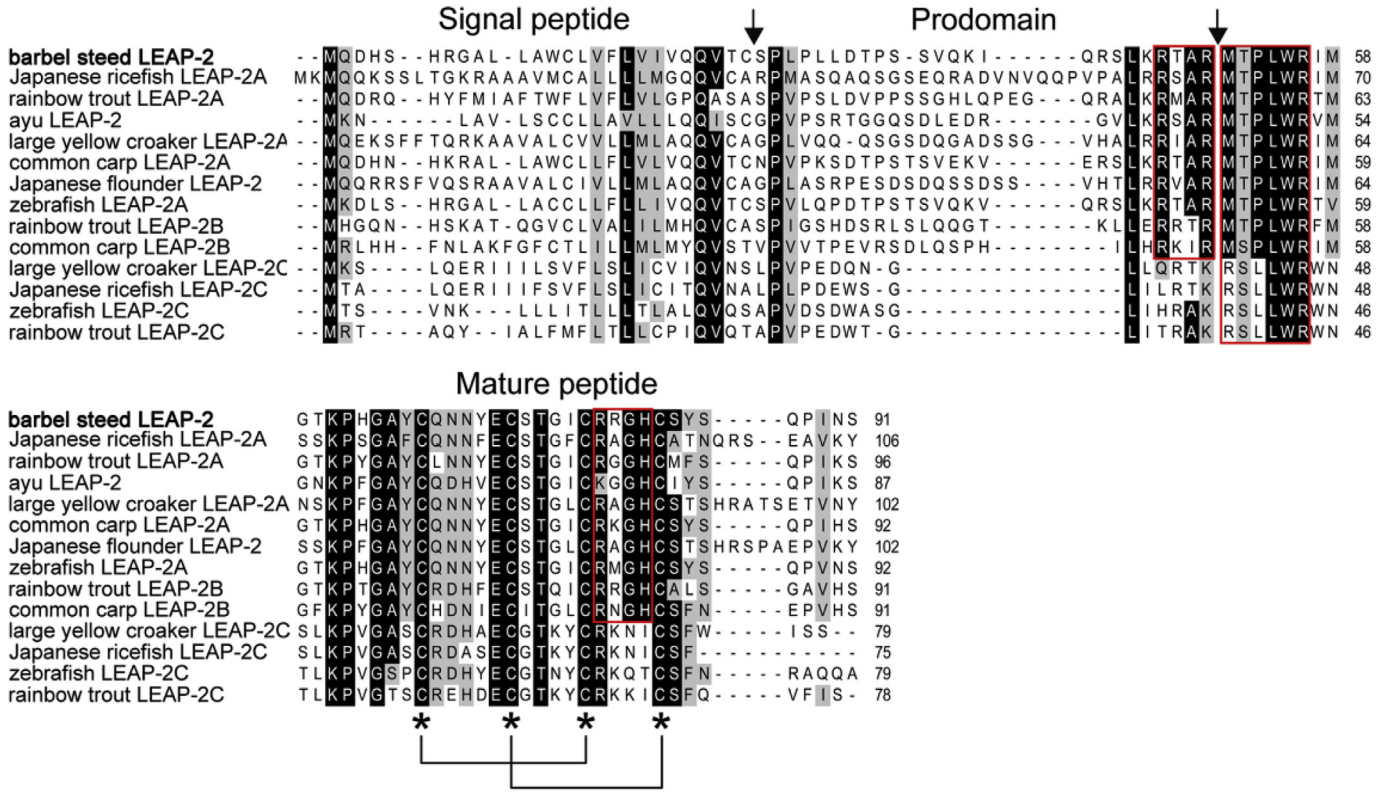

Fig. 1. Multiple alignment of the amino acid sequences of barbel steed LEAP-2 and its homologues. The threshold for shading is 70%. Similar residues are marked in grey, identical residues are marked in black, and alignment gaps are marked as '-'. The predicted cleavage site for the signal peptide or mature peptide is marked with '↓'. The boxes indicate "RXXR", "MTPLWR", "RSLLWR", and "RXGH" motifs, respectively. The four conserved cysteine residues in the mature peptide are indicated by '*'. Two cysteine residues joined by a solid line represent a disulphide bond. Accession numbers of the sequences used are as follows: Japanese ricefish LEAP-2A (XM_004079958); rainbow trout LEAP-2A (NM_001124464); ayu LEAP-2 (KJ412462); large yellow croaker LEAP-2A (KJ024787); common carp LEAP-2A (KC551971); Japanese flounder LEAP-2 (EU586111); zebrafish LEAP-2A (BC162807); common carp LEAP-2B (KC551972); rainbow trout LEAP-2B (NM_001124465); large yellow croaker LEAP-2C (KJ024789); Japanese ricefish LEAP-2C (XM_004074820); zebrafish LEAP-2C (XM_009295063); and rainbow trout LEAP-2C (GQ870279). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

molecular weight (MW) of 10.3 kDa and a putative isoelectric point (*p*I) of 9.50. The LEAP-2 protein contains a signal peptide, a prodomain, and a mature peptide. The mature peptide of LEAP-2 has a putative MW of 4.7 kDa and a *p*I of 8.88. An alignment of barbel steed LEAP-2 with selected teleost LEAP-2 proteins showed that the functionally mature peptide is highly conserved in teleosts, whereas the signal peptide and prodomain are variable (Fig. 1). There is a conserved sequence "RXXR" before the cleavage site separating the prodomain and the mature peptide in fish LEAP-2A and LEAP-2B group. The N termini of mature peptides are composed of six highly conserved amino acids, "MTPLWR" in fish LEAP-2A and LEAP-2B group and "RSLLWR" in fish LEAP-2C, respectively. In the mature peptide region, we also found that there is a conserved amino acid sequence "RXGH" in fish LEAP-2A and LEAP-2B group (Fig. 1). In addition, all LEAP-2 contain four cysteine residues that are conserved in all the fish LEAP-2 homologues examined, and form two disulphide bonds (barbel steed LEAP-2: Cys67 with Cys78, and Cys73 with Cys83).

