Fish and Shellfish Immunology 92 (2019) 256–264

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Molecular characterization of the NK-lysin in a teleost fish, *Boleophthalmus pectinirostris*: Antimicrobial activity and immunomodulatory activity on monocytes/macrophages

Fei-Fei Ding[a,b,1], Chang-Hong Li[a,b,1], Jiong Chen[a,b,c,*]

[a] State Key Laboratory for Quality and Safety of Agro-products, Ningbo University, Ningbo, 315211, China
[b] Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Meishan Campus, Ningbo University, Ningbo, 315832, China
[c] Key Laboratory of Applied Marine Biotechnology of Ministry of Education, Meishan Campus, Ningbo University, Ningbo, 315832, China

ARTICLE INFO

Keywords:
Antibacterial activity
Edwardsiella tarda
Monocyte/macrophage
mRNA expression
Mudskipper
NK-Lysin
Sequence analysis

ABSTRACT

NK-lysin (NKL) is a cationic host defense peptide that plays an important role in host immune responses against various pathogens. However, the immunomodulatory activity of NKL in fishes is rarely investigated. In this study, we characterized a cDNA sequence encoding an NK-lysin homolog (BpNKL) from the fish, mudskipper (*Boleophthalmus pectinirostris*). Sequence analysis revealed that BpNKL is most closely related to tiger puffer (*Takifugu rubripes*) NKL. BpNKL transcript was detected in all the tested tissues, with the highest level in the gill, followed by the spleen and kidney. Upon *Edwardsiella tarda* infection, the mRNA expression of *BpNKL* in the mudskipper was significantly upregulated in the spleen, kidney, and gill. A shortened peptide derived from BpNKL, BpNKLP40, was then chemically synthesized and its biological functions were investigated. BpNKLP40 exhibited a direct antibacterial activity against some Gram-negative bacteria, including *E. tarda*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio harveyi*, and induced hydrolysis of *E. tarda* genomic DNA. Intraperitoneal injection of 1.0 μg/g BpNKLP40 significantly improved the survival of mudskipper following *E. tarda* infection and reduced the bacterial burden in tissues and blood. Moreover, 1.0 μg/ml BpNKLP40 treatment had an enhanced effect on the intracellular killing of *E. tarda* by monocytes/macrophages (MO/MΦ) as well as on the mRNA expression of pro-inflammatory cytokines in MO/MΦ. In conclusion, our study reveals that BpNKL plays a role against *E. tarda* infection in the mudskipper by not only directly killing bacteria but also through an immunomodulatory activity on MO/MΦ.

1. Introduction

Antimicrobial peptides (AMPs) are so-called because of their capacity of directly killing microbes; these peptides are now referred as host defense peptides (HDPs) due to their additional immunomodulatory properties [1,2]. HDPs are conserved in living beings as primitive components of the innate immune response [3] and suppress infections by direct antimicrobial properties, modulation of host immune responses, or both [4]. In recent decades, a large number of researches have focused on the immunomodulatory properties of cationic HDPs, such as cathelicidin, defensin, and NK-lysin (NKL) [5–8].

NKLs (or granulysin in humans), which were first isolated from porcine small intestines, are cationic HDPs of 74–78 amino acids produced by cytotoxic T lymphocytes and natural killer cells and stored in cytolytic granules [9,10]. NKLs are the members of the Saposin-like protein superfamily with a conserved surfactant protein B (SP–B) domain, in which six cysteine residues form three disulfide bonds [11]. NKLs exhibit a broad range of activity against microbial pathogens, such as *Escherichia coli* and *Staphylococcus aureus* [12]. They act on bacterial membranes and their target of action are not only the bacterial membranes but also DNA in the cytoplasm [13]. NKLs also have immunomodulatory activities, for example, a chicken (*Gallus gallus*) NKL-derived peptide, cNK-2, could induce the expression of CCL4, CCL5, and IL-1β in macrophage cell line HD11 or induce CCL4 and CCL5 in primary monocytes via activating the MAPK signaling pathway [8].

Till date, homologs of NKLs have been identified and studied in many fish species, such as half-smooth tongue sole (*Cynoglossus*

* Corresponding author. Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Meishan Campus, Ningbo University, Ningbo, 315832, China.
E-mail addresses: chenjiong@nbu.edu.cn, jchen1975@163.com (J. Chen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2019.06.021
Received 15 April 2019; Received in revised form 9 June 2019; Accepted 10 June 2019
Available online 12 June 2019

semilaevis) [14], zebrafish (*Danio rerio*) [15], channel catfish (*Ictalurus punctatus*) [16], common carp (*Cyprinus carpio*) [17], Nile tilapia (*Oreochromis niloticus*) [18], turbot (*Scophthalmus maximus*) [19], Atlantic salmon (*Salmo salar*) [20], and large yellow croaker (*Larimichthys crocea*) [21]. Some fish have several isoforms of NKLs, for example, the common carp has six NKL genes [17]. Tissue-specific expression pattern of NKLs differ in different fish species, for example, the highest level of NKL2 transcripts in common carp were found in the spleen, followed by the head kidney, heart, and gills [17], while the highest level of NKL2 transcripts in the large yellow croaker were found in the head kidney, followed by the spleen and gill [21]. It is observed that NKL expression responds to defense against invading pathogens in fish. For instance, NKL transcripts increased instantly in the spleen, intestine, head kidney, and nonspecific cytotoxic cells of Nile tilapia subsequent to *Streptococcus agalactiae* infection [18]. Fish NKLs mainly act as direct microbe-killing molecules [21–25]. Recent reports demonstrated that turbot NKL performed its antiviral activity by participation in the inhibition of the fusion of viral particles to the *Epithelioma papulosum cyprinid* (EPC) cells [25] and in autophagy of turbot erythrocytes [19]. Overexpression of NKL *in vivo* or intraperitoneal administration with NKL could increase fish survival and decrease the pathogen load [14,18,22,24]. In addition, fish NKLs may also have an immunomodulatory activity, for example, intraperitoneal injection of NKLP27 (27 aa peptide from half-smooth tongue sole NKL1) induced the expression of immune genes such as IL-1β, IL-8, and CXC chemokine (CXCe1) in the spleen and kidney of half-smooth tongue sole [14,22]; stimulation with NK1 and NK2 (NKL derived peptides) induced the expression of IL-1β and IL-8 in head kidney leukocytes of Atlantic salmon *in vitro* [20].

The mudskipper (*Boleophthalmus pectinirostris*) is an amphibious fish distributed along the Northwestern Pacific coast. Data from the comparative analyses of whole-genome sequencing of four representative species of mudskippers have provided insights into the terrestrial adaptation of amphibious fishes [26]. Considering the pivotal roles of HDPs in fish immune response against pathogens, it would be promising to investigate the biological functions of mudskipper HDPs involved in diseases. In the present study, we studied an NKL homolog (BpNKL) from the mudskipper. The tissue mRNA expression of BpNKL in healthy mudskipper fish upon *Edwardsiella tarda* infection was determined. Furthermore, the direct bactericidal activity of a chemically synthesized peptide BpNKLP40 was determined *in vitro*, and its effect on survival and bacterial load of the mudskipper infected with *E. tarda* was investigated *in vivo*. In addition, the immunomodulatory effects of BpNKLP40 on regulating the function of monocytes/macrophages (MO/MΦ) were also analyzed.

2. Materials and methods

2.1. Fish maintenance

Healthy mudskippers, weighing 30–40 g each, were obtained from a commercial farm in Ningbo city, China. The fish were kept in brackish water (with salinity of 10‰) tanks at 24–26 °C in a filtered-water recirculating system and acclimatized to laboratory conditions for two weeks before carrying out the experiments. Prior to tissue dissection, fish were anesthetized with 0.03% (v/v) ethylene glycol monophenyl ether and sacrificed. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

2.2. Sequence analysis of BpNKL cDNA

The cDNA sequence encoding BpNKL was obtained from NCBI's nucleotide database under the accession number, XM_020919797. The cleavage sites of signal peptides were predicted by the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/). Disulfide bonds were predicted by Prosite (http://prosite.expasy.org/), and the protein domain architecture was predicted using the SMART program (http://smart.emblheidelberg.de/). Multiple alignments were analyzed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [27]. The related NKL sequences are listed in Table 1.