The phylogenetic tree generated indicated that teleost LEAP-2 sequences cluster into three groups, named LEAP-2A, LEAP-2B, and LEAP-2C, and are distinct from those of the mammalian group (Fig. 2). Barbel steed's LEAP-2, belongs to the LEAP-2A group of other teleost fish, closer to the zebrafish LEAP-2A (Fig. 2).

3.2. Constitutive expression of the barbel steed LEAP-2 gene

The constitutive expression of LEAP-2 gene was assessed in different tissues of barbel steed was analysed using RT-qPCR. The expression was detectable in an array of tissues, with the highest levels being detected in the liver, moderate levels observed in the mid intestine, and relatively low levels detected in other tissues (Fig. 3). We found that the expression level of the LEAP-2 gene in the liver was 2.95-fold that in the head kidney.

3.3. Expression of the LEAP-2 gene in response to LPS challenge

In response to LPS treatment, expression of the LEAP-2 gene was significantly upregulated in all examined tissues in a time-dependent manner (Fig. 4). The LEAP-2 transcripts in liver, mid intestine, and gill were upregulated at 4 hpt (Fig. 4A, D, E), whereas the upregulation of LEAP-2 transcripts in spleen and head kidney occurred at 8 and 24 hpt, respectively (Fig. 4B and C). In the liver, spleen, and gill, there was an inverted U-shaped tendency in the upregulation of LEAP-2 gene expression post-LPS treatment. Highest expression levels of LEAP-2 in the spleen and gill were detected at 8 hpt and in the liver at 12 hpt. At the highest expression levels, LEAP-2 mRNA in liver, spleen, and gill after stimulation was upregulated by 18.2-fold, 7.6-fold, and 33.2-fold respectively (Fig. 4A, B, E). Expression of the LEAP-2 gene in the head kidney and mid intestine increased and reached a peak at 24 hpt, with the highest expression levels showing 11.0-fold and 12.0-fold upregulation, respectively (Fig. 4C and D).