Table 1
NKL sequences used for multiple alignment and phylogenetic analyses.

Accession number	Species		Protein
	Latin name	English name	
XM_020919797	*Boleophthalmus pectinirostris*	mudskipper	NKL
KY801205	*Dicentrarchus labrax*	European sea bass	NKL
NM_001303323	*Larimichthys crocea*	large yellow croaker	NKL
MF113051	*Lateolabrax japonicus*	Japanese sea bass	NKL
XM_018663395	*Latescal carifer*	Asian sea bass	NKL
XM_003962706	*Takifugu rubripes*	tiger puffer	NKL
KU705506	*Scophthalmus maximus*	turbot	NKL
XM_020591071	*Monopterus albus*	swamp eel	NKL1
XM_015969930	*Nothobranchius furzeri*	turquoise killfish	NKL
MF678822	*Oreochromis niloticus*	Nile tilapia	NKL
AGM21637	*Cynoglossus semilaevis*	half-smooth tongue sole	NKL
XM_020487202	*Oncorhynchus kisutch*	coho salmon	NKL
XM_014129907	*Salmo salar*	Atlantic salmon	NKL
XM_021574735	*Oncorhynchus mykiss*	rainbow trout	NKL
XM_004082250	*Oryzias latipes*	Japanese ricefish	NKL
XM_026230159	*Carassius auratus*	goldfish	NKL
XM_019120973	*Cyprinus carpio*	common carp	NKL1
KX034213	*C. carpio*	common carp	NKL2
XM_019071085	*C. carpio*	common carp	NKL3
XM_019092430	*C. carpio*	common carp	NKL4
LN590809	*C. carpio*	common carp	NKL5
XM_021473084	*Danio rerio*	zebrafish	Nkl
KP100115	*D. rerio*	zebrafish	Nkla
KP100116	*D. rerio*	zebrafish	Nklb
KP100117	*D. rerio*	zebrafish	Nklc
KP100118	*D. rerio*	zebrafish	Nkld
KT877168	*Ctenopharyngodon idella*	grass carp	NKL
XM_012822640	*Clupea harengus*	Atlantic herring	NKL
NM_001200208	*Ictalurus punctatus*	channel catfish	NKL1
DQ153186	*I. punctatus*	channel catfish	NKL2
DQ153187	*I. punctatus*	channel catfish	NKL3
XM_019485307	*Alligator mississippiensis*	American alligator	NKL
KT962967	*Gallus gallus*	chicken	NKL
NM_001075143	*Bos taurus*	cattle	Gran
XM_010810101	*B. taurus*	cattle	NKL
NM_001278755	*Sus scrofa*	pig	NKL
NM_001302758	*Homo sapiens*	human	Gran

2.3. In vivo bacterial challenge and sample collection

In vivo bacterial infection was performed as previously described [28]. Briefly, *E. tarda* strain, Et-CD [29], was cultured in Tryptic Soy Broth (TSB) medium at 28 °C with shaking, and harvested from the mid-logarithmic phase. The bacterial cells were washed and resuspended thrice in sterile PBS to obtain a final concentration of 1.0×10^5 colony-forming units (CFU)/ml. Fish were injected intraperitoneally with 1.0×10^4 CFU of live *E. tarda* per fish, and sterile PBS was used as a control. Tissue samples including gills, spleen, and kidney were collected at 4, 8, 12, and 24 h post injection (hpi) from the *E. tarda*-infected or healthy mudskippers. The samples were frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction to perform real-time quantitative polymerase chain reaction (qPCR). The tissues of healthy fish, including the liver, muscle, gill, brain, skin, heart, spleen, kidney, and intestine were also collected for qPCR analysis.

Table 2
Oligonucleotide primers used for the qPCR.

Gene	Accession number	Primer	Sequence (5′–3′)	Amplicon size (bp)
NKL	XM_020919797	BpNKL-F	AGTTTGTGAACAAGCACCTCG	124
		BpNKL-R	GCTGGGCTGTTGTAGTAGAG	
IL-1β	KX492895	IL-1β-F	ACGAGTGGTGAATGTGGTCA	163
		IL-1β-R	GAACTGAGGTTGTGCTGCAA	
TNF-α	KX492896	TNF-α-F	GGACAACAACGAGATCGTGA	155
		TNF-α-R	GTTCCACCGTGTGACTGATG	
IFN-γ	NM_001360715	IFN-γ-F	ACCCTGCACAACATTACGAC	186
		IFN-γ-R	GCTGGAGGAATCCTGAATTTA	
IL-10	XM_020936977	IL-10-F	GTGGAGGGGTTCCCTCTAAG	179
		IL-10-R	GTGCGGAGGTAAAAGCTCAG	
TFG-β	XM020928521	BpTGF-β-F	TCAAAGGACACTTGCACAGC	183
		BpTGF-β-R	CAGGGCCAAGATCTGTGAAT	
18S rRNA	KX492897	18S–F	GGCCGTTCTTAGTTGGTGGA	112
		18S-R	CCCGGACATCTAAGGGCATC	

2.4. qPCR analysis of mRNA expression

Total RNA extraction, DNase I digestion, and first-strand cDNA synthesis were carried out as previously reported [30]. qPCR was performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR premix Ex Taq II (TaKaRa, Dalian, China) as previously described [30]. The primers used are listed in Table 2. The qPCR amplification conditions were as follows: 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; followed by melting curve analysis at 95 °C for 30 s, 72 °C for 30 s, and 95 °C for 30 s. Relative gene expression of BpNKL in healthy or *E. tarda*-infected samples was calculated using the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method respectively, and the data were normalized against Bp18S rRNA. Each qPCR run was performed with four samples and repeated three times.

2.5. Minimal inhibitory concentration (MIC) assay

In previous studies, the core region of the SP-B domain of NKLs, which was thought to be responsible for the antibacterial activity of NKLs, had been used in bioactivity studies [8,11,21–23,31]. Therefore, we chemically synthesized the core region peptide within the BpNKL SP-B domain (SIKAKLLAVCKNIGLLKSLCQKFVNKHLGVLIEELTTTDD) with one disulfide bond (Cys78 with Cys88) (over 95% purity, SynPeptide, Shanghai, China) and named it as BpNKLP40. Antibacterial activity of the synthetic BpNKLP40 was assayed against eight Gram-negative bacteria (*E. tarda*, *Aeromonas hydrophila*, *E. coli*, *Vibrio harveyi*, *Vibrio anguillarum, Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio vulnificus*) and three Gram-positive bacteria (*Streptococcus iniae*, *Listeria monocytogenes*, and *S. aureus*). A modified two-folds microdilution method was used to determine the MIC values of the various agents as previously described [32]. MIC was calculated as the lowest concentration of BpNKLP40 that inhibited the growth of the tested bacteria. Briefly, serial dilutions of the peptides (100, 50, 25, 12.5, 6.25, 3.125, and 1.563 μg/ml) were prepared and an aliquot of 80 μl of each solution was added to wells of a 96-well plate. The solution without BpNKLP40 was used as the blank control. The bacteria were grown to mid-logarithmic phase and diluted in suitable media to a final inoculum concentration of 1.0×10^5 CFU/ml. A 20-μl aliquot of this bacterial suspension was added to each well of the above 96-well plate and the plates incubated at appropriate temperatures for 24 h. MIC was calculated by visual verification of bacterial sedimentation and also by measuring the absorbance with the SpectraMax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, USA) at 600 nm. The experiment was repeated four times, each performed in triplicate.