3.4. In vitro antibacterial activity of barbel steed LEAP-2

The synthetized mature peptide of barbel steed LEAP-2 was observed to have divergent antimicrobial activity against a panel of heterogenous bacteria. The MICs of the synthetic LEAP-2 peptide are

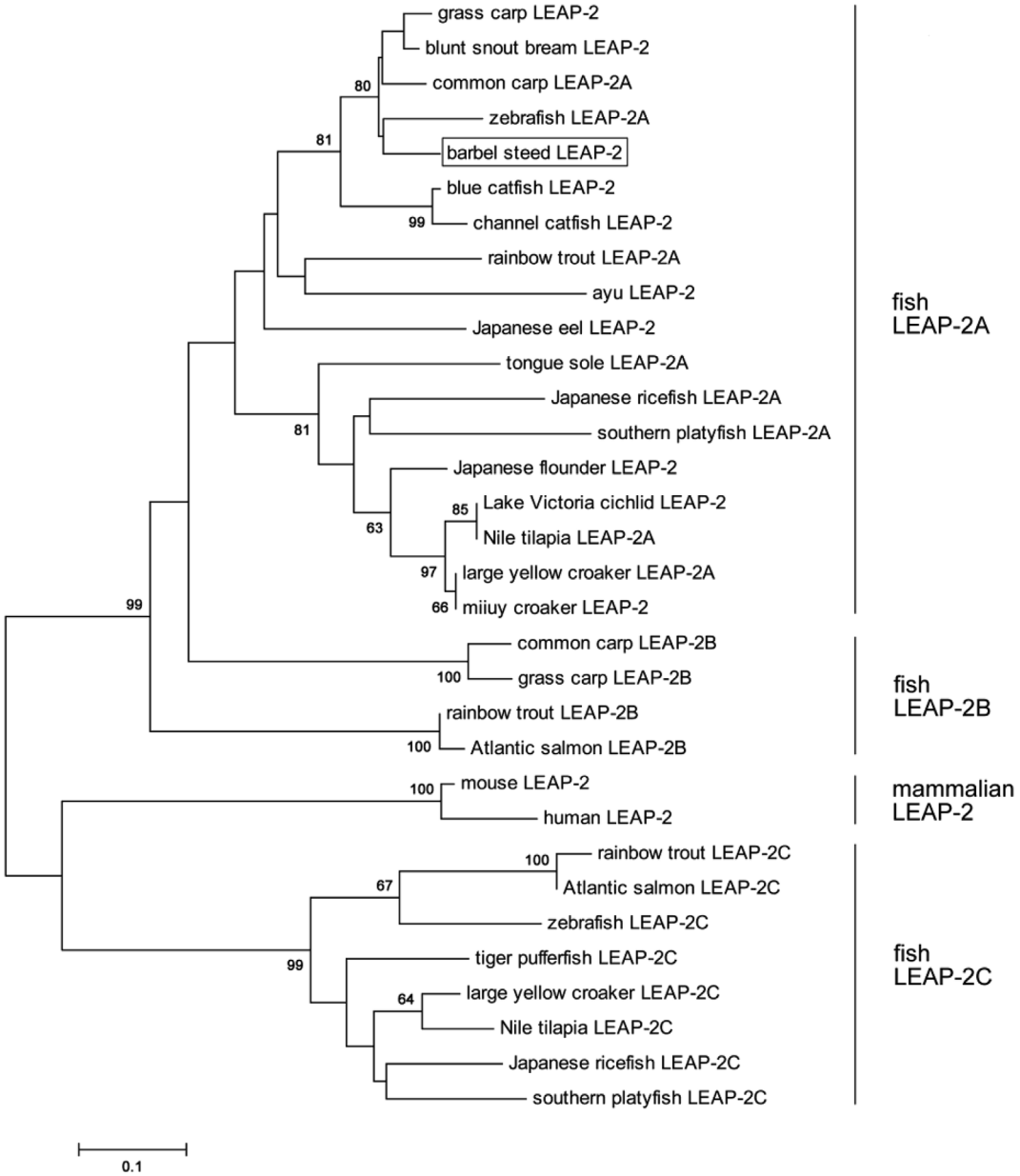

Fig. 2. Phylogenetic tree showing the evolutionary relationship of barbel steed LEAP-2 with other known LEAP-2s. The tree was constructed using the Neighbour-Joining method in MEGA6 program. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; only value over 60% are shown). The scale bar shows the number of substitutions per base. Accession numbers of sequences used are as follows: grass carp LEAP-2 (FJ390414); blunt snout bream LEAP-2 (JQ344324); common carp LEAP-2A (KC551971); common carp LEAP-2B (KC551972); rainbow trout LEAP-2A (NM_001124464); rainbow trout LEAP-2B (NM_001124465); rainbow trout LEAP-2C (GQ870279); Lake Victoria cichlid LEAP-2 (XM_005750928); large yellow croaker LEAP-2A (KJ024787); large yellow croaker LEAP-2C (KJ024789); ayu LEAP-2 (KJ412462); Japanese flounder LEAP-2 (EU586111); Japanese ricefish LEAP-2A (XM_004079958); Japanese ricefish LEAP-2C (XM_004074820); zebra mbuna LEAP-2C (XM_004538202); southern platyfish LEAP-2A (XM_005806413); southern platyfish LEAP-2C (XM_005810019); blue catfish LEAP-2 (AY845142); channel catfish LEAP-2 (AY845141); tiger pufferfish LEAP-2A (XM_003966863); tiger pufferfish LEAP-2C (XM_011604959); zebrafish LEAP-2A BC162807; zebrafish LEAP-2C (XM_009295063); Atlantic salmon LEAP-2A (XM_014138936); Atlantic salmon LEAP-2B (XM_014129528); Atlantic salmon LEAP-2C (GQ870280); Nile tilapia LEAP-2A (XM_003457723); Nile tilapia LEAP-2C (XM_013270789); Japanese eel LEAP-2 (KP893812); half-smooth tongue sole LEAP-2A (XM_008320540); half-smooth tongue sole LEAP-2C (XM_008324342); miiuy croaker LEAP-2 (KJ000088); mudskipper LEAP-2 (KX355130); mouse LEAP-2 (BC089593); rat LEAP-2 (C086950); and human LEAP-2 (BC070199).

shown in Table 2. The peptide displayed strongest antimicrobial activity towards *V. anguillarum* and *A. hydrophila*, with an MIC value of 12.5 μg/mL, whereas chloramphenicol showed MIC values of 6.25 and 1.563 μg/mL, respectively. However, the peptide exhibited no significant bactericidal activity against *V. parahaemolyticus* or *S. iniae*.

3.5. Effect of LEAP-2 on iNOS enzymatic activity and respiratory burst

The ability of LEAP-2 to modulate the nitric oxide response was examined by stimulating the activity of iNOS in MO/MΦ. After a 12 h exposure of MO/MΦ to LPS, the iNOS enzymatic activity was induced up to 7.73-fold, whereas LEAP-2, by itself, could also induce the iNOS activity. The iNOS activity of MO/MΦ in 1 or 10 μg/mL LEAP-2-treated groups increased up to 4.77-fold or 4.18-fold, respectively (Fig. 5A). The combination of LPS and LEAP-2 was found to further enhance the induced activity up to 13.59-fold and 13.12-fold, respectively (Fig. 5A). Respiratory burst reflects the oxygen-dependent killing of bacteria [29]. We analysed the changes in respiratory burst in barbel steed MO/MΦ in

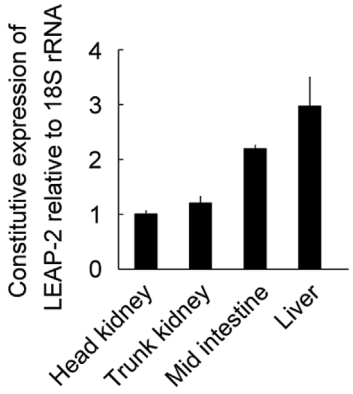

Fig. 3. RT-qPCR was performed to analyse the constitutive expression of LEAP-2 in different tissues (liver, trunk kidney, head kidney, and mid intestine) of barbel steed. The LEAP-2 transcript levels were normalized to those of 18S rRNA. Data are expressed as means ± SEM. $n = 4$.