2.6. Hydrolysis of bacterial genomic DNA (gDNA) by BpNKLP40

Hydrolysis of *E. tarda* gDNA by BpNKLP40 was assayed as previously described [32,33]. Bacterial gDNA was extracted using a Mini BEST Universal gDNA Extraction Kit Ver. 5.0 (TaKaRa), and its concentration was determined using a NanoDrop instrument (Thermo Fisher Scientific, Wilmington, USA). To determine the effect of different doses of BpNKLP40 on the hydrolysis of purified gDNA of *E. tarda*, 800 ng gDNA in 20 μl PBS was mixed with increasing amounts of the BpNKLP40 peptide (0, 25, 50, 75, and 100 μg/ml). After incubation at room temperature for 30 min, 2 μl 10 × loading buffer (TaKaRa) was added to the mixture, and 20 μl of each reaction mixture was applied to a 1.0% agarose gel in 0.5 × Tris-acetate-EDTA buffer. To determine the effect of BpNKLP40 on gDNA hydrolysis of live bacteria, *E. tarda* was cultured and adjusted to a final concentration of 1.0×10^{10} CFU/ml in PBS. The *E. tarda* suspension (100 μl) was mixed with 100 μg/ml BpNKLP40, or BSA (control) and incubated at 28 °C for 1, 2, 4, or 6 h. After incubation, *E. tarda* gDNA was extracted and applied to 1% w/v agarose gel.

2.7. Fish survival assay

The survival assay was performed as previously described [32]. Mudskippers were divided into four groups of 16 fish each for the survival assay. Fish were intraperitoneally injected with 1.0×10^4 CFU of live *E. tarda* in 100 μl PBS each. Thirty minutes later, fish in three experimental groups were further injected intraperitoneally with 0.1, 1.0, or 10.0 μg/g BpNKLP40, while the control group was intraperitoneally injected with a same volume of sterile PBS. During the next 7 d, the fish were monitored every 24 h for death or moribund state. The Kaplan-Meier method was used to analyze the 7-day survival rate.

2.8. Bacterial load assay

Two groups each of six mudskippers were used for the bacterial load assay. The fish were intraperitoneally injected with 1.0×10^4 CFU of live *E. tarda*, according to the determined 50% lethal dose (LD_{50}) of *E. tarda* in mudskippers (about 1.0×10^4 CFU/fish). Thirty minutes later, fish in the experimental groups were intraperitoneally injected with 1.0 μg/g BpNKLP40, while the control group was injected with sterile PBS. At 24 hpi, the liver, spleen, kidney, and blood samples were collected. The tissue (0.1 g fresh tissue) and blood (0.1 ml) samples were homogenized in 1 ml sterile TSB. The homogenates were serially diluted in sterile TSB, plated onto separate tryptic soy agar (TSA) plates, and incubated at 28 °C for 18 h. For each sample, the colonies were counted and normalized to 0.1 g tissue weight or 0.1 ml blood volume.

2.9. Cell preparation

The mudskipper kidney-derived MO/MΦ were isolated and cultured

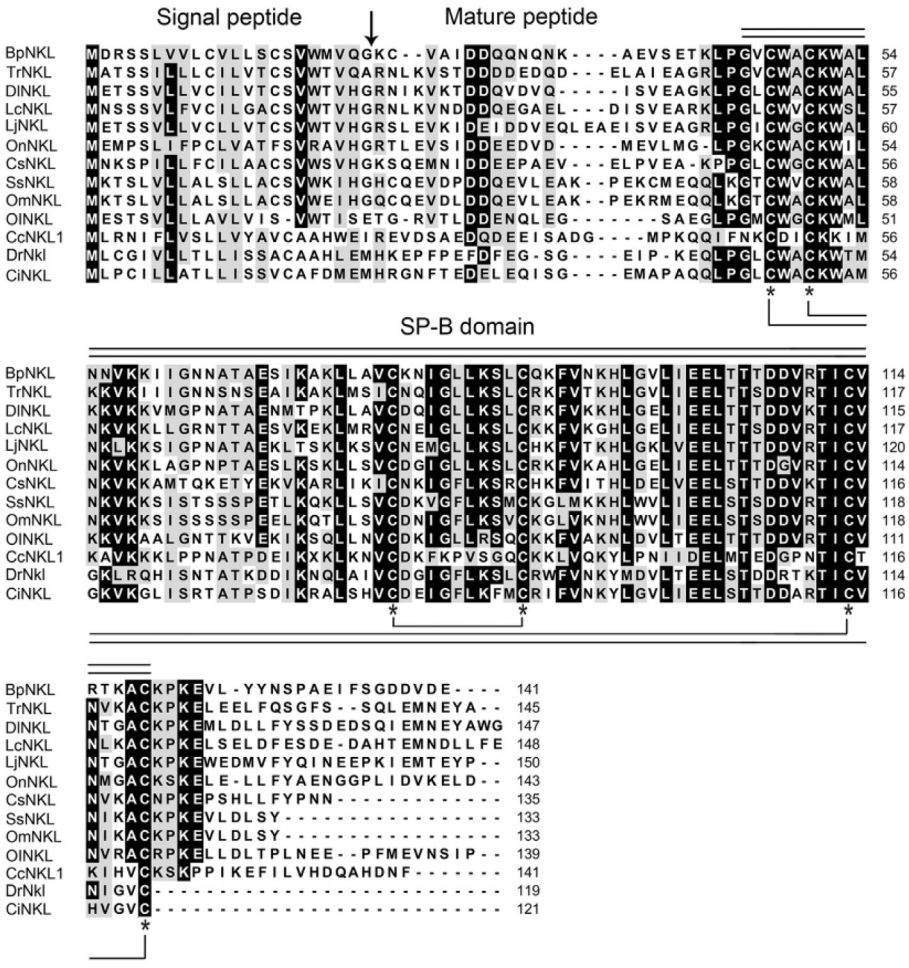

Fig. 1. Multiple alignment of the amino acid sequences of mudskipper and related fish NKL sequences. Threshold for the shaded amino acids is > 70%, similar residues are marked with a gray shadow, identical residues with a black shadow, and alignment gaps with "-". Predicted cleavage site for the signal peptide or mature peptide is marked as "↓ ". Conserved six cysteine residues are marked as "*". Two cysteine residues joined by a solid line represent a disulfide bond. Abbreviations are as follows: BpNKL, mudskipper NKL; TrNKL, tiger puffer NKL; DlNKL, European sea bass NKL; LcNKL, large yellow croaker NKL; LjNKL, Japanese sea bass NKL; OnNKL, Nile tilapia NKL; CsNKL, half-smooth tongue sole NKL; SsNKL, Atlantic salmon NKL; OmNKL, rainbow trout NKL; OlNKL, Japanese ricefish NKL; CcNKL1, common carp NKL1; DrNkl, zebrafish Nkl; CiNKL, grass carp NKL. Accession numbers of these sequences are listed in Table 1.

as previously described [28]. Fish kidney leukocyte-enriched fractions were obtained using Ficoll-Hypaque PREMIUM (1.077 g/ml) (GE healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The cells were then seeded in 35-mm dishes at a density of 2×10^7/ml. Non-adherent cells were washed off, and the adherent cells were incubated in complete medium (RPMI 1640, 5% mudskipper serum, 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) at 24 °C with 5% CO_2. The purity of the mudskipper MO/MΦ isolated was greater than 95% as measured by Wright-Giemsa staining.

2.10. Phagocytosis assay

Phagocytosis of mudskipper MO/MΦ was performed as previously described [34]. Briefly, *E. tarda* in the logarithmic phase of growth were labeled with fluorescein isothiocyanate (FITC) (Sigma, Saint Louis, USA) and designated as FITC-*E. tarda*. Mudskipper kidney-derived MO/MΦ were seeded in 35-mm plates (2×10^6/ml), and were pretreated with 1.0 μg/ml BpNKLP40 for 8 h and the control group treated with a same volume of PBS. Later, heat-killed FITC-*E. tarda* were added to the medium at a multiplicity of infection (MOI) of 20 and incubated for another 30 min, after which the cells were washed extensively with sterile PBS to remove extracellular particles. Trypan blue (0.4%) was used to quench the fluorescence outside the cell membranes. Finally, the cells were harvested and resuspended in FACS buffer (PBS, 0.2% BSA, 0.1% sodium azide). The engulfed bacteria were examined using a Gallios Flow Cytometer (Beckman Coulter, Miami, USA). Relative mean fluorescence intensity (MFI) of bacteria engulfed by the cells was analyzed using FlowJo software. MFI of PBS- or BpNKLP40-treated group was expressed as a fold-change relative to the MFI of the group without added bacteria, and the MFI of PBS-treated group was assigned a value unit of 100. Four independent experiments were performed.

2.11. Bacterial killing assay

Bacterial killing assay for mudskipper MO/MΦ was performed as previously described [32]. Briefly, fish MO/MΦ (2×10^6/ml) were pretreated with 1.0 μg/ml BpNKLP40 for 8 h, while the control group was treated with a same volume of PBS. Subsequently, the cells were washed with sterile PBS and infected with live *E. tarda* at an MOI of 10. Bacterial uptake by MO/MΦ was allowed to proceed for 30 min at 24 °C in the atmosphere of 5% CO_2, and non-internalized *E. tarda* were removed by washing extensively with sterile PBS. One set of samples (the uptake group) was collected and lysed in 1% Triton X-100 solution and plated onto TSA plates to acquire bacterial uptake values. Another set of samples (the kill group) was further incubated for 1.5 h to allow bacterial killing to proceed before cell lysis. After incubation at 28 °C for 18 h, the colonies on the plates were counted. Bacterial survival rate was determined by dividing the CFU of the kill group by that of the uptake group. Four independent experiments were performed.

2.12. Cytokine mRNA expression analysis in MO/MΦ

The mudskipper MO/MΦ (2×10^6/ml) were pretreated with 1.0 μg/ml BpNKLP40 for 8 h followed by infection with live *E. tarda* at an MOI of 10. The control group received a similar volume of PBS. The cells were harvested at 4, 8, 12, and 24 h post-treatment, and mRNA expression level of five cytokines, including TNF-α, IL-1β, IFN-γ, IL-10, and TGF-β was examined by qPCR. The primers used are listed in

Table 2.

2.13. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was conducted by one-way ANOVA using SPSS version 13.0 (SPSS Inc., Chicago, USA). $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Molecular characterization of BpNKL

cDNA sequence of BpNKL was obtained from the DDBJ/EMBL/GenBank databases under the accession number XM_020919797. The sequence possessed an open reading frame (ORF) of 426 nucleotides, which was predicted to encode a 141 aa polypeptide with a calculated molecular weight (MW) of 15.54 kDa and an isoelectric point (pI) of 9.77. Sequence analyses revealed that BpNKL possesses a 22 aa N-terminal signal peptide and a SP-B domain (aa position 45–119), in which six cysteine residues forming three disulfide bonds (Cys47-Cys119, Cys50-Cys113, and Cys78-Cys88) (Fig. 1). Multiple alignment of BpNKL amino acid sequence with other known NKL sequences of fish revealed that the SP-B domain was highly conserved in teleosts, while the N- and C-terminus were variable (Fig. 1).

Sequence comparison showed that BpNKL shared the highest aa identity (55.4%) with tiger puffer NKL. Phylogenetic tree analysis of complete aa sequences of NKLs showed that fish NKLs grouped together to form a large cluster distinct from the mammal-bird-reptile cluster and that BpNKL is most closely related to tiger puffer NKL (Fig. 2).

3.2. Alteration of the BpNKL transcripts in tissues of mudskipper upon E. tarda infection

The relative mRNA expression levels of BpNKL in various tissues were detected using qPCR. BpNKL transcripts could be detected in all the tested tissues of healthy mudskipper, with the highest level detected in the gill, following by the spleen and kidney (Fig. 3A). Upon *E. tarda* infection, BpNKL transcripts rapidly altered in the three immune-related tissues (the gill, spleen, and kidney) than those in control group (Fig. 3B–D). In the gill, BpNKL transcript increased 5.64 fold at 4 hpi, then gradually decreased but was still significantly higher than the control till 24 hpi (Fig. 3B). In the spleen and kidney, BpNKL transcripts increased at 4 hpi (spleen: 1.86 fold; kidney: 2.80 fold), reaching a peak at 8 hpi (spleen: 4.29 fold; kidney: 5.42 fold), and gradually decreasing to control levels at 12 and 24 hpi (Fig. 3C and D).

3.3. In vitro antibacterial activity of BpNKLP40

The MIC of BpNKLP40 was determined against a panel of bacteria. BpNKLP40 exhibited a narrow spectrum, displaying antibacterial activities against four Gram-negative bacteria, including *E. tarda*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. harveyi,* with MIC values of 6.25, 6.25, 12.5, and 50 μg/ml, respectively (Table 3).

3.4. Hydrolysis of gDNA of E. tarda by BpNKLP40

The gDNA of *E. tarda* was incubated with different concentrations of BpNKLP40 before being subjected to agarose gel electrophoresis to determine the effect of different dosages of BpNKLP40 on DNA hydrolysis. The gDNA band gradually diminished in intensity with the increasing amounts of BpNKLP40 added and almost disappeared at the concentration of 100 μg/ml BpNKLP40 (Fig. 4A). Subsequently, we determined the effect of BpNKLP40 on the gDNA hydrolysis of live *E. tarda*. BpNKLP40 was incubated with *E. tarda* at a concentration of 100 μg/ml for various time-periods, and the gDNA was extracted before being subjected to electrophoresis. Results showed that treatment with

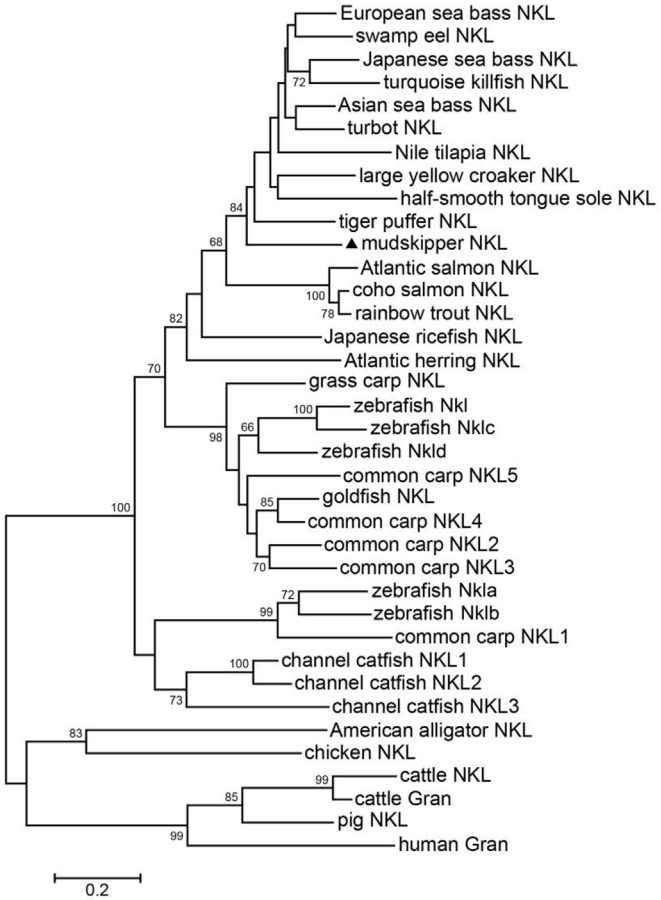

Fig. 2. Phylogenetic (Neighbor-joining) analysis of complete amino acid sequences of NKLs using the MEGA6.0 program. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; shown only when the values were > 60%). The scale bar shows the number of substitutions per base. The sequences used in the analysis are listed in Table 1.

BpNKLP40 caused degradation of the intracellular gDNA in a time-dependent manner, whereas treatment with BSA had no apparent effect (Fig. 4B).

3.5. Effect of BpNKLP40 treatment on the survival of E. tarda-infected mudskipper

Survival analysis was conducted to investigate the *in vivo* effect of BpNKLP40 on mudskipper against *E. tarda* infection. Fish without BpNKLP40 treatment or with 0.1 μg/g of BpNKLP40 treatment died by day 7, while fish treated with 1.0 or 10.0 μg/g BpNKLP40 achieved survival rates of 31.3% or 18.5%, respectively, on day 7 (Fig. 5). Statistically significant difference was observed between the saline- and 1.0 μg/g BpNKLP40-treated groups (Fig. 5).