response to LEAP-2 stimulation, and found that this burst was induced in MO/MΦ after 12 h of incubation with LEAP-2 or PMA. After treatment with PMA, the respiratory burst of MO/MΦ was induced up to 3.67-fold, whereas the respiratory burst of MO/MΦ in 1 or 10 μg/mL LEAP-2-treated groups increased up to 2.50-fold or 1.76-fold, respectively (Fig. 5B). In contrast to the LEAP-2 or PMA treatment, MO/MΦ treated with the combination of PMA and LEAP-2 showed a significant increase in the production of reactive oxygen intermediates (Fig. 5B). The respiratory burst of MO/MΦ increased up to 6.79-fold or 5.89-fold, respectively (Fig. 5B).

3.6. Effect of LEAP-2 on cytokine gene expression in MO/MΦ

The effects of LEAP-2 on the induction of pro-inflammatory cytokines were examined by measurement of its influence on the expression of IFN-γ, TNF-α, and IL-1β. As shown in Fig. 6, LPS or LEAP-2 treatment significantly increased the expression of IFN-γ, TNF-α, and IL-1β in barbel steed MO/MΦ. After treatment with LPS, the gene expression of IFN-γ, TNF-α, and IL-1β was induced up to 84.11-fold, 24.28-fold, and 205.71-fold, respectively. The gene expression of IFN-γ, TNF-α, and IL-