3.6. Effect of BpNKLP40 on bacterial load in tissues of E. tarda-infected mudskipper

Bacterial load was evaluated in the immune tissues and blood of *E. tarda*-infected mudskipper following treatment with 1.0 μg/g BpNKLP40. The BpNKLP40-treated group showed a significant reduction of *E. tarda* load in the liver, spleen, kidney, and blood compared with that of the saline-treated group (Fig. 6).

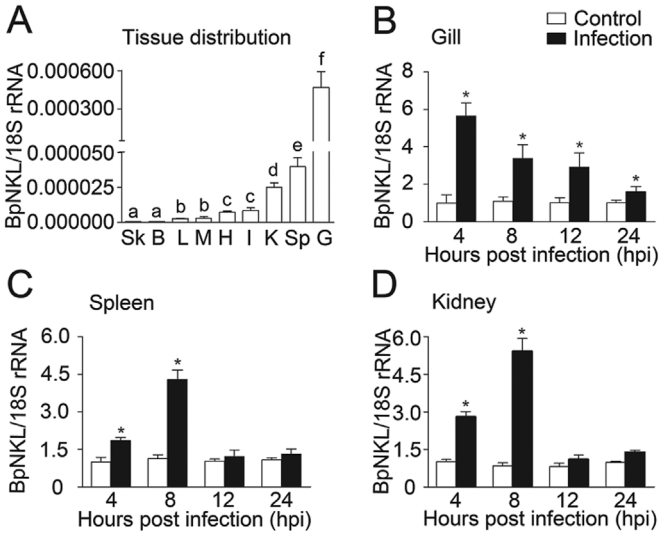

Fig. 3. mRNA expression analysis of BpNKL in healthy mudskipper and mudskipper challenged with *E. tarda*. (A) BpNKL mRNA expression levels relative to that of 18S rRNA were calculated using the $2^{-\Delta CT}$ method. Sk: skin, B: brain, L: liver, M: muscle, H: heart, I: intestine, K: kidney, Sp: spleen, and G: gill. Values denoted by different letters are significantly different when compared by ANOVA ($P < 0.05$). (B–D) BpNKL mRNA expression levels relative to that of 18S rRNA were calculated using the $2^{-\Delta\Delta CT}$ method. Tissues were collected at different time-points after bacterial infection. Data are expressed as mean ± SEM of the results from four fish. *$P < 0.05$.

3.7. Effect of BpNKLP40 on phagocytosis and bacterial killing activity of MO/MΦ

MO/MΦ play a pivotal role in the immune responses of fish against invasion by most pathogens, and phagocytosis and intracellular bacterial killing are the main biological functions of MO/MΦ [35]. Consequently, we further investigated the effect of BpNKLP40 on these functions in mudskipper MO/MΦ. After BpNKLP40 treatment, no significant effect was seen on the phagocytosis of FITC-*E. tarda* by MO/MΦ compared to that on PBS-treated control (Fig. 7A and B), suggesting that BpNKLP40 had no effect on the phagocytic function of MO/MΦ. As an intracellular bacterium, during evolution, *E. tarda* has learned strategies to counter-balance the host's immune defense mechanisms, in order to secure survival or multiplication inside MO/MΦ. We found that the survival rate of *E. tarda* (44.33 ± 6.87%) in BpNKLP40-treated mudskipper MO/MΦ was significantly lower than that in the PBS group (79.98 ± 9.38%) (Fig. 7C and D), suggesting that treatment with 1.0 μg/ml BpNKLP40 could efficiently inhibit the survival of *E. tarda* in MO/MΦ.

3.8. Effect of BpNKLP40 on the mRNA expression of BpTNF-α, BpIL-1β, BpIFN-γ, BpIL-10, and BpTGF-β in MO/MΦ following E. tarda infection

MO/MΦ are a major source of many cytokines involved in immune responses of fish. We therefore investigated the effect of BpNKLP40 on mRNA expression of some cytokines in mudskipper MO/MΦ following *E. tarda* infection. When mudskipper MO/MΦ were treated with 1.0 μg/ml BpNKLP40, the mRNA expression of BpTNF-α, BpIL-1β, and BpIFN-γ (pro-inflammatory cytokines) were significantly upregulated at all the tested time-points, while that of BpIL-10 and BpTGF-β (anti-inflammatory cytokines) showed a tendency towards downregulation (Fig. 8).

4. Discussion

NKL, an important HDP, has been demonstrated to possess broad-spectrum antimicrobial properties and immunomodulatory activities [8,11,12]. NKL isoforms have been reported in some fish, such as large yellow croaker, channel catfish, and zebrafish [15,21,36]. In this study, the cDNA sequence for a putative NKL gene was characterized in mudskipper by genome analysis. The predicted aa sequence had the prominent characteristics of NKL. BpNKL shares the highest aa identity (up to 55.4%) with tiger puffer NKL. Phylogenetic tree analysis showed that BpNKL grouped with other known fish NKLs and was most closely related to tiger puffer NKL. The high conservation of the protein suggests that this molecule plays an important role in the evolution of vertebrates.

NKL displays constitutive expression in most fish tissues, mainly in the gills, spleen, head kidney, and trunk kidney [15,17,21,23,31,36,37]. The mRNA expression of NKL in fish could be induced by pathogenic infections, such as bacteria, parasites, or viruses [15,17,21,23,37]. However, the alteration of NKL expression was quite variable between fish species or between different isoforms. For example, NKL1 transcript in the head kidney was downregulated at 6 h post *Cryptocaryon irritans* infection; but no significant change of NKL2 transcript was observed at 6 hpi [21,23]. In the present study, BpNKL transcript was constitutively expressed in all the tested tissues of healthy mudskipper with higher levels detected in the gill, spleen, and kidney, which were similar to those reported previously in other teleosts. Upon *E. tarda* infection, BpNKL transcript in the gill, spleen, and kidney were immediately and significantly upregulated, suggesting that BpNKL is involved in the early immune response of mudskipper against *E. tarda*.

Some studies have reported that mature peptides of fish NKLs act to directly kill various types of microorganisms *in vitro* [16,17,21,23,31]. Japanese flounder NKL could effectively inhibit the growth of gram-negative bacteria, including *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Photobacterium damselae* subsp. *piscicida* [31]. Both the

Table 3
MIC values of BpNKLP40 against bacteria.

Bacteria	Strains	Culture temperature	BpNKLP40 MIC (μg/ml)	Kanamycin MIC (μg/ml)
Gram-positive bacteria				
Streptococcus iniae	ATCC29178	28 °C	–	6.25
Listeria monocytogenes	ATCC19115	37 °C	–	1.563
Staphylococcus aureus	ATCC6538	37 °C	–	12.5
Gram-negative bacteria				
Edwardsiella tarda	Et-CD	28 °C	6.25	25
Aeromonas hydrophila	ATCC7966	28 °C	–	6.25
Escherichia coli DH5α	K12	37 °C	–	50
Vibrio harveyi	ATCC33866	28 °C	50	1.563
Vibrio anguillarum	ATCC19264	28 °C	–	25
Vibrio parahaemolyticus	ATCC33847	28 °C	6.25	50
Vibrio alginolyticus	ATCC 17749	28 °C	12.5	25
Vibrio vulnificus	ATCC 279562	28 °C	–	25

"-": no inhibition detected at 100 μg/ml.

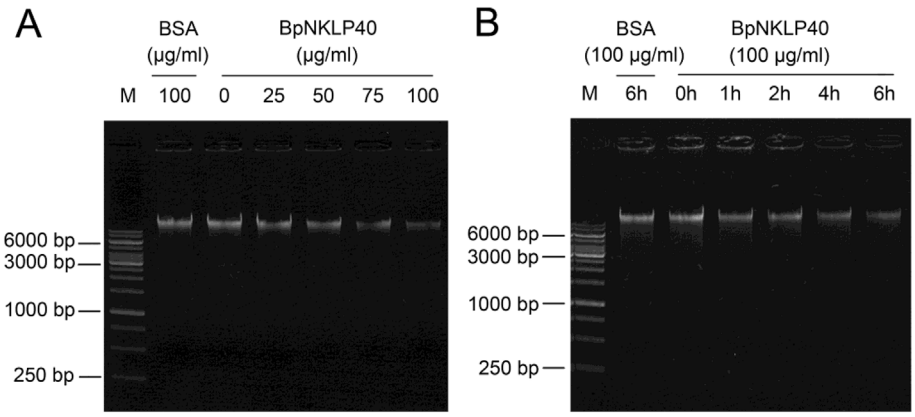

Fig. 4. Hydrolytic activity of BpNKLP40 on *E. tarda* gDNA. (A) Various concentrations of BpNKLP40 were incubated with 800 ng gDNA of *E. tarda* at room temperature for 30 min before subjecting the gDNA to electrophoresis using a 1.0% agarose gel. (B) Live *E. tarda* were incubated with 100 μg/ml BpNKLP40 for 0, 1, 2, 4, and 6 h or with a same volume of BSA for 6 h before subjecting the gDNA to electrophoresis using a 1.0% agarose gel.

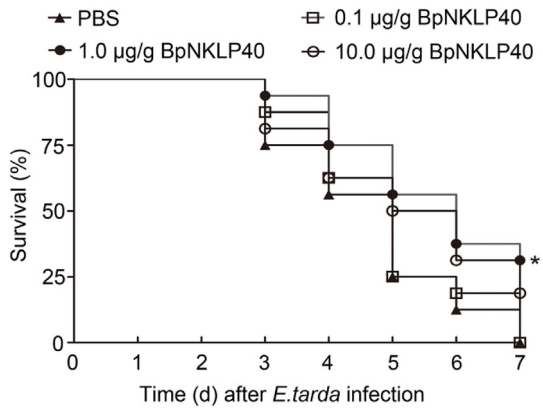

Fig. 5. Effects of BpNKLP40 treatment on the survival rate of *E. tarda*-infected mudskipper. The fish were intraperitoneally injected with equal volumes of saline, 0.1, 1.0, and 10.0 μg/g BpNKLP40 at 30 min after *E. tarda* infection, and mortality was monitored daily for 7 days ($n = 16$). $^*P < 0.05$.

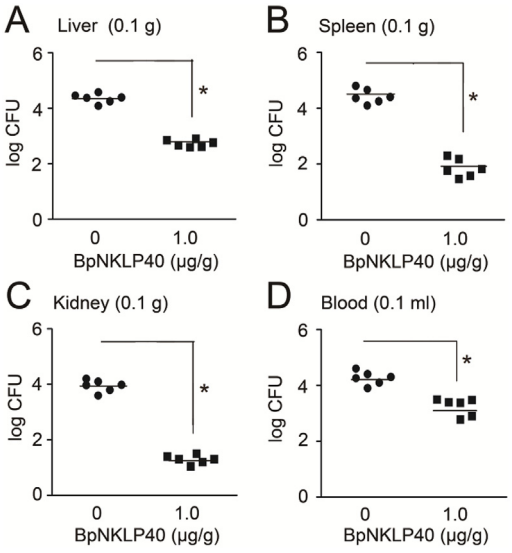

Fig. 6. Effect of BpNKLP40 treatment on bacterial load in tissues of *E. tarda*-infected mudskipper. Each fish was first injected intraperitoneally with 1.0×10^4 CFU of *E. tarda* before being injected with an equal volume of either saline or 1.0 μg/g BpNKLP40 after 30 min. The mudskippers were euthanized 24 h later; the liver, spleen, kidney, and blood were collected and homogenized before culturing on TSA plates. CFUs were normalized to 0.1 ml of blood and 0.1 g tissue weight (for the liver, spleen, and kidney). Data represent the bacterial load in the liver, spleen, kidney, and blood (n = 6). $^*P < 0.05$.

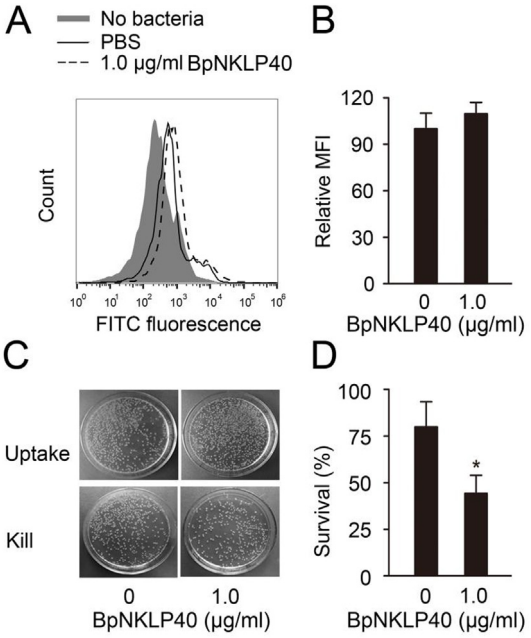

Fig. 7. Effect of BpNKLP40 treatment on the phagocytosis and bacterial killing activity of mudskipper MO/MΦ. (A and B) Effect of BpNKLP40 on the phagocytosis of FITC-*E. tarda* by mudskipper MO/MΦ. MO/MΦ were pretreated with PBS or 1.0 μg/ml BpNKLP40 for 8 h. FITC-*E. tarda* was added at an MOI of 20 and incubated for additional 30 min. Phagocytosis of FITC-*E. tarda* was determined using flow cytometric analysis. Mean fluorescent intensity (MFI) was presented as fold-change relative to the PBS-treated group, which was assigned a value of 100. (C and D) Effect of BpNKLP40 on the bacterial killing activity of *E. tarda* by mudskipper MO/MΦ. MO/MΦ were pretreated with PBS or 1.0 μg/ml BpNKLP40 for 8 h before live *E. tarda* were added at an 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 the killing of bacteria. The histograms represent the effect of BpNKLP40 on the survival of bacteria in mudskipper MO/MΦ. Data are expressed as mean ± SEM (n = 4). $^*P < 0.05$.

NKL1 and NKL2 of large yellow croaker exhibited antibacterial activity against *S. aureus*, *E. coli*, and *V. harveyi* [21,23]. In this study, BpNKLP40 demonstrated antibacterial activity against four Gram-negative bacteria, including *E. tarda*, *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. Moreover, the MIC of BpNKLP40 for *E. tarda* and *V. parahaemolyticus* was extremely low. This result suggested that BpNKLP40 was highly efficacious in eliminating such bacteria. Several HDPs not only carry out their functions by perturbing the bacterial cell membrane [38] but also enter the cytoplasm of target microbes to exert antimicrobial activity by interacting with microbial nucleic acids and other target factors, thus causing cell death [13,39,40]. For example, half-smooth tongue sole NKLP27 destroyed cell membrane integrity,

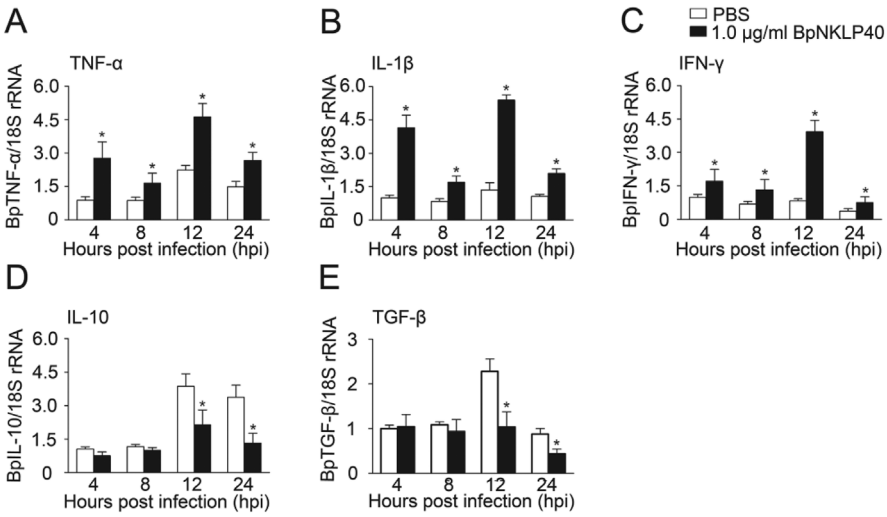

Fig. 8. Effect of BpNKLP40 on the mRNA expression of some cytokines in mudskipper MO/MΦ challenged with *E. tarda*. MO/MΦ were pretreated with 1.0 μg/ml BpNKLP40 for 8 h before being infected with live *E. tarda* at an MOI of 10. mRNA expression of BpTNF-α (A), BpIL-1β (B), BpIFN-γ (C), BpIL-10 (D), and BpTGF-β (E) were analyzed by qPCR at 4, 8, 12, and 24 hpi. Transcript levels of cytokines at different time-points were normalized to that of Bp18S rRNA. Data are expressed as mean ± SEM (n = 4). *$P < 0.05$.