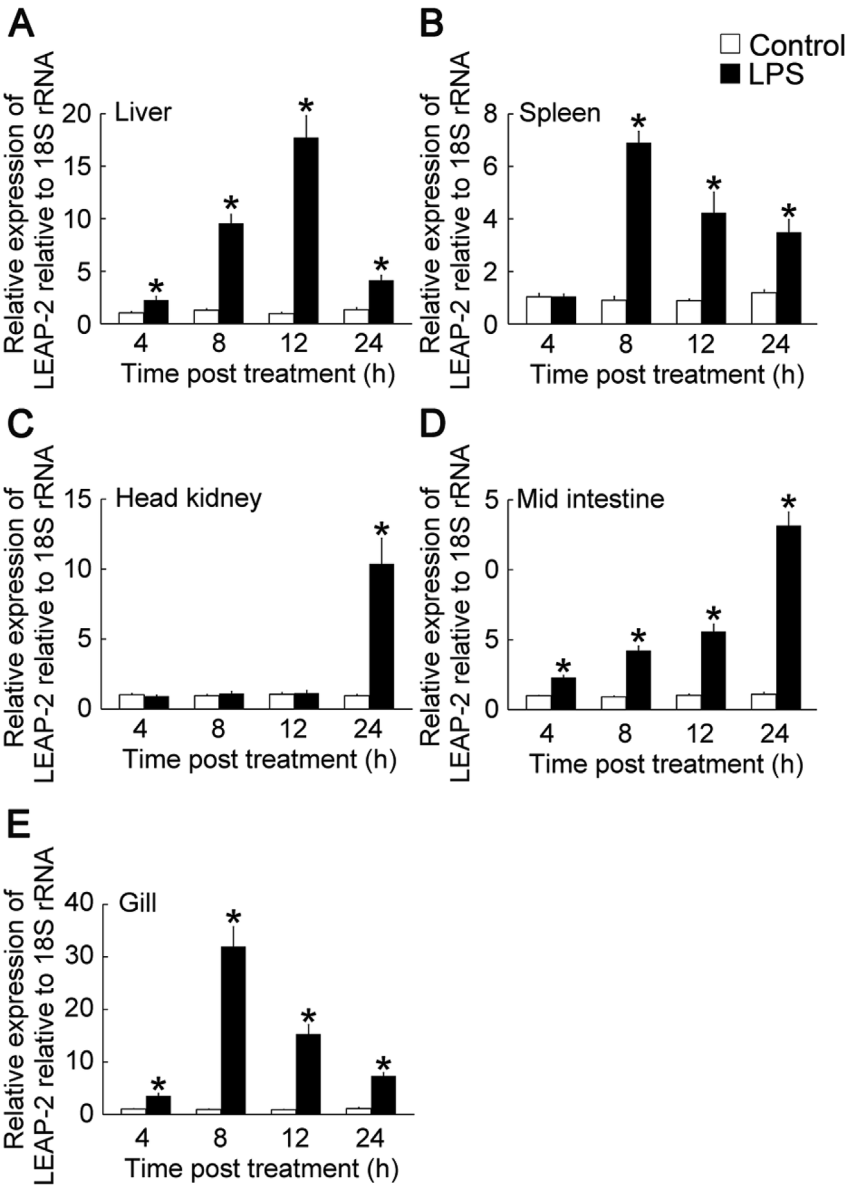

Fig. 4. Changes in expression of the LEAP-2 gene in barbel steed tissues post-lipopolysaccharide (LPS) stimulation. The LEAP-2 transcript levels were normalized to those of 18S rRNA. Data are expressed as means ± SEM. $n = 4$. *$P < 0.05$.

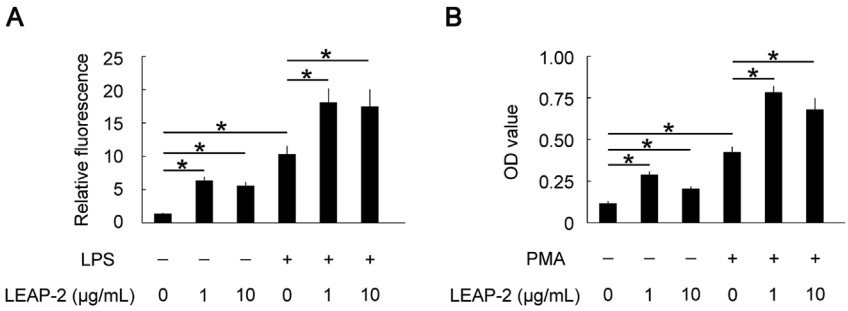

Fig. 5. Effect of LEAP-2 on iNOS enzymatic activity and respiratory burst of barbel steed monocytes/macrophages (MO/MΦ). (A) Barbel steed MO/MΦ were treated with LEAP-2 (0, 1, or 10 μg/mL), lipopolysaccharide (LPS) (10 μg/mL), or the combination of these for 12 h. PBS was used in the control treatment. iNOS enzymatic activity was plotted as relative fluorescence units. (B) MO/MΦ were pre-treated with or without LEAP-2 (0, 1, or 10 μg/mL) for 12 h before treatment with 0.1 μg/mL phorbol 12-myristate 13-acetate (PMA). The respiratory burst was determined by measuring the optical density at 620 nm. Data are expressed as means ± SEM. n = 4. *P < 0.05.