penetrated into the cytoplasm, and induced the degradation of gDNA [22]. Our study revealed that BpNKLP40 had a hydrolytic effect on *E. tarda* gDNA, which is consistent with previous reports. Therefore, our study suggests that BpNKL may play a pivotal role in killing the invasive *E. tarda*. Furthermore, BpNKLP40 had a stronger effect against *E. tarda*, *V. parahaemolyticus*, and *V. alginolyticus* compared to commercial antibiotics (kanamycin), suggesting the potential use of the fish-derived HDP to improve disease resistance in fish.

NKLs have been reported to contribute to immunity against pathogenic microorganisms in fish [14,18,22,24] Both NKL1 overexpression and intraperitoneal administration of NKLP27 *in vivo* decreased the microbial burden in the kidney and spleen of half-smooth tongue sole upon *V. anguillarum* or megalocytivirus RBIV-C1 infections [14,22]. NKL overexpression *in vivo* significantly increased the survival rate of Nile tilapia upon *S. agalactiae* infection and decreased the bacterial burden in the spleen [18]. Moreover, intraperitoneal injection of a synthetic peptide, Nkl71-100, a short peptide containing the active core of turbot NKL, significantly increased the survival rate of *Philasterides dicentrarchi*-infected turbot and decreased the parasite burden in the peritoneum [24]. In our study, we found that the mudskipper, intraperitoneally injected with 1.0 μg/g BpNKLP40, showed a significantly higher survival rate (31.3%) than that in fish treated with saline, while 0.1 μg/g or 10.0 μg/g BpNKLP40 treatment had no significant effect (0%,18.5%, respectively) on the fish survival. In a previous report, the higher concentration of 300 μg/ml cNK-2 had a significant cytotoxic effect on chicken HD11 cells, but the lower concentrations (10, 20, 50, and 100 μg/ml) displayed no obvious effect in the cell viability [8]. Therefore, the cytotoxic effect of 10 μg/g of BpNKLP40 may be the cause of the lower protective effect against *E. tarda* -infection compared with 1 μg/g of BpNKLP40. The effect of 1.0 μg/g BpNKLP40 on the fish survival rate was accompanied by a decline in the bacterial burden of tissues and blood, which concurred with some previous reports. This result illustrated that BpNKL improved the outcome of mudskipper upon *E. tarda* infection.

MO/MΦ in vertebrates are important components of the mononuclear phagocytic system and play diverse roles during infection, inflammation, and tissue injury and repair [41]. In addition to the direct bactericidal ability of NKLs, NKLs may also have regulatory effects of on immune cells such as MO/MΦ. A recent study showed that cNK-2, a chicken NKL derived peptide, induced the expression of CCL4, CCL5 and IL-1β in chicken HD11 and CCL4 and CCL5 in primary monocytes [8]. In the present study, the effect of BpNKLP40 on phagocytosis, bacterial killing, and cytokine mRNA expression of mudskipper MO/MΦ were investigated. We found that 1.0 μg/ml of BpNKLP40 treatment had no significant effect on the phagocytosis of FITC-*E. tarda* by mudskipper MO/MΦ; however, it enhanced the bacteria-killing ability of mudskipper MO/MΦ. BpNKLP40 treatment rapidly upregulated the mRNA expression of pro-inflammatory cytokines (BpIL-1β, BpTNF-α, and BpIFN-γ) and downregulated the mRNA expression of anti-inflammatory cytokines (BpIL-10 and BpTGF-β) in mudskipper MO/MΦ upon *E. tarda* stimulation. The effect induced by BpNKLP40 during *E. tarda* infection in MO/MΦ (increase of pro-inflammatory but inhibition of anti-inflammatory cytokines) seems to be that corresponding to a macrophage polarization toward an M1 profile. Human granulysin had been reported to present a similar effect in T helper (Th) cell populations, with the increasing levels of pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, and IL-12 after granulysin treatment [42]. These results suggest that BpNKL exerts a pro-inflammatory effect on mudskipper MO/MΦ.

In summary, we characterized and investigated the antimicrobial and immunomodulatory properties of an NKL homolog from mudskipper. mRNA expression of BpNKL was upregulated in mudskipper tissues upon *E. tarda* infection. Treatment with suitable concentration of a synthetic peptide, BpNKLP40, effectively decreased the tissue bacterial burden of mudskipper infected by *E. tarda* and improved survival of the diseased fish. BpNKLP40 demonstrated not only a direct antibacterial activity against some gram-negative bacteria, such as *E. tarda*, but also enhanced the killing of *E. tarda* and induced mRNA expression of pro-inflammatory cytokines in MO/MΦ. However, further investigations are needed to verify the underlying mechanisms that account for the immunomodulatory activity of BpNKL.

Acknowledgements

The project was supported by the Program for the National Natural Science Foundation of China (31772876, 31402333), the Zhejiang Provincial Natural Science Foundation of China (LZ18C190001; LY17C190003), the Scientific Innovation Team Project of Ningbo (2015C110018), the Scientific Research Foundation of Graduate School of Ningbo University (G19122), and the K.C. Wong Magna Fund in Ningbo University.

References

[1] R.E. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.
[2] S. Cotton, S. Donnelly, M.W. Robinson, J.P. Dalton, K. Thivierge, Defense peptides secreted by helminth pathogens: antimicrobial and/or immunomodulator molecules? Front. Immunol. 3 (2012) 269.
[3] J.M. Ageitos, A. Sánchez-Pérez, P. Calo-Mata, T.G. Villa, Antimicrobial peptides (AMPs): ancient compounds that represent novel weapons in the fight against bacteria, Biochem. Pharmacol. 133 (2017) 117–138.

[4] B. Bommarius, H. Jenssen, M. Elliott, J. Kindrachuk, M. Pasupuleti, H. Gieren, et al., Cost-effective expression and purification of antimicrobial and host defense peptides in *Escherichia coli*, Peptides 31 (2010) 1957–1965.

[5] F. Niyonsaba, H. Ushio, M. Hara, H. Yokoi, M. Tominaga, K. Takamori, et al., Antimicrobial peptides human β-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells, J. Immunol. 184 (2010) 3526–3534.

[6] A. Sonawane, J.C. Santos, B.B. Mishra, P. Jena, C. Progida, O.E. Sorensen, et al., Cathelicidin is involved in the intracellular killing of mycobacteria in macrophages, Cell Microbiol. 13 (2011) 1601–1617.

[7] M. Wan, O. Soehnlein, X. Tang, A.M. van der Does, E. Smedler, P. Uhlén, et al., Cathelicidin LL-37 induces time-resolved release of LTB4 and TXA2 by human macrophages and triggers eicosanoid generation *in vivo*, FASEB J. 28 (2014) 3456–3467.

[8] W.H. Kim, H.S. Lillehoj, W. Min, Evaluation of the immunomodulatory activity of the chicken NK-lysin-derived peptide cNK-2, Sci. Rep. 7 (2017) 45099.