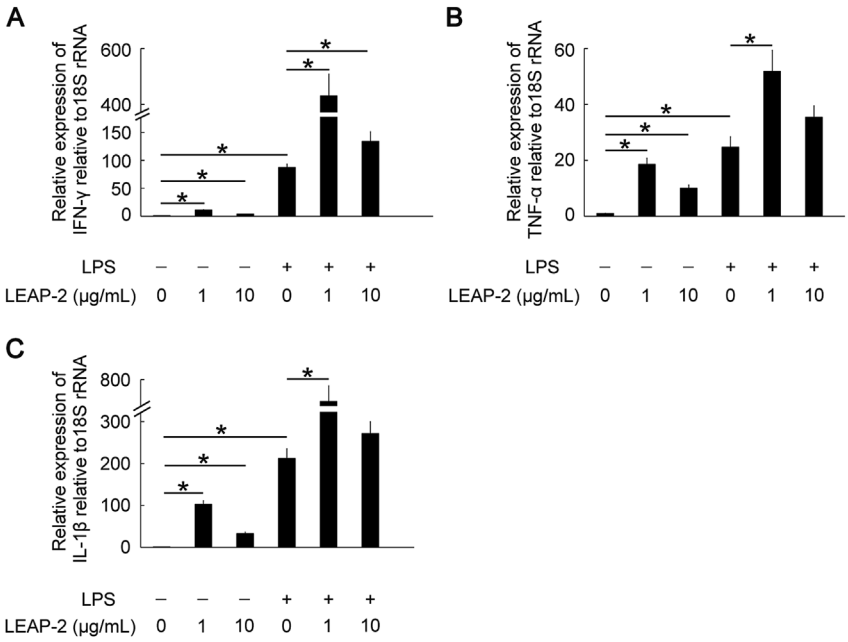

Fig. 6. Effects of LEAP-2 on the expression of IFN-γ, TNF-α, and IL-1β in barbel steed MO/MΦ. The barbel steed MO/MΦ were treated with LEAP-2 (0, 1, or 10 μg/mL), lipopolysaccharide (LPS) (10 μg/mL), or a combination of these for 12 h. PBS was used in the control treatment. Expression of cytokine genes IFN-γ (A), TNF-α (B), and IL-1β (C) was analysed by RT-qPCR. Data are expressed as means ± SEM. n = 4. *P < 0.05.

1β in 1 μg/mL LEAP-2-treated groups increased up to 11.06-fold, 18.25-fold, and 99.55-fold, respectively. Increases in the levels of IFN-γ, TNF-α, and IL-1β mRNAs were further induced in response to combined treatment with LEAP-2 and LPS compared with the induction caused by LPS or LEAP-2 alone (Fig. 6). The expression levels of IFN-γ, TNF-α, and IL-1β genes were maximally increased up to 415.16-fold, 50.93-fold, and 675.14-fold, respectively.

4. Discussion

LEAP-2 is a small, cysteine-rich, cationic HDP, which is the second liver-expressed HDP to be identified in vertebrates, and plays an important role in the host innate immune system [11]. Previous studies demonstrated that LEAP-2 promotes bacterial clearance via direct antimicrobial effects and immunomodulatory functions [19,23,30]. In the present study, the cDNA sequence of a putative LEAP-2 gene was identified in barbel steed. The predicted amino acid sequence of the identified LEAP-2 comprises of a signal peptide and a prodomain, followed by the mature peptide. As reported for most of the examined teleosts, the mature peptide of LEAP-2 was found to contain four conserved cysteines, which form two pairs of disulphide bonds that are necessary for secondary structure conformation [12]. Our phylogenetic analysis indicated that barbel steed LEAP-2 belongs to the fish LEAP-2A group and is phylogenetically most closely related to the zebrafish LEAP-2A. Taken together, the results from phylogenetic and amino acid sequence analyses indicate that this molecule may play an important role in the evolution of vertebrates.

We found that barbel steed LEAP-2 is constitutively expressed in healthy fish tissues, mainly in the liver, which is consistent with the observations in other fish species [15,17,20,30]. In rainbow trout, both LEAP-2A and LEAP-2B genes were found to be constitutively expressed only in the liver [15]. Contrastingly, in the miiuy croaker, the LEAP-2 gene displayed constitutive expression in all the examined tissues, including liver, spleen, intestines, head kidney, gill, heart, eye, fin, muscle, and brain, with expression being the highest in the liver [20]. We found that in response to LPS treatment, expression of the LEAP-2 gene was upregulated in all examined tissues in barbel steed, as has been previously demonstrated in other fish infected with pathogens [15,17,19,22,23,30]. For example, in rainbow trout infected with *Aeromonas salmonicida* for 2 and 8 days, both LEAP-2A and 2B were strongly induced in the intestine, and weak expression of both genes was also detected in the skin [15]. In ayu, LEAP-2 expression was found to increase in liver, gill, spleen, kidney, and heart in response to infection with *V. anguillarum* [30]. These findings suggest that LEAP-2 is intimately involved in the innate immunity of fish.