[9] M. Andersson, H. Gunne, B. Agerberth, A. Boman, T. Bergman, R. Sillard, et al., NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity, EMBO J. 14 (1995) 1615–1625.

[10] J. Kumar, S. Okada, C. Clayberger, A.M. Krensky, Granulysin: a novel antimicrobial, expert opin, Invest. Drugs 10 (2001) 321–329.

[11] L. Yang, J. Johansson, R. Ridsdale, H. Willander, M. Fitzen, H.T. Akinbi, et al., Surfactant protein B propeptide contains a saposin-like protein domain with antimicrobial activity at low pH, J. Immunol. 184 (2010) 975–983.

[12] J. Andrä, D. Monreal, G. Martinez de Tejada, C. Olak, G. Brezesinski, S.S. Gomez, et al., Rationale for the design of shortened derivatives of the NK-lysin-derived antimicrobial peptide NK-2 with improved activity against Gram-negative pathogens, J. Biol. Chem. 282 (2007) 14719–14728.

[13] J.X. Yan, K.R. Wang, W. Dang, R. Chen, J.Q. Xie, B.Z. Zhang, et al., Two hits are better than one: membrane-active and DNA binding-related double-action mechanism of NK-18, a novel antimicrobial peptide derived from mammalian NK-lysin, Antimicrob. Agents Chemother. 57 (2013) 220–228.

[14] M. Zhang, H. Long, L. Sun, A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens, Dev. Comp. Immunol. 40 (2013) 258–265.

[15] P. Pereiro, M. Varela, P. Diaz-Rosales, A. Romero, S. Dios, A. Figueras, et al., Zebrafish Nk-lysins: first insights about their cellular and functional diversification, Dev. Comp. Immunol. 51 (2015) 148–159.

[16] S.R. Cai, J. Wang, K.Y. Wang, D.F. Chen, X.W. Dong, T. Liu, et al., Expression, purification and antibacterial activity of NK-lysin mature peptides from the channel catfish (*Ictalurus punctatus*), Appl. Sci. 6 (2016) 240.

[17] G.L. Wang, M.C. Wang, Y.L. Liu, Q. Zhang, C.F. Li, P.T. Liu, et al., Identification, expression analysis, and antibacterial activity of NK-lysin from common carp *Cyprinus carpio*, Fish Shellfish Immunol. 73 (2018) 11–21.

[18] Y. Huang, Q. Zheng, J. Niu, J. Tang, B. Wang, E.D. Abarike, et al., NK-lysin from *Oreochromis niloticus* improves antimicrobial defence against bacterial pathogens, Fish Shellfish Immunol. 72 (2018) 259–265.

[19] P. Pereiro, A. Romero, P. Díaz-Rosales, A. Estepa, A. Figueras, B. Novoa, Nucleated teleost erythrocytes play an Nk-lysin- and autophagy-dependent role in antiviral immunity, Front. Immunol. 8 (2017) 1458.

[20] J. Acosta, F. Roa, I. González-Chavarría, A. Astuya, R. Maura, R. Montesino, et al., *In vitro* immunomodulatory activities of peptides derived from *Salmo salar* NK-lysin and cathelicidin in fish cells, Fish Shellfish Immunol. 88 (2019) 587–594.

[21] Q.J. Zhou, J. Wang, Y. Mao, M. Liu, Y.Q. Su, Q.Z. Ke, et al., Molecular structure, expression and antibacterial characterization of a novel antimicrobial peptide NK-lysin from the large yellow croaker *Larimichthys crocea*, Aquaculture 500 (2019) 315–321.

[22] M. Zhang, M.F. Li, L. Sun, NKLP27: a teleost NK-lysin peptide that modulates immune response, induces degradation of bacterial DNA, and inhibits bacterial and viral infection, PLoS One 9 (2014) e106543.

[23] Q.J. Zhou, J. Wang, M. Liu, Y. Qiao, W.S. Hong, Y.Q. Su, et al., Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish *Larimichthys crocea*, Fish Shellfish Immunol. 55 (2016) 195–202.

[24] R. Lama, P. Pereiro, M. Costa, J. Encinar, R.M. Medina-Gali, L. Pérez, et al., Turbot (*Scophthalmus maximus*) Nk-lysin induces protection against the pathogenic parasite *Philasterides dicentrarchi* via membrane disruption, Fish Shellfish Immunol. 82 (2018) 190–199.

[25] A. Falco, R.M. Medina-Gali, J.A. Poveda, M. Bello-Perez, B. Novoa, J.A. Encinar, Antiviral activity of a turbot (*Scophthalmus maximus*) NK-lysin peptide by inhibition of low-pH virus-induced membrane fusion, Mar. Drugs 17 (2019) 87.

[26] X.X. You, C. Bian, Q.J. Zan, X. Xu, X. Liu, J.M. Chen, et al., Mudskipper genomes provide insights into the terrestrial adaptation of amphibious fishes, Nat. Commun. 5 (2014) 5594.

[27] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.

[28] F. Guan, X.J. Lu, C.H. Li, J. Chen, Molecular characterization of mudskipper (*Boleophthalmus pectinirostris*) hypoxia-inducible factor-1α (HIF-1α) and analysis of its function in monocytes/macrophages, PLoS One 12 (2017) e0177960.

[29] X.P. Wang, C.P. Lu, Mice orally vaccinated with *Edwardsiella tarda* ghosts are significantly protected against infection, Vaccine 27 (2009) 1571–1578.

[30] 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. 40 (2019) 77–88.

[31] I. Hirono, H. Kondo, T. Koyama, N.R. Arma, J.Y. Hwang, R. Nozaki, et al., Characterization of Japanese flounder (*Paralichthys olivaceus*) NK-lysin, an antimicrobial peptide, Fish Shellfish Immunol. 22 (2007) 567–575.

[32] J. Chen, L. Nie, J. Chen, Mudskipper (*Boleophthalmus pectinirostris*) hepcidin-1 and hepcidin-2 present different gene expression profile and antibacterial activity and possess distinct protective effect against *Edwardsiella tarda* infection, Probiotics Antimicrob. Proteins 10 (2018) 176–185.

[33] H.X. Li, X.J. Lu, C.H. Li, J. Chen, Molecular characterization of the liver-expressed antimicrobial peptide 2 (LEAP-2) in a teleost fish, *Plecoglossus altivelis*: antimicrobial activity and molecular mechanism, Mol. Immunol. 65 (2015) 406–415.

[34] J. Chen, Q. Chen, X.J. Lu, J. Chen, The protection effect of LEAP-2 on the mudskipper (*Boleophthalmus pectinirostris*) against *Edwardsiella tarda* infection is associated with its immunomodulatory activity on monocytes/macrophages, Fish Shellfish Immunol. 59 (2016) 66–76.

[35] B. Magnadóttir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2006) 137–151.

[36] Q. Wang, Y. Wang, P. Xu, Z. Liu, NK-lysin of channel catfish: gene triplication, sequence variation, and expression analysis, Mol. Immunol. 43 (2006) 1676–1686.

[37] Q. Wang, B. Bao, Y. Wang, E. Peatman, Z. Liu, Characterization of a NK-lysin antimicrobial peptide gene from channel catfish, Fish Shellfish Immunol. 20 (2006) 419–426.

[38] C.L. Townes, G. Michailidis, J. Hall, The interaction of the antimicrobial peptide cLEAP-2 and the bacterial membrane, Biochem. Biophys. Res. Commun. 387 (2009) 500–503.

[39] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.

[40] J.D. Hale, R.E. Hancock, Alternative mechanisms of action of cationic antimicrobial peptides on bacteria, Expert Rev. Anti Infect. Ther. 5 (2007) 951–959.

[41] X.J. Lu, J. Chen, Specific function and modulation of teleost monocytes/macrophages: polarization and phagocytosis, Zool. Res. 40 (2019) 146–150.

[42] P. Tewary, D. Yang, G. de la Rosa, Y. Li, M.W. Finn, A.M. Krensky, et al., Granulysin activates antigen-presenting cells through TLR4 and acts as an immune alarmin, Blood 116 (2010) 3465–3474.