In recent years, several *in vitro* studies have attributed direct antibacterial activity to fish LEAP-2 [17,19,20,23,30]. For example, synthetic peptides of mudskipper LEAP-2 have been demonstrated to exhibit distinct antimicrobial activity against *V. vulnificus* and *V. alginolyticus in vitro* [23]. In the present study, we found that barbel steed LEAP-2 showed effective antimicrobial activity against most of the examined bacteria, exhibiting a broad antimicrobial spectrum. Moreover, the 12.5 μg/mL MIC of barbel steed LEAP-2 against *V. anguillarum* and *A. hydrophila* was relatively low, thereby indicating that this LEAP-2 could effectively counter these pathogens. *A. hydrophila* is the main pathogen of cyprinid fish [31], and our *in vitro* bacteriostatic

experiments showed that barbel steed LEAP-2 can effectively inhibit the proliferation of this bacterium, indicating that this peptide has potential value in the control of *A. hydrophila* infection. Some *in vivo* studies have demonstrated that fish LEAP-2s can significantly decrease mortality attributable to bacterial infection [19,30]. For example, ayu intraperitoneally injected with mature peptides of LEAP-2 show a significant decrease in mortality caused by *V. anguillarum* infection, with this effect being characterized by a lowered bacterial burden and a decline in pro-inflammatory cytokine expression [30]. Similar phenomena have been observed in large yellow croaker LEAP-2 [19].

In the present study, we focused on determining whether LEAP-2 is involved in the M1-type polarization of MO/MΦ. The M1-type macrophages in fish are characterized by the production of ROS, NO, and pro-inflammatory cytokines, including IL-1β and TNF-α [32,33]. In our initial evaluation of the effect of LEAP-2 on M1-type polarization of MO/MΦ, we assessed the induction of iNOS expression in response to stimulation with LEAP-2, and accordingly found that LEAP-2 alone, or in combination with LPS, could effectively enhance the iNOS activity. iNOS is one of three key enzymes generating NO from the amino acid L-arginine [34]. The production of large amounts of NO by iNOS is believed to be one of the most important inflammatory reactions in activated macrophages [35]. With respect to the respiratory burst, we found a significantly increased respiratory burst activity in MO/MΦ in response to treatment with both LEAP-2 alone and in combination with PMA. Similar results have been obtained in mudskippers, which indicated that LEAP-2 could effectively enhance the respiratory burst of MO/MΦ in response to exposure to *E. tarda* [23]. It is known that macrophages kill most bacteria by phagocytosis, producing several types of ROS and reactive nitrogen species, and the respiratory burst reflects the oxygen-dependent killing of bacteria [29]. Indeed, the stimulation of head kidney-derived MO/MΦ with LEAP-2 alone, or in combination with LPS or PMA, in addition to promoting enhanced iNOS activity and respiratory burst, also showed a synergistic induction of the expression of pro-inflammatory cytokines genes (IFN-γ, TNF-α, and IL-1β). These results thus indicate the involvement of LEAP-2 in the M1-type polarization of MO/MΦ. As is known, macrophages can be polarized to classically activated inflammatory macrophages (M1) and alternatively activated anti-inflammatory macrophages (M2) in response to microenvironmental signals, such as pathogens, inflammatory mediators, and certain other factors [36], and in mammals, several studies have shown that HDPs are involved in these polarization events [6,7]. In teleost, some studies have only shown that HDPs have an immunomodulatory effect on MO/MΦ. For example, both mudskipper LEAP-2 and ayu cathelicidin can enhance the respiratory burst and bacterial killing efficiency and regulate the cytokine expression of MO/MΦ. The present study is, to the best of our knowledge the first to demonstrate the effect of a teleost LEAP-2 on macrophage polarization.

In summary, we characterized a LEAP-2 homologue from the barbel steed. The barbel steed LEAP-2 was shown to have a broad antibacterial activity against various species of bacteria. Different fish LEAP-2s are known to have different antibacterial spectra, which may be associated with differences in mature peptide sequences and related changes in spatial configuration. Further, we found that in response to LPS treatment, expression of the barbel steed LEAP-2 gene was induced in all examined tissues, as has been observed in other fish infected with pathogens, such as rainbow trout, in which both LEAP-2A and 2B were induced in the intestine and skin in response to infection with *A. salmonicida* [15]. Finally, barbel steed LEAP-2 was found to be involved in the M1-type polarization of MO/MΦ, as indicated by the enhanced iNOS activity and respiratory burst, and the up-regulation of representative genes in MO/MΦ. However, further studies are needed to determine the detailed mechanisms and signalling pathways underlying the regulatory roles of LEAP-2 in the immune response of MO/MΦ.

